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DIVERGENCE OF STREAMLINES APPROACHING A PECTINATE INSECT ANTENNA: CONSEQUENCES FOR CHEMORECEPTION

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Abstract—Pectinate (feathery) antennae have high resistance to air flow, and therefore most of the air approaching an antenna is diverted around it and is not available for chemical sampling by the sensory hairs on that antenna. The small fraction (approximately 10–20%) of approaching air that passes through the air spaces or gaps in the antenna decelerates and the streamlines diverge as the air approaches the antenna. Sampling a small fraction of air that is decelerating and diverging has consequences for chemoreception that are described here for the first time. The behavior of the air is predicted from application of a fluid mechanical law: the principle of continuity. As this small fraction of air decelerates and flows through the air gaps in the antenna, it will be "stretched" in the plane perpendicular to the air flow. Therefore, the air may be sampled by the sensory hairs at a greater spatial resolution than expected from the distribution of the odorant molecules in the air upstream of the antenna. However, the slowing down of odorant-laden air as it passes through an antenna will not change the perceived temporal characteristics of the chemical stimulus (e.g., the rate of odorant filament encounter). This distortion or stretching of the air sample is expected to develop within about one antennal width upstream of the antenna, as verified by examining wakes of simple physical models.

Key Words—Antenna, insect, flow, chemoreception, pheromone, pheromone plume, spatial heterogeneity.

INTRODUCTION

The pectinate insect antennae have thousands to tens of thousands of sensory hairs arrayed in complex arrangements in three-dimensional space (e.g., Steinbrecht, 1970). Whereas such a large number of chemosensory hairs should facilitate the

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capture of odorant molecules from the air, Vogel (1983) noted that such a dense array of hairs would have a high resistance to air movement and, therefore, decrease the amount of air sampled by those hairs. This has been verified for the pectinate antennae of two insect species: only 10–20% of the approaching air passed through the pectinate antennae of the luna moth, *Actias luna* (L.) (Vogel, 1983) and the silkworm moth, *Bombyx mori* (L.) (Zhang, 2001; Zhang and Loudon, unpublished). A "trade-off" exists between the amount of air processed and the efficiency with which that air is sampled by the sensory hairs. For pectinate antennae, this small fraction of air is sampled with great efficiency (Loudon and Koehl, 2000). There will be additional consequences of this particular air flow pattern that have not been considered in the literature until now.

Some general features of this diverging and decelerating air flow pattern may be predicted by applying the fluid mechanical "principle of continuity." The principle requires that air approaching a pectinate antenna will decelerate and, thus, its streamlines will diverge (splay downstream). For air containing a spatially-heterogeneous odorant, divergence will distort the plume, affecting its interception by chemosensory structures. Odorants have a patchy distribution in natural environments, with filaments of concentrated odorant interspersed with clean air (Murlis and Jones, 1981; Murlis, 1986; Murlis et al., 1990). The filamentous nature of odorants is used during orientation to odor sources by some insect species (Vickers and Baker, 1994; Willis et al., 1994; Fadamiro and Baker, 1997; Baker et al., 1998; Vickers et al., 2001).

The principle of continuity is a statement of the conservation of mass applied to a moving fluid such as air or water. If only 10–20% of the air approaching a pectinate antenna passes through, then the approaching air must diverge (the streamlines will splay) (Figure 1A and B). By definition, a streamline is tangent to the local flow at every point, which means that a streamline will not be crossed even though it is not a solid barrier. Therefore, conservation laws may be applied to a volume enclosed within a set of streamlines (a "streamtube"), and the mass of air entering this volume must be identical to the mass of air leaving this volume during the same time period (if air moleculer are not created, destroyed, or accumulated within this volume). Air volume may be substituted for mass, assuming air of constant density (valid under these subsonic, biologically-relevant conditions). It follows that the product of cross-sectional area and average velocity will be constant at every downstream location (Figure 1A). Therefore, as the cross-sectional area increases (as the streamlines diverge), the average velocity will decrease. If the flow pattern is not changing over time, a streamline will indicate the path that will be traced by an individual fluid particle as it moves downstream in the flow (see Vogel 1994, for more explanation). Any patches or filaments of odorant will be distorted in space as each patch follows its own set of streamlines, slowing and diverging. The principle of continuity predicts that patches of odorant in air approaching a pectinate antenna will be stretched in the plane perpendicular to

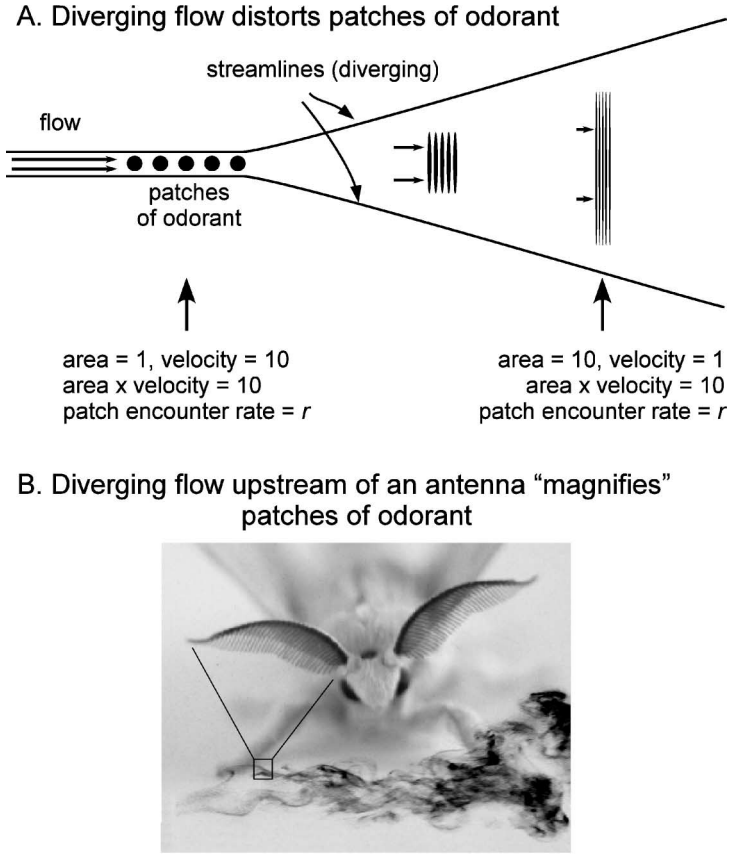

FIG. 1. (A) Patches of odorant, shown at left as black circles, become distorted in shape as the streamlines of the air diverge and the flow slows. Arrows indicate the direction of the air flow, with speed represented by arrow length. The representative values for area, velocity, and patch encounter rate demonstrate the predictions from the principle of continuity: the constant product of area × velocity (between streamlines) and the unchanging patch encounter rate. (B) The picture of the moth shows how an odorant plume will be magnified (stretched) as it is sampled by a pectinate antenna.

the direction of air flow and shortened in the direction of air flow (Figure 1A). A stretched, thinner patch of odorant will have the opportunity to strike a larger number of sensory hairs, but the patch encounter rate by an individual sensory hair is not expected to change. This is because the odorant patches are thinned and slowed by the same factor as they approach an antenna (Figure 1A).

Although the principle of continuity predicts that the air approaching a pectinate antenna will decelerate and diverge, it does not predict how far upstream of

the antenna this divergence will occur. The farther upstream the divergence occurs, the greater the opportunity for the thinner, stretched filaments (Figure 1A) to be eroded by diffusion and the less likely to be detected by the antennae as discreet filaments. It is not known where streamlines start to diverge upstream of a porous bluff body ("bluff" means nonstreamlined), such as a pectinate antenna.

In general, the location of the divergence upstream of a body will depend on the geometry and the magnitude of the Reynolds number (a dimensionless number characterizing the ratio of inertial to viscous effects in a fluid). The Reynolds number is defined as $Re = \rho L v/\mu$, where ρ is the density of the fluid, L is the characteristic length, v is the velocity, and μ is the kinematic viscosity of the fluid. The characteristic length of the object is usually measured perpendicular to the direction of flow, and the velocity of the fluid is measured with respect to the object's frame of reference (Vogel, 1994). Most of the literature on porous structures is for higher Reynolds number cases (Elder, 1959; Lau and Baines, 1968; Castro, 1971; Koo and James, 1973; Castro, 1976) in which the upstream divergence is extremely close to the body. For lower Reynolds numbers (relevant to insect antennae), diverging flow could develop farther upstream because of the increased importance of viscous effects.

To estimate where the flow diverges around porous structures for lower Reynolds numbers, we measured the flow around physical models of arrays of platinum wires. Because of the technical difficulties with accurate measurements of upstream streamlines, we measured downstream flow because it allowed us to identify unambiguously the fluid parcel that had moved through the porous array. These downstream measurements may be used to predict upstream streamlines because upstream and downstream flow patterns will be symmetrical for low Reynolds number flow ($Re < 1$). The downstream convergence will start to extend farther from the body than the upstream divergence as the Reynolds number increases above one (White, 1991). Therefore, measurements for this higher Reynolds number range ($Re > 1$) based on the downstream convergence will be a conservative estimate (in this case, overestimate) of the extent of the upstream divergence.

Dimensionless modeling was used (matching the Reynolds number of the physical model with the relevant Reynolds number for antennae) to enable comparison of the flow around the model array with air flow around insect antennae. A shift from air to water at the same Reynolds number range will not change the flow pattern geometry, which is the logic behind dimensionless modeling. The implications of the principle of continuity as described here are relevant for chemoreception in air or water.

METHODS AND MATERIALS

Physical Models and Flow Chamber. The physical model consisted of an array of platinum wires inside a fluid-filled enclosed chamber (with a glass top

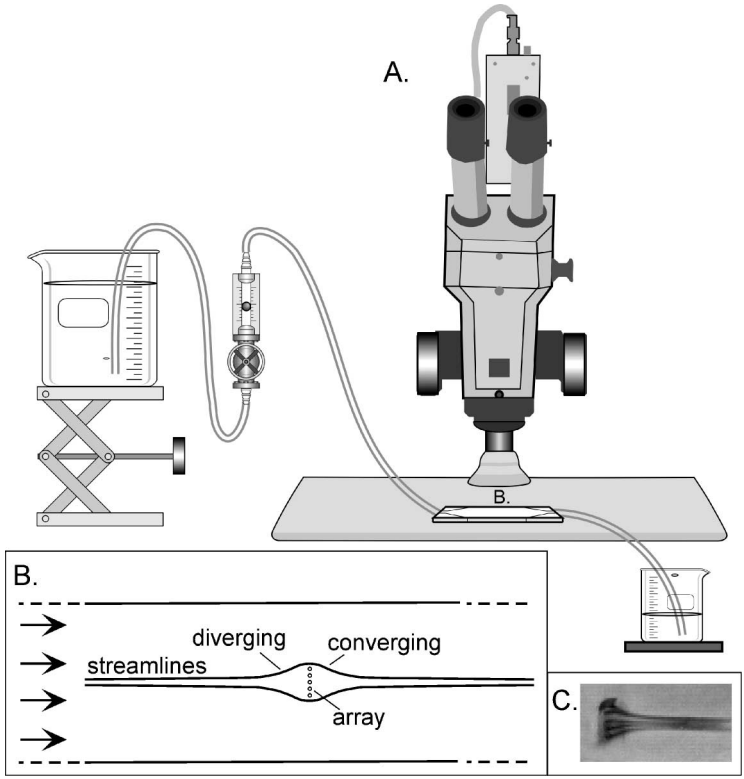

FIG. 2. Flow visualization technique: A—Experimental setup showing location of visualization chamber on microscope. B—Camera/microscope view of chamber showing array (5 parallel wires viewed end-on) and two hypothetical streamlines diverging upstream of the array (with symmetrical downstream convergence). C—Frame from videotape showing streaklines of marked thymol blue solution downstream of the array.

for viewing) (Figure 2). The array had five parallel wires (wire diam = 0.3 mm, length 3.0 mm, array width 4.28 mm). The platinum wires of the physical model were used as the anodes to mark the fluid at the array itself; a single platinum wire elsewhere in the chamber served as the cathode. The applied voltage was 1.7 V. This array was centered in a chamber of 3.0-mm height and 28-mm width (Figure 2A). Therefore, the wires completely spanned the height of the chamber to make the flow two-dimensional, and the array spanned 20% of the total cross-sectional area of the chamber. To generate higher resistance to flow (lower porosity) as a second treatment, a nylon mesh sheath was placed over the array of wires.

Gravity-driven flow of the solution through the chamber was controlled and measured by a flow meter (Manostat flow meter #2, New York). For each array

type, three different volumetric flow rates were tested, each corresponding to a different Reynolds number. For these Reynolds numbers, the flow was laminar (not turbulent). Three Reynolds numbers were used for each array type: 0.5, 1, and 3 (using chamber height, 3 mm, as characteristic length L). The volumetric flow rates were kept within 5% of the target flow rate. The complete range of fluid speeds inside the chamber ranged from 0.02 cm/sec to 0.138 cm/sec. The range of fluid temperatures was 19–23°C.

Physical Properties of Fluid Used for Flow Visualization

An aqueous solution of the pH indicator thymol blue (0.05 g thymol blue and 1.52 g NaCl in 500 ml water) was used to visualize the flow. This fluid can be "marked" electrolytically when titrated just below the pH at which it changes color from amber to blue (pH 8, Baker, 1966; Sparrow et al., 1970). We added salt (NaCl) to the original published recipe (only thymol blue in water) in order to increase the electrical conductivity of the solution and decrease the amount of hydrogen bubbles generated. The fluid was marked at the array of wires and the resulting streaklines in the chamber were videotaped (Panasonic WV-CL700 camera mounted on a Nikon Stemi SV6 dissecting microscope). The advantage of this flow visualization technique is that the marked fluid does not differ in physical properties from the unmarked fluid, and therefore will accurately represent its behavior. The marked fluid eventually reverts back to its original unmarked state; this change was imperceptible over the time period of our measurements. We tested this by repeatedly digitizing marked patches of fluid as the fluid moved downstream. The digitized areas did not change over the videotaping period (up to a minute). This was tested for three replicates for each of three different flow rates. The area of the marked fluid was not significantly affected by flow speed (ANCOVA, $P = 0.87$, $N = 9$) or distance downstream ($P = 0.98$).

The physical properties of the solution were determined within the range of fluid temperatures used in the experiments (19–23°C). The density of the solution was determined by weighing three 100 ml samples (in a 100 ml volumetric flask) at 20°C. The average density of the solution was 0.99923 ± 0.00056 g/ml (mean $\pm$ 1 SD, $N = 3$), 0.09% greater than pure water at that same temperature (0.99823 g/ml, Weast et al., 1988). Therefore, the density of pure water was used for the density of the thymol blue solution in all subsequent calculations. The viscosity of the solution was measured for three replicates at each of two temperatures (20°C and 25°C) using a Gilmont falling ball viscosimeter (Barnant Company, Barrington, IL) and it was always within 2% of the value reported for pure water (Weast et al., 1988). Comparable measurements performed on deionized water were of similar accuracy (within 3% of the published value for pure water). Therefore, the viscosity of pure water was used for the viscosity of the thymol blue solution in all subsequent calculations.

Video Analysis

Regular speed video (30 frames/sec) was digitized using motion analysis software (Motus, Peak Performance Inc., Englewood, CO). The distance between the two outermost streamlines ("width of marked fluid") was digitized at multiple locations (up to 18 locations; 3 replicates for each *Re* and resistance) downstream of the array at 1.2 or 0.25 mm intervals (the finer spatial scale was used for the less porous/higher resistance array). From these digitized points, we were able to calculate the change in width of the marked fluid as it moved farther downstream from the array. These widths were normalized to a 0–1 scale where "1" is the width of the marked fluid at the array for each porosity. When we were no longer able to measure further change in the width (successive widths differed by a pixel or less and no longer showed a monotonic trend), we considered the streamlines to have "converged" to a constant distance apart.

Statistical Analysis and Mathematical Manipulations

All statistics were performed with SAS software (version 8.2, SAS Institute Inc., Cary, NC). Exponential fits to the widths of the marked fluid (Figure 3) were calculated by using Excel's built-in exponential regression option after subtracting the constant asymptotic value (for large x). Streamlines around a sphere at low

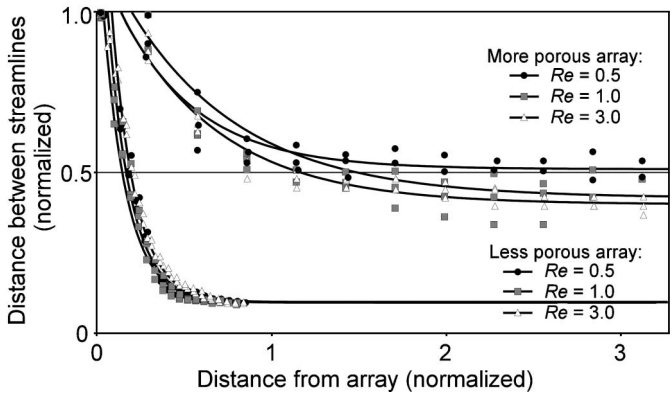

FIG. 3. Streamlines of the fluid that had passed through the array converged more rapidly downstream for the less porous array (with higher resistance to flow). The porosity (the normalized distance between streamlines far downstream of the array) did not differ within the Reynolds number range (0.5–3.0) for the less porous array, but decreased slightly with Reynolds number for the more porous array. Each symbol represents a single replicate, and the lines indicate best exponential fits to the data for each Reynolds number and array type. The distance between streamlines was normalized to the width of the marked fluid at the array, whereas the distance downstream was normalized to array width.

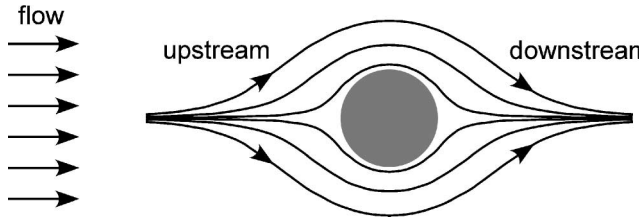

FIG. 4. Streamlines around a sphere at low Reynolds number (calculated from Stokes' equations) show the upstream/downstream symmetry expected for small bodies or in slow flow.

Reynolds number were calculated from Stokes' equations of the velocity flow field (Equation 3-214 in White, 1991) by a stepwise iterative simulation calculating the next position of a fluid particle from its current position and velocity vector (Figure 4).

RESULTS

Physical Modeling

The marked fluid downstream of the five-membered array (Figure 2C) was used to identify the locations of the streamlines of the fluid that had passed through the array (marked at the array elements). The distance between the two outermost streamlines decreased downstream of the array asymptotically to an approximately constant value (Figure 3). This value was compared to the distance between the same streamlines at the array. The streamline width ratio (width far downstream: width at array) was used to estimate the porosity of the array (the amount of approaching fluid that passes through the array). The average porosity of the high resistance array was $9.7 \pm 0.08\%$ (mean $\pm$ 1 SD, $N = 3$ *Re*) and the average porosity of the low resistance array was $44 \pm 6\%$ (mean $\pm$ 1 SD, $N = 3$ *Re*). The porosities were significantly different for the two different array resistances; the porosity of the low resistance array decreased slightly with increasing Reynolds number, whereas the porosity of the high resistance array was independent of Reynolds number for the range of parameters and resistances used (two-way ANOVA, $P < 0.001$ for array resistance, $P = 0.04$ for Reynolds number, $P = 0.04$ for the *Re* $\times$ resistance interaction term; $N = 3$ replicates for each of 3 Reynolds numbers for each resistance level).

Convergence took place within one array diameter downstream for the less porous array (entire *Re* range 0.5–3) and within two array diameters for the more porous array (entire *Re* range 0.5–3). That is, the distance between the outermost streamlines did not continue to decrease farther downstream.

DISCUSSION

It is not intuitive that a parcel of air approaching a pectinate antenna will be "stretched" in the plane perpendicular to flow, although this streamline divergence is mandated by the fluid mechanical principle of continuity. This divergence of the flow has important consequences for chemoreception by pectinate antennae because it will affect the spatial pattern with which the odorant molecules encounter the sensory hairs on an antenna. For pectinate antennae, the parcel of air will be stretched by a factor of $5x$–$10x$ because these antennae typically sample only about 10–20% of the approaching air. For example, a patch of odorant that is 1 mm^2 across (upstream) could be enlarged to 5–10 mm^2 across when it arrives at the sensory hairs. From the perspective of the sensory hairs, it appears as if the patch is magnified; i.e., a larger number of sensory hairs will encounter a patch of odorant than would be expected from the size of the patch upstream. Whether this stretching is two-dimensional (within the plane perpendicular to flow) vs. one-dimensional (along a single axis within that plane), is not known and could differ for antennae of different morphology. Note that simply changing the shape of an odorant patch (and not its volume) will not change the odorant concentration in that patch. The patch volume remains constant because as the patch is stretched in the plane perpendicular to flow, it will be shortened in the direction parallel to flow (Figure 1).

Over time, diffusion will reduce the differences in concentration between the odorant patches and the intervening clean air, making that air sample more homogeneous. The elongation and thinning of the patches that occurs during streamline divergence (e.g., Figure 1) will greatly increase the surface area of the patches and, therefore, the rate of homogenization of the air. If the streamline divergence is far upstream of an antenna, there will be more time for this diffusion-driven homogenization to occur, and the potential information in the filamentous odorant pattern could be lost or diminished far more rapidly. In addition, this homogenization may lower the concentration to a level below the detection threshold of the sensory array. To obtain an accurate picture of the extent to which a patchy or filamentous odorant pattern may linger or be obliterated, it is important to determine how far upstream of an antenna the streamline divergence will take place. The principle of continuity does not specify the location of streamline divergence, but only that it will occur.

It is reasonable, as a first approximation, to assume that flow around a slightly porous structure (such as a pectinate insect antenna) might resemble flow around a solid object with the same outer dimensions. The velocity flow field around a solid sphere at low Reynolds number is a classic solution in fluid mechanics (Stokes' equations, Equation 3-214 in White, 1991; see streamlines calculated from these equations in Figure 4). However, flow through or around porous structures may sometimes exhibit complex or unexpected behavior, such as the pulsatile flow

generated by vortex shedding of a cylindrical array for certain combinations of geometry and flow conditions (Leonard, 1992), or the "pathological behavior" of the mathematical solutions for low Reynolds number flow through porous pipes (referring to lack of solutions, or multiple sets of double solutions, p. 148, White, 1991). In low Reynolds number flow, objects may have surprisingly large velocity boundary layers or may "feel" other objects or walls from very far away (Happel and Brenner, 1965; Loudon et al., 1994). The lack of published detail on streamline divergence upstream of porous objects in low Reynolds number flow supplied the motivation for making direct estimates of streamlines using physical modeling.

Theoretically, the distance between diverging (or converging) streamlines around an isolated body in an unbounded medium (Figure 4) will continue to approach a limit indefinitely, making it impossible to define a location at which divergence starts. In practice, however, it is useful to consider when most (90%) of the divergence (or convergence) has occurred. This definition is similar to the recommendation made by Vogel (1994, 2003) to use 90% of free stream velocity for identifying the depth of the velocity boundary layer (rather than the 99% definition used by fluid mechanical engineers). For example, streamlines passing closely around a solid sphere at low *Re* ($Re < 1$, streamlines passing within 5% of the sphere's diameter), will have converged 90% within one radius downstream of the sphere's surface (Figure 4, innermost streamlines).

Results from the physical modeling make it clear that most of the divergence of the air is likely to take place immediately upstream of a pectinate antenna for biologically relevant antennal morphologies and air flows. Therefore, there will be little chance for the increased homogenization of filaments and clean air caused by far-upstream diverging flow and distorted odorant patches. A pectinate antenna has porosity more similar to the "less porous" array used for these measurements, and, therefore, the divergence of the air is expected within about one array diameter upstream.

The experimental Reynolds number range (0.5–3) corresponds to cases of extremely slow flow or very small antennae, such as 1-mm wide antennae in air flows of a few cm/sec (calculated from the formula for *Re* given above, using $\rho/\mu = 15 \times 10^{-6}$ m^2/sec). For larger Reynolds number flow (larger antennae or faster air flow), the upstream divergence is expected to be even closer to the antenna (within one antennal width). No evidence was seen for complex or unexpected flow behavior; the streamlines converged smoothly downstream in a manner similar to streamlines downstream from a solid sphere in low Reynolds number flow (compare Figures 3 and 4).

Some pectinate antennae are flat (planar) and are held perpendicular to the oncoming air, similar to our experimental array (e.g., luna moth antennae, *A. luna*), whereas others are curved or bent in a "V" shape, such as antennae from gypsy moths [*Lymantria dispar* (L.)] and silkworm moths (*B. mori*), and are held with the

concave "V" oriented approximately upstream. While details of the streamlines passing through these antennae remain unexplored, experiments with curved or tilted screens placed in a flow field show that the streamlines approaching a screen surface tend to bend such that the flow always passes perpendicularly through the screen (Elder, 1959; Lau and Baines, 1968; Koo and James, 1973). In an analogous manner, the streamlines flowing through a curved or bent pectinate antenna are likely to deflect and pass through the mesh-like structure at right angles.

A physical model will capture the essential flow behavior at the antennal level if its Reynolds number (based on the whole antenna) and porosity are matched by the model, and if its geometry is similar. Therefore, the divergence pattern reported here will be most closely approximated by the flow approaching flat pectinate antennae, and will be similar to the flow approaching curved or "V" shaped pectinate antennae. Whereas the flow through an antenna could be affected by the presence of nearby surfaces including the head of the insect, we do not expect the streamlines to diverge in a qualitatively different manner from that described here. Flow through an antenna positioned into the flow is less likely to be affected by the proximity of the head.

The results presented in this paper are of interest when the odorants in the environment are of a patchy distribution and where the patches are much smaller than the antennae. If the odorant concentration is uniform everywhere (which is approximately true when the patch size greatly exceeds the size of the antenna), any divergence or mixing (within the patch) will have no effect on the odorant concentration at the sensory hairs. Sensory hairs on pectinate antennae are individually innervated (Steinbrecht, 1987, 1999) but are so small and close to each other that spatial heterogeneity in the odorant concentration at their spatial scale will only be meaningful in fast air flows, such as those generated during flying; i.e., for slow air speeds, the "random walks" taken by individual odorant molecules passing between sensory hairs exceed the distance between adjacent hairs (Loudon and Koehl, 2000). There is behavioral evidence for the importance of small scale sampling (within an antenna) by species with filamentous antennae (Liu and Haynes, 1992; Baker et al., 1998) although this is not yet demonstrated for an insect with pectinate antennae. How filamentous antennae stretch or distort an odorant pattern is not known, but the work of Schneider et al. (1998) with the sexually dimorphic antennae from *Manduca sexta* (L.) suggests that this small difference in antennal morphology will change the way in which chemicals are sampled. It is clear that pectinate antennae sample a "magnified" piece of the olfactory landscape, without much homogenization of this magnified patch. The prediction that the temporal pattern of filament interception will remain unchanged has recently been tested and verified by electroantennography on *B. mori* and *L. dispar* antennae (Bau, et al., in review).

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EFFECT OF PBAN ON PHEROMONE PRODUCTION BY MATED *Heliothis virescens* AND *Heliothis subflexa* FEMALES

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Abstract—Mated female *Heliothis virescens* and *H. subflexa* were induced to produce sex pheromone during the photophase by injection of pheromone biosynthesis activating neuropeptide (PBAN). When injected with 1 pmol *Hez*-PBAN, the total amount of pheromone that could be extracted from glands of mated females during the photophase was similar to that extracted from virgin females in the scotophase. The PBAN-induced profile of pheromone components was compared between mated, PBAN-injected females and virgin females during spring and fall. Virgin females exhibited some differences in the relative composition of the pheromone blend between spring and fall, but no such temporal differences were detected in PBAN-injected, mated females. Because the temporal variation in pheromone blend composition was greater for virgin females than for PBAN-injected females, PBAN can be used to determine a female's native pheromone phenotype. This procedure has the advantages that pheromone glands can be extracted during the photophase, from mated females that have already oviposited.

Key Words—*Heliothis virescens*, *Heliothis subflexa*, sex pheromone, PBAN, temporal variation.

INTRODUCTION

The sex pheromones of many moths are 12–18 carbon unsaturated derivatives of fatty acids, with an oxygen-containing functional group. Species specificity of the pheromone signal is achieved by the presence of specific pheromone components,

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and the relative amounts of these components in the pheromone blend. For example, most noctuid moths in the genera *Heliothis* and *Helicoverpa* use (*Z*)-11-hexadecenal (Z11-16:Ald) as the major component of their pheromone blends, but vary the types and relative amounts of minor components (e.g., Roelofs et al., 1974; Tumlinson et al., 1975, 1982; Klun et al., 1980a,b, 1982; Teal et al., 1981, 1984; Pope et al., 1982, 1984; Ramaswamy et al., 1985; Vetter and Baker, 1984; Heath et al., 1991; Choi et al., 2002). The closely related *Heliothis virescens* (*Hv*) and *H. subflexa* (*Hs*) both produce small amounts of hexadecanal (16:Ald), (*Z*)-7-hexadecenal (Z7-16:Ald), and (*Z*)-11-hexadecenol (Z11-16:OH). In addition, *Hv* females produce tetradecanal (14:Ald) and (*Z*)-9-tetradecenal (Z9-14:Ald) that are generally not found in *Hs*, whereas *Hs* females produce (*Z*)-9-hexadecenal (Z9-16:Ald) and three acetates, (*Z*)-7-hexadecenyl acetate (*Z*7-16:OAc), (*Z*)-9-hexadecenyl acetate (*Z*9-16:OAc), and (*Z*)-11-hexadecenyl acetate (*Z*11-16:OAc). Whereas the acetates are completely absent in the glands of *Hv*, Z9-16:Ald can be found in trace quantities. Even though a number of studies have been conducted to determine the pheromone compositions of *Hv* (Roelofs et al., 1974; Tumlinson et al., 1975, 1982; Klun et al., 1980; Pope et al., 1982; Heath et al., 1991) and *Hs* (Teal et al., 1981; Tumlinson et al., 1982; Klun et al., 1982; Heath et al., 1991), the female pheromone component emission ratios and rates, and the relative importance of various blend components to males, have not been fully resolved (Vickers, 2002).

Well-characterized pheromone systems in closely related, interfertile congeners offer a powerful tool for studying the genetic bases of both pheromone production and male response to different pheromone components. Understanding the genetic mechanisms underlying sex pheromone production can in turn enhance our understanding of the evolution of divergent sexual communication systems (e.g., Löfstedt, 1990, 1993; Butlin, 1995; Linn and Roelofs, 1995; Phelan, 1997; Coyne and Orr, 1998). Teal and colleagues (Teal and Oostendorp, 1995a,b; Teal and Tumlinson, 1997) recognized that *Hv* and *Hs* offer such a model: these are closely related species that can be hybridized (Laster, 1972; Prosholt and LaChance, 1974; Sheck and Gould, 1993, 1995, 1996), although their pheromone compositions differ. Recently, we initiated studies aimed at identifying genes that regulate the species-specific pheromone profiles of *Hv* and *Hs*. By hybridizing these species, followed by backcrossing to one of the parents, genes from one species have been introgressed into the genetic background of the other species. We used amplified fragment length polymorphism (AFLP) analysis to genotype each female (Sheck et al., unpublished), while her pheromone phenotype was determined by chemical analysis of the pheromone gland. Central to this approach were the requirements that each female be sampled for both DNA and pheromone, and that she produce viable progeny for the next round of genetic crosses. However, this approach is confounded by the temporal dynamics of pheromone production: females must mate and oviposit to produce progeny, but pheromone production is reduced

dramatically after mating. Preliminary data showed that in some backcrosses, the glands of less than half of the females contained more than 50 ng of pheromone (A. Groot, unpublished data), even though the glands were extracted during the scotophase, when the pheromone titer is maximal. When gland extracts contained less than 50 ng of total pheromone, we found it impossible to accurately analyze the amount of the minor pheromone components.

Several approaches can be used to obtain pheromone before females mate, without interfering with subsequent mating and oviposition. These include (a) headspace collection and analysis, (b) solid phase microextraction (SPME) analysis, or (3) non-debilitating microdissection and extraction of a portion of a virgin female's pheromone gland. However, all are laborious, time-consuming, expensive, or preclude storage of many samples prior to pheromone analysis. Because pheromone biosynthesis activating neuropeptide (PBAN) stimulates sex pheromone production in female moths (Raina et al., 1989), it appeared promising for determining the pheromone phenotype of individual females by stimulating pheromone production *after* oviposition. Indeed, a common assay of its activity is to induce pheromone production during the photophase, when the pheromone titer is normally minimal in nocturnal moths (reviews: Rafaeli, 2002; Rafaeli and Jurenka, 2003). Furthermore, Teal et al. (1993) demonstrated that PBAN-injected *Hv* and *Hs* females were induced to produce sex pheromone during the photophase; Teal and Oostendorp (1995b) extended these observations to *Hv-Hs* hybrid females. However, only Z11-16:Ald, the major sex pheromone component, was analyzed by Teal and colleagues. Because our genetic analysis required quantification of all the pheromone components, it was important to establish whether PBAN elevated production of all pheromone components in naturally occurring ratios.

Several studies have examined variation of the pheromone blends in virgin *Hs* and *Hv* females. Pope et al. (1982) determined the temporal variation in the pheromone blend emitted by *Hv* throughout the night, and Heath et al. (1991) examined the periodicity of both pheromone gland content and its emission in both *Hv* and *Hs*. Both studies found variation in the alcohol/aldehyde ratios during the scotophase, and maximal pheromone titers and release between the 4th and 8th hour of the scotophase, although *Hs* seemed to produce maximum amounts of pheromone earlier in the scotophase than *Hv* (Heath et al., 1991). Pheromone production and composition may vary not only during the night, but also during the flight season. In this study, we aimed to determine whether the blend composition in PBAN-induced mated females at a given sampling time was similar to that found in untreated virgin females, and whether any differences between the two types of females were comparable in magnitude to differences across sampling times among virgin females. We compared the amount of pheromone produced, as well as the pheromone profile, in the glands of both *Hs* and *Hv* during two periods: spring and fall.

METHODS AND MATERIALS

Insects. Hv and *Hs* were reared on artificial diet as described in Sheck and Gould (1993, 1995). Pupae were separated by sex, and placed under a reversed light cycle (14L:10D, lights off from 04:00 to 14:00). Newly eclosed adults were collected daily and placed in plastic containers (diameter 11 cm, height 8 cm) with sugar water.

Pheromone Gland Extractions: Virgin Females. In April and May, 2002 (denoted "spring treatment"), the glands of 14 *Hs* and 7 *Hv* females were dissected 2–4 d after eclosion and 4–7 hr into the scotophase. The glands were placed in conical vials containing 50 μl hexane and 20 ng of 1-pentadecanol acetate as internal standard. After 20–60 min, the glands were removed and the extracts were stored at -20°C until analysis. Variation of pheromone composition was evaluated by repeating these extractions in September and October 2002 (denoted "fall treatment"), during which glands of 12 *Hs* females and 18 *Hv* females were dissected in a similar way.

PBAN-Injections and Pheromone Gland Extractions: Mated Females. Upon eclosion, single *Hs* and *Hv* females were confined with conspecific males. The mating buckets were checked daily for oviposition, and females were allowed an additional 4 d to oviposit before they were injected with PBAN. Males were kept in the mating buckets the entire period, which allowed for multiple matings.

A stock solution of *Hez*-PBAN (Peninsula Laboratories, San Carlos, CA) (200 pmol/μl in 50% methanol and 1 N HCl) was diluted in saline (Kurtti and Brooks, 1976) within 24 hr of injection to 1pmol/μl. Females were injected during the photophase with 1 pmol PBAN in 1 μl, using a 10 μl syringe (Hamilton, Reno, NV) with a 31 gauge needle that was inserted ventrally between the 8th and the 9th abdominal segments. One hr after injection, the pheromone glands were dissected and extracted as described for virgin females. This procedure was conducted in the spring and in the fall, in the same periods as the virgin females. A total of 37 mated *Hs* females were subjected to this procedure (23 and 14 in the spring and fall, respectively), while a total of 23 mated *Hv* females were used and analyzed (9 and 14 in spring and fall, respectively).

Gas Chromatography (GC) and GC-Mass Spectrometry (MS). The hexane extracts were reduced to 0.5–1.5 μl under a gentle stream of N_2. Each sample was injected into a pulsed splitless injector held at 240°C in an HP6890 GC (Agilent, Palo Alto, CA), and separated using a 30 m $\times$ 0.25 mm $\times$ 0.5 μm Stabilwax column (Restek, Bellefonte, PA) programmed from 60°C (with a 2 min hold) to 180°C at 30°C/min, then to 230°C at 2°C/min, during which all the pheromone components eluted. The column was then heated to 245°C at 20°C/min and held at 245°C for 5 min to clean it before the next analysis. The FID detector was held at 240°C. The amount of each pheromone component was calculated relative to the 20 ng of internal standard. The components we quantified were: 14:Ald, Z9-14:Ald, 16:Ald,

Z7-16:Ald, Z9-16:Ald, Z11-16:Ald, Z7-16:OAc, Z9-16:OAc, Z11-16:OAc, and Z11-16:OH. Most chromatographic analyses did not separate Z7-16:Ald from Z9-16:Ald. Therefore, we combined the peak areas of these compounds and denoted the combination as Z7/9-16:Ald, even though Z7-16:Ald is present only in low amounts in both species, and Z9-16:Ald is present in relatively large amounts in *Hs*.

The chemical identities of all pheromone components were confirmed by GC-MS. Aliquots of these extracts were injected into an HP5890 GC, and separated using a 30 m $\times$ 0.25 $\times$ 0.5 μm DB-Wax column or a DB-225 column (both from J&W Scientific, Folsom, CA) temperature programmed from 60°C (with a 1 min hold) to 230°C at 10°/min and held for 10 min. The eluted compounds were detected with an HP5972 mass selective detector, and mass spectra were compared to those of known standards injected in the same manner. Further GC-MS analysis of the 14-carbon aldehydes was conducted on a G1800A GCD (Agilent, Palo Alto, CA) equipped with a 60 m $\times$ 0.25 mm $\times$ 0.25 μm DB-23 column, programmed from 130°C (1.5 min hold) to 170°C at 6.5°/min, then to 210°C at 2.5°/min, and to 240°C at 10°/min, followed by an 11 min hold.

Statistical Analyses. First, the total amount of pheromone extracted from the pheromone glands of virgin and PBAN-injected mated *Hs* and *Hv* at the two sampling periods was compared between species, treatments (virgin vs. PBAN-injected, mated females) and periods (spring vs. fall). The total amounts of pheromone were log-transformed and subjected to 3-factor analysis of variance (ANOVA) in which the factors species, treatment, and period were treated as fixed. ANOVA was carried out using the GLM procedure of SAS (SAS, 2002). Specific comparisons between virgin females and PBAN-injected mated females were carried out as appropriate, depending on the presence of interaction between treatments and the other two factors. To test for differences in variability among individuals in the total pheromone amounts, a generalization of the Lev1:med test (Conover et al., 1981; Boos and Brownie, 1989) was used to test for main and interaction effects of the factors species, treatment, and period, on the measure of variation corresponding to the average absolute deviation from the median. This test is robust to non-normality of the data, and is essentially a 3-factor ANOVA on $Z_{ij} = |Y_{ij} - median_i|$, where Y_{ij} is the total pheromone for the jth individual in the ith group, and $median_i$ is the median of the ith group, a group being one of the eight species-treatment-period combinations.

Second, the impact of PBAN on ratios of pheromone components was tested. The relative percentage of each of the components was calculated by setting the total amount of all pheromone components to 100%. The three C_{16} acetates were summed and treated as one component, the sum of acetates, to reduce the number of comparisons. All values were then log-transformed to stabilize the variance. When we compared *Hv* with *Hs*, large differences were found between the means and the variances of most pheromone components. Therefore, only intraspecific differences were analyzed further. Within each species, the following comparisons

were made, using multivariate ANOVA (MANOVA): (a) Differences in overall pheromone composition between treatments, (b) Differences in overall pheromone composition between periods, and (c) Interaction effects between treatments and periods. These overall comparisons were followed by a univariate ANOVA for each component separately, with separation of means using Tukey's adjustment for multiple comparisons. Comparisons between virgin females and PBAN-injected mated females in the same period were of primary interest, but comparisons across periods were also of interest to provide information about the magnitude of temporal differences.

RESULTS

Our GC and GC-MS results confirmed the presence of 14:Ald and Z9-14:Ald in *Hs* females (data not shown). The retention times of each of these two compounds on both polar (Stabilwax) and non-polar (HP-1) GC columns were coincident with the retention times of the respective authentic sample. The parent ions of 14:Ald and Z9-14:Ald were not detected, but for 14:Ald, *m/z* 194 ($M^+ - 18$) and 168 ($M^+ - 44$) were diagnostic. The mass spectrum of Z9-14:Ald showed $M^+ - 18$ at *m/z* 192, which is characteristic of C_{14} monounsaturated aldehydes. Based on these results, together with the earlier identification by Klun et al. (1982), we are confident that both 14:Ald and Z9-14:Ald were present in *Hs* females.

Injection of 1 pmol of *Hez*-PBAN into mated, post-oviposition *Hs* and *Hv* females during the photophase resulted in a total amount of extractable pheromone in the range of that found in virgin females during the scotophase (Table 1). The only significant effects were the Species × Period and Species × Treatment × Period interactions ($P = 0.004$ and $P < 0.001$, respectively). Pheromone glands of virgin *Hs* females in the spring contained significantly more pheromone than those of PBAN injected, mated *Hs* females in the same period, but the difference between virgin *Hs* females in the spring and fall (i.e., a temporal effect) was of similar magnitude to the treatment difference in the spring (Table 1). In contrast to the significant temporal effect for virgin *Hs* females, the PBAN-induced pheromone titers in mated females of both species were not influenced by the sampling period. More importantly, the amount of pheromone extracted during the photophase from glands of mated, post-oviposition females injected with PBAN was sufficient, i.e., $\gg$50 ng, for accurate analyses of pheromone blend composition.

Analysis of absolute deviations from the median using the Lev1:med test (Conover et al., 1981; Boos and Brownie, 1989) yielded a marginally significant ($P = 0.058$) treatment main effect. Mean absolute deviations presented in Table 2 suggest that variation in the amount of pheromone produced among females of the same species within a period was higher in virgin females than in PBAN-induced mated females. Values in Table 1 for the coefficient of variation also suggest that PBAN reduced variation in pheromone titers.

TABLE 1. AMOUNT OF PHEROMONE EXTRACTED FROM THE GLANDS OF VIRGIN FEMALES, AND MATED FEMALES INJECTED WITH 1 PMOL PBAN AFTER THEY HAD OVIPOSITED FOR 4 DAYS[a]

Source	Period	*N*	Total pheromone per female (ng), mean ± SEM (CV)	
H. subflexa	Spring	13	Virgin	414 ± 72 (63) **a**
		23	Mated + PBAN	201 ± 21 (50) **b**
	Fall	13	Virgin	166 ± 41 (90) **b**
		14	Mated + PBAN	231 ± 28 (46) **ab**
H. virescens	Spring	7	Virgin	196 ± 44 (59) **A**
		9	Mated + PBAN	260 ± 37 (43) **A**
	Fall	18	Virgin	317 ± 46 (61) **A**
		14	Mated + PBAN	279 ± 35 (47) **A**

[a]Within a species, means without a letter in common differ significantly ($P < 0.05$), using Tukey's procedure for multiple comparisons on log-transformed total amounts. ng: nanogram, SEM: Standard error of the mean, CV: Coefficient of variation.

When comparing the overall profiles per species using MANOVA, a significant difference was found between virgin females and PBAN-injected mated females, averaged over periods, for both *Hv* and *Hs* (Table 3). Overall pheromone profile differences between periods were not found in *Hv*, but were significant in *Hs*. Overall interaction effects between treatment and periods were found for both *Hv* and *Hs* (Table 3).

To determine which components contributed to these overall differences, results of separate analyses on each component are presented in Table 3. In *Hv*, significant treatment main effects were found for 14:Ald and Z11-16:OH (see Table 3 and Figure 1). Examining the means for these components showed that,

TABLE 2. VARIATION, MEASURED BY THE MEAN ABSOLUTE DEVIATION FROM THE MEDIAN, IN TOTAL PHEROMONE AMOUNTS EXTRACTED FROM THE GLANDS OF VIRGIN FEMALES, AND MATED FEMALES INJECTED WITH 1 PMOL PBAN AFTER THEY HAD OVIPOSITED FOR 4 DAYS

Source	Period	*N*	*z* mean ± SEM[a] (ng)	
H. subflexa	Spring	13	Virgin	175 ± 53
		23	Mated + PBAN	82 ± 13
	Fall	13	Virgin	103 ± 39
		14	Mated + PBAN	84 ± 16
H.virescens	Spring	7	Virgin	91 ± 30
		9	Mated + PBAN	75 ± 26
	Fall	18	Virgin	147 ± 29
		14	Mated + PBAN	107 ± 18

[a]Mean and SEM of $Z_{ij} = |Y_{ij} - \text{median}_i|$, or the absolute deviation from the median, a measure of variation within a group of the total amount of pheromone extracted (Conover et al., 1989; Boos and Brownie, 1991). See text for further explanation.

TABLE 3. MANOVA PER SPECIES TO TEST FOR OVERALL[a] DIFFERENCES IN PHEROMONE COMPOSITION, FOLLOWED BY UNIVARIATE ANALYSES FOR EACH OF THE COMPONENTS (EXPRESSED AS A PERCENTAGE OF TOTAL PHEROMONE AMOUNT). *P*-VALUES ARE REPORTED FOR TREATMENTS, PERIODS, AND INTERACTION EFFECTS

	H. subflexa			*H. virescens*		
	Treatment	Periods	Treatment × Period	Treatment	Periods	Treatment × Period
MANOVA	<0.001	<0.001	0.001	<0.001	0.30	0.035
Univariate tests						
Compound[b]						
14:Ald	<0.001	0.002	ns	<0.001	ns	0.033
Z9-14:Ald	ns	ns	0.001	ns	ns	ns
16:Ald	ns	<0.001	0.001	ns	ns	ns
Z7/9-16:Ald	0.034	0.020	ns	ns	ns	ns
Z11-16:Ald	0.040	0.007	ns	ns	ns	0.012
Acetates[c]	0.017	0.030	ns	—	—	—
Z11-16:OH	ns	0.023	ns	0.011	0.017	0.009

Note. ns: $P > 0.05$.
[a] Comparing pheromone profiles as a whole, instead of per compound.
[b] All analyses were carried out on log-transformed values.
[c] Absent in *Hv* females.

although statistically significant, the difference in the relative percentages was small for 14:Ald, i.e., 0.8%. The difference in Z11-16:OH between virgin and PBAN-injected *Hv* was bigger (6.7%), which is due to the relatively high amount of Z11-16:OH in virgin *Hv* females in spring.

In *Hs,* significant differences were found for all components except Z11-16:OH (Table 3). The percentages of 14:Ald, 16:Ald, and Z11-16:Ald were significantly lower in fall than in spring, whereas the percentages of Z9-14:Ald and Z7/9-16:Ald were significantly higher in fall than in spring (see Figure 1). Also, significant differences were found between virgin *Hs* females and PBAN-injected, mated *Hs* females (Table 3). Despite these differences, the relative percentages of the different compounds found in virgin or PBAN-injected mated *Hs* females overlapped in at least one of the periods (Figure 1).

DISCUSSION

Our results demonstrate that mated *Hs* and *Hv* females injected with 1 pmol PBAN during photophase produced pheromone at levels and ratios comparable to those produced by virgin females during the scotophase. It is likely, based on dose-response studies (Raina et al., 1989; Abernathy et al., 1996), that injection of more PBAN would have stimulated mated females to produce more pheromone.

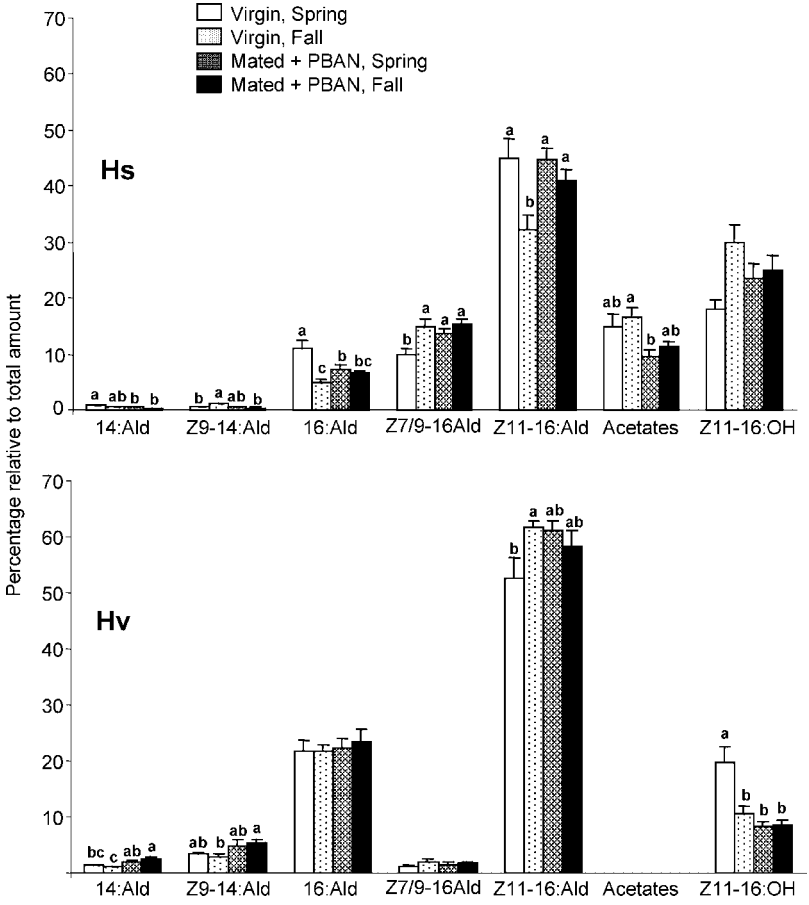

FIG. 1. Pheromone profiles (mean ±SEM) of *H. subflexa* (***Hs***, upper graph) and *H. virescens* (***Hv***, lower graph) when the relative abundance of each component is calculated as a percentage of the total amount of pheromone. Different letters within each component indicate significant differences ($P < 0.05$), using a Tukey adjustment for multiple comparisons. No letters: not significant. See text for further explanation.

Our results also demonstrate that PBAN-induced mated females produce and accumulate pheromone of comparable blend quality to that of virgin females. This is not surprising given our current understanding of the enzymatic steps affected by PBAN. In most species, including *H. zea*, PBAN appears to affect steps in or prior to fatty acid synthesis (e.g., Jurenka et al., 1991; Jurenka, 1996; Teal et al., 1996; reviews: Tillman et al., 1999; Rafaeli, 2002; Rafaeli and Jurenka, 2003). In

some species, reduction of the fatty acyl moiety to alcohol appears to be controlled by PBAN (Arima et al., 1991; Ozawa et al., 1993; Fabrias et al., 1994). One study using *Sesamia nonagrioides* (Mas et al., 2000) found an activation by PBAN of acetyl transferase, converting alcohols to acetates. In heliothines, it appears that PBAN controls either the biosynthesis of fatty acids, their reduction, or both.

Pheromone composition is usually reported either relative to the mass of the major component (here, Z11-16:Ald), or as a percentage of each component relative to the total mass of pheromone recovered. The first approach, expressing the secondary components as a percentage of the main component, may facilitate comparisons between species that differ in the number of secondary components. The second approach, expressing all components as a percentage of the total amount of pheromone produced, can show intraspecific treatment effects on the main component. We calculated and analyzed the relative amounts of the different pheromone components in both ways. Because similar trends emerged, we have shown only the pheromone profiles in which all components are expressed as a percentage of the total amount of pheromone produced.

The fact that we found statistically significant differences in the relative amount of 14:Ald produced in both species may not be biologically significant, because the quantities produced were very low. The overall significant difference in Z11-16:OH between virgin *Hv* and PBAN-induced mated *Hv* females, and the overall significant difference in the amount of acetates produced between virgin *Hs* and PBAN-injected mated *Hs* females, are harder to explain. However, the difference in relative percentages of these components between virgin females and PBAN-induced mated females was no greater than the difference between periods (i.e., temporal changes) for virgin females of the same species.

We did not find any of the acetates in *Hv* females, in agreement with previous analyses of *Hv* (Roelofs et al., 1974; Tumlinson et al., 1975, 1982; Klun et al., 1980; Pope et al., 1982; Heath et al., 1991). However, acetate esterases, which convert acetates to alcohols (Bjostad and Roelofs, 1983; Roelofs and Wolf, 1988; Wolf and Roelofs, 1989), appear to be present in both *Hv* and *Hs*. When Teal et al. (1989) applied acetate esters to the surface of the pheromone glands of both *Hs* and *Hv* females, they found more of the corresponding alcohols and aldehydes in *Hv* than in *Hs*. Whether acetyl transferases that convert alcohols to acetate esters (Bjostad and Roelofs, 1983; Morse and Meighen, 1986) are present in *Hv* as well remains to be determined.

Both species had Z11-16:OH, but it was present in much higher amounts in *Hs* than in *Hv*. In *Hs*, this alcohol has been found to be an essential component for the attraction of conspecific males (Heath et al., 1991; Vickers, 2002), whereas *Hv* females apparently do not emit this compound (Teal et al., 1986; Heath et al., 1991).

We confirmed an earlier report by Klun et al. (1982) that 14:Ald and Z9-14:Ald were present in *Hs* females. Because other studies did not find these compounds in *Hs* (Teal et al., 1981; Tumlinson et al., 1982; Heath et al., 1991),

it was questionable whether they were produced by *Hs* at all (Vickers, 2002). Not only did we consistently find both compounds in both virgin and PBAN-injected mated *Hs* females, but the percentage of 14:Ald relative to the main component was similar to that found in virgin *Hv* females. In *Hv,* these compounds, especially Z9-14:Ald, are essential for attracting conspecific males (Vickers et al., 1991). *Hs* males do not seem to be attracted or repelled by Z9-14:Ald (Vickers, 2002), although receptor cells responding to Z9-16:Ald also respond to Z9-14:Ald in *Hs* (Baker et al., 2004).

Z7/9-16:Ald were almost absent in *Hv*, but present in relatively large amounts in *Hs*. Z9-16:Ald is one of the major pheromone components that provides species-specificity; *Hs* males are not attracted to a blend lacking this component (Vickers, 2002), whereas attraction of *Hv* males is independent of the presence or absence of Z9-16:Ald (Tumlinson et al., 1982; Teal et al., 1986; Vickers et al., 1991). Z9-16:Ald can be formed through Δ9-desaturase acting on the 16:acid, or through Δ11-desaturase acting on the 18:acid (e.g., Choi et al., 2002). A Δ11-desaturase is present in both species, because it is involved in formation of the major pheromone component, Z11-16:Ald (Jurenka, 1996; Choi et al., 2002). We hypothesize that in *Hv,* low amounts of Z9-16:Ald are formed through Δ11-desaturase acting on the 18:acid, whereas *Hs* females possess a Δ9-desaturase which allows them to produce Z9-16:Ald through 16:acid as well. We are currently investigating whether *Hv* and *Hs* differ in how the Δ9-components are produced.

Because there was considerable similarity in blend composition between virgin and PBAN-injected mated females, we conclude that PBAN can be used in determining ratios of pheromone components in backcross females. PBAN injection has several major advantages over other possible methods, because glands can be extracted during the photophase, from relatively old (>6 d old) and/or mated females, which makes this method time- and age-independent.

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EFFECT OF REARING CONDITIONS ON PRODUCTION OF STERNAL GLAND SECRETION, AND IDENTIFICATION OF MINOR COMPONENTS IN THE STERNAL GLAND SECRETION OF THE PREDATORY STINK BUG *Eocanthecona furcellata*

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Abstract—Production of the male specific compound, 6,10,13-trimethyltetradecyl isovalerate by the predatory stink bug *Eocanthecona furcellata* (Wolff) was dramatically affected by rearing conditions. Male bugs kept isolated after eclosion produced an average of 1,948 ng of 6,10,13-trimethyltetradecyl isovalerate per bug, whereas male bugs reared in groups of 5–8 bugs produced an average of only 4 ng of 6,10,13-trimethyltetradecyl isovalerate per bug. Same-sex or mixed-sex pairs of bugs produced less than 50 ng per bug. Male bugs kept isolated for 1 wk and then grouped for 1 wk produced 3 ng of 6,10,13-trimethyltetradecyl isovalerate per bug, whereas male bugs grouped first and then isolated produced 135 ng of 6,10,13-trimethyltetradecyl isovalerate. A total of 11 minor components in relative amounts of less than 1% of the major 6,10,13-trimethyltetradecyl isovalerate were found in the sternal gland secretion. These included 6,10,13-trimethyltetradecanol, acetate, propionate, and butyrate esters of 6,10,13-trimethyltetradecanol, and isovalerate or valerate esters of homologs of 6,10,13-trimethyltetradecanol.

Key Words—*Eocanthecona furcellata*, Pentatomidae, 6,10,13-trimethyltetradecyl isovalerate, 6,10,13-trimethyltetradecyl acetate, 6,10,13-trimethyltetradecyl propionate, 6,10,13-trimethyltetradecyl butyrate, sternal gland, rearing conditions.

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INTRODUCTION

In the study of semiochemicals from the predatory stink bug *Eocanthecona furcellata* (Wolff), 6,10,13-trimethyltetradecyl isovalerate extracted from sternal glands (SG) was found to be present in only 5% of mature virgin male bugs (Ho et al., 2003). In a related species, *Perillus bioculatus* (Fabricius), production of 6,10,13-trimethyltetradecyl isovalerate as an attractant pheromone was highest when males were starved first and then fed. It was proposed that stimulation of isovalerate production after feeding occurred in order to attract conspecifics to food sources (Aldrich et al., 1986). In another report (Aldrich and Lusby, 1986), a subset of male bugs of *Mineus strigipes* Herrich–Schaeffer and *Oplomus severus* Breddin with SG setae never produced a detectable amount of secretion, but starvation for 2–3 d after ecdysis followed by feeding was the most effective way of stimulating secretion.

In our initial attempts to obtain enough of the SG secretion from *E. furcellata* for bioassays, starving followed by feeding failed to stimulate the secretion of SG compounds from *E. furcellata*. Because 6,10,13-trimethyl-1-tetradecanol from the SG of *Stiretrus anchorago* (Fabricius) was found to be an aggregation pheromone (Kochansky et al., 1989), and SG of males of the male predatory stink bug *E. furcellata* produce compounds similar to that of *S. anchorago*, we hypothesized that the SG secretion of *E. furcellata* may be an aggregation pheromone as well. We further postulated that isolated bugs should produce more aggregation pheromone than bugs in groups because isolated bugs would use the chemical to attract conspecifics. The male-specific SG secretion may also have a role as a sex pheromone to attract female bugs. Thus, we hypothesized that male bugs grouped with females should produce minimal SG secretion to attract the opposite sex, whereas males grouped with males would still produce more SG secretion.

In this report, the amount of 6,10,13-trimethyltetradecyl isovalerate from SGs of virgin male bugs reared under different conditions, including being reared in isolation or in groups, and being reared in single or mixed sex pairs, was quantified and compared. Furthermore, because relatively large amounts of SG secretion could be collected from male bugs reared in isolation, other minor compounds in the SG secretion, mainly homologs of the major compound—6,10,13-trimethyltetradecyl isovalerate, were also identified.

METHODS AND MATERIALS

Insects. Bugs were reared on blowfly larvae, *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) in the laboratory under a 14:10 L:D regime at 23 $\pm$ 3°C and 70% relative humidity. The bugs were fed on alternate days *ad libitum*. Blowfly larvae were purchased from a fishing supply store. The colony was

maintained as described earlier in the literature (Chu and Chu, 1975a,b). Nymphs were kept in transparent plastic cups of 7 cm height $\times$13 cm diam. cup in groups of 10 bugs per cup. Adults were kept under different conditions as described later in this paper. The cups were covered with cheesecloth and a sugar–water impregnated cotton ball was placed at the top of the cup as the water source for the bugs.

Bugs Under Different Conditions. For the group-reared treatment, adults of the same sex were kept in groups of 5–8 bugs in 7 cm height $\times$13 cm diam. cup containers after eclosion. For the isolated rearing treatment, adults were kept individually in 250 ml transparent ice-cream cups after eclosion. For tests with pairs of bugs, two bugs, either both males of the same age or one male and one female of the same age, were kept in 250 ml ice-cream cups after eclosion. All these bugs were analyzed for the contents of the SG secretions 2 wk after eclosion.

To test the effects of grouping on previously isolated bugs, five bugs were kept individually for 1 wk and then brought together in a 7 cm height $\times$13 cm diam. cup for 1 wk before the extraction of SG compounds. For the converse test of grouped and then isolated adults, the bugs were kept in groups of five in the 7 cm height $\times$13 cm diam. cup for 1 wk, and then isolated individually in the 250 ml ice cream cups for 1 wk.

Extraction of Sternal Gland Contents of Adults. The bugs from various treatments were anesthetized with CO_2, and the ventral surface was washed with 40 μl of hexane. The extracts were analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). The amount of 6,10,13-trimethyltetradecyl isovalerate was quantified vs. benzophenone as an internal standard as described by Ho et al. (2003).

Chemical Analysis. The extracts were analyzed by splitless coupled GC–MS with a Thermo Quest Trace GC, interfaced with a Finnigan Trace MS (electron impact ionization, 70 eV). The GC was held at 60°C for 1 min, then programmed at 10°C/min to 300°C, with injector and transfer line temperatures of 200 and 250°C, respectively. A DB-1 column (30 m $\times$ 0.32 mm ID, J & W Scientific, Folsom, CA) was used in the GC–MS analyses with helium carrier gas.

Hydrolysis of 6,10,13-Trimethyltetradecyl Isovalerate. Ten micrograms of 6,10,13-trimethyltetradecyl isovalerate (authentic sample from Dr. J. R. Aldrich) were hydrolyzed in 200 μl of ethanol and 20 μl of 1N NaOH at 50°C for 8 hr. The hydrolyzed product, 6,10,13-trimethyltetradecanol, was extracted into hexane and analyzed by GC-MS, then used for esterification as described below.

Formation of Esters of 6,10,13-Trimethyltetradecanol. Acetyl chloride (50 μl) was added to a solution of 6,10,13-trimethyltetradecanol (1 μg) in hexane at 0°C. After 5 min, water was added to the reaction mixture, and the product was extracted with hexane. After filtration through anhydrous $MgSO_4$, the extract was concentrated for GC-MS analysis. Analogous procedures were used to esterify aliquots of the 6,10,13-trimethyltetradecanol solution with propionyl chloride, isobutyryl chloride, butyryl chloride, and valeryl chloride, respectively.

TABLE 1. AMOUNT OF 6,10,13-TRIMETHYLTETRADECYL ISOVALERATE FOUND IN THE SG OF VIRGIN MALE *E. furcellata* REARED UNDER DIFFERENT CONDITIONS. PAIRS OF TREATMENTS WERE COMPARED WITH *t*-TESTS

Rearing condition	Amount of 6,10,13-trimethyltetradecyl isovalerate (ng) (mean ± SE)	*P*
Grouped	4 ± 3 ($N = 20$)	$P < 0.001$
Isolated	1948 ± 525 ($N = 27$)	
Grouped first, then isolated	135 ± 109 ($N = 24$)	$P = 0.07$
Isolated first, then grouped	3 ± 3 ($N = 19$)	
Paired with another male	22 ± 13 ($N = 12$)	$P = 0.57$
Paired with another female	48 ± 43 ($N = 15$)	

RESULTS

Amounts of Isovalerate from Bugs Reared under Different Conditions. The amounts of 6,10,13-trimethyltetradecyl isovalerate produced by male bugs reared in isolation, in pairs, or in groups are shown in Table 1. Almost 500 times as much isovalerate was extracted from bugs reared in isolation as compared to bugs reared in groups (*t*-test, $P < 0.001$). Although the mean amount of isovalerate that was produced by bugs that were grouped first and then isolated was more than 40 times greater than the mean amount produced by bugs that were isolated first and then grouped, the two groups were not significantly different ($P = 0.07$), probably due to the extreme variability in the amounts produced per bug. There was also no difference in the mean amount of isovalerate recovered from males paired with other males or with females ($P = 0.57$).

Identification of Homologs of 6,10,13-Trimethyltetradecyl Isovalerate. Several compounds with mass spectra similar to that of 6,10,13-trimethyltetradecyl isovalerate were found in the SG extracts. Spectral data, possible structures, percentage relative to the most abundant compound, 6,10,13-trimethyltetradecyl isovalerate, and Kovats retention indices are listed in Table 2, and a chromatogram of the gland extract is shown in Figure 1. Detailed identification data are presented in subsequent paragraphs.

Peak 1: The compound gave a base peak of m/z 97, a molecular ion at m/z 256, and an M-18 peak (m/z 238), very similar to the mass spectrum of 6,10,13-trimethyltetradecanol in the SG secretion of *Oplomus dichrous* (Aldrich and Lusby, 1986). The mass spectrum and GC retention time matched those of an authentic standard generated by hydrolysis of 6,10,13-trimethyltetradecyl isovalerate, confirming the structure.

Peak 2: The mass spectrum of this compound was similar to that of 6,10,13-trimethyltetradecyl isovalerate, but its GC retention time was shorter. A prominent fragment at m/z 61 (McLafferty, 1980) suggested that it might be the acetate analog

TABLE 2. RELATIVE PERCENTAGES AND MASS SPECTRAL DATA OF COMPOUNDS IN THE SG SECRETION OF MALE *E. furcellata*. DIAGNOSTIC IONS ARE SHOWN IN BOLD

Peak No.[a]	Retention time (min)	Compound	Relative percentage[b]	Mass spectral data	Kovats index[c]
1	15.35	6,10,13-trimethyltetradecanol	0.12 ± 0.03	57 (96), 69 (62), 83 (33), **97 (100)**, 111 (19), 126 (14), 139 (1.6), 168 (1), 182 (3), 210 (0.1), **238 (1), 256 (0.01, M^+)**	1817
2	16.58	6,10,13-trimethyltetradecyl acetate	0.16 ± 0.08	43 (100), 57 (97), **61 (21)**, 69 (53), 83 (29), 97 (76), 111 (18), 126 (9), 182 (3), 213 (3), **256 (1), 298 (0.03, M^+)**	1940
3	17.33	IVb - 2 CH_2	0.04 ± 0.02	57 (100), 69 (44), 85 (68), **97 (88), 103 (78)**, 112 (13), 168 (2), 182 (2), **255 (0.3), 312 (0.3, M^+)**	2016
4	17.45	6,10,13-trimethyltetradecyl propionate	0.38 ± 0.09	57 (100), 69 (32), **75 (30), 97 (54)**, 111 (13), 126 (6), 154 (1.3), 182 (2.3), 210 (0.2), 238 (0.07), **283 (0.41), 312 (0.02, M^+)**	2028
5	18.17	IVa - CH_2	0.66 ± 0.07	57 (100), 69 (44), 85 (75), **97 (80), 103 (77)**, 111 (14), 126 (5), 168 (2), 182 (1), 196 (1), 224 (1), **269 (1), 326 (trace, M^+)**	2101
6	18.24	6,10,13-trimethyltetradecyl butyrate	0.06 ± 0.02	57 (100), 71 (69), **89 (60), 97 (93)**, 111 (18), 126 (8), 182 (3), 207 (0.29), **326 (0.02, M^+)**	2110
7	18.32	IVb - CH_2	0.18 ± 0.05	57 (100), 69 (42), 85 (52), **97 (75), 103 (78)**, 111 (11), 168 (2), 182 (0.13), 196 (0.75), 224 (0.6), **269 (0.7), 326 (0.03, M^+)**	2120
IV	18.72	6,10,13-trimethyltetradecyl isovalerate	100	57 (88), 69 (44), 85 (45), **97 (100), 103 (64)**, 111 (46), 126 (25), 182 (11), 210 (1), 238 (1), **283 (2), 325 (0.26), 340 (0.02, M^+)**	2163
8	19.01	IV isomer (IVa)	1.03 ± 0.07	57 (100), 69 (36), 85 (41), **97 (69), 103 (58)**, 111 (12), 126 (5), 182 (1.3), 210 (0.6), 238 (0.5), **283 (0.6), 340 (0.03, M^+)**	2205
9	19.13	IV isomer (IVb)	0.08 ± 0.02	57 (100), 69 (49), 85 (62), **97 (82), 103 (78)**, 111 (15), 126 (4), 182 (1), 210 (0.6), 238 (0.4), **283 (1.2), 340 (0.43, M^+)**	2217
10	19.51	IV + CH_2	0.02 ± 0.00	57(100), 71 (32), 85 (37), **97 (50), 103 (68)**, 111 (21), 126 (11), 182 (3), 196 (1), 252 (0.6), **297 (0.4), 354(trace, M^+)**	2263
11	19.82	IVa + CH_2	0.34 ± 0.05	57 (100), 71 (42), 85 (58), **97 (57), 103 (52)**, 111 (21), 126 (7), 182 (1), 196 (0.7), 224 (0.42), 252 (0.34), **297 (0.51), 354 (0.08, M^+)**	2299
12	20.37	IV + 2 CH_2	0.19 ± 0.02	45 (100), 57 (81), 69 (29), 85 (35), **97 (49), 103 (45),** 111 (13), 125 (9), 182 (0.77), 210 (0.5), 238 (0.47), 266 (0.23), **311 (0.05), 368 (0.01, M^+)**	2367

[a] Peak number corresponds to the peaks indicated in Figure 1.
[b] Relative percentage is to the major compound (6,10,13-trimethyltetradecyl isovalerate, abbreviated IV). $N = 3$, mean ± SE.
[c] Kovats indices calculated in relation to straight-chain alkanes.

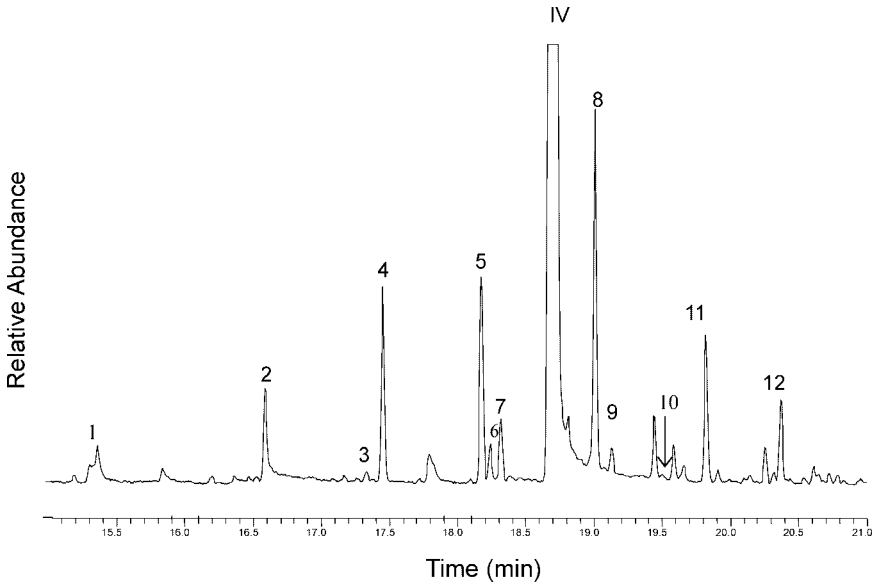

FIG. 1. Total ion chromatogram of the SG secretion of male *E. furcellata*. Compounds: **1**: 6,10,13-trimethyltetradecanol; **2**: 6,10,13-trimethyltetradecyl acetate; **3**: IVb - $2CH_2$; **4**: 6,10,13-trimethyltetradecyl propionate; **5**: IVa $-$ CH_2; **6**: 6,10,13-trimethyltetradecyl butyrate; **7:** IVb $-$ CH_2; **IV**: 6,10,13-trimethyltetradecyl isovalerate; **8**: IVa isomer; **9**: IVb isomer; **10**: IV $+$ CH_2; **11**: IVa $+$ CH_2; **12**: IV $+$ $2CH_2$. Column: DB-1. GC conditions are described in "Methods and Materials."

of 6,10,13-trimethyltetradecyl isovalerate, and the identification was confirmed with an authentic standard.

Peak 4: Because of the distinct ion at m/z 75, characteristic of fragmentation of a propionate ester (McLafferty, 1980), and a mass spectrum similar to that of 6,10,13-trimethyltetradecyl isovalerate, peak 4 was identified as 6,10,13-trimethyltetradecyl propionate, again confirmed with an authentic standard.

Peak 6: The fragment ion at m/z 89, characteristic of a butyrate or isobutyrate ester (McLafferty, 1980), and an otherwise similar mass spectrum to that of 6,10,13-trimethyltetradecyl isovalerate, suggested that this compound was 6,10, 13-trimethyltetradecyl butyrate or isobutyrate. The peak was confirmed as 6,10,13-trimethyltetradecyl butyrate with a standard.

Peaks 8 and 9: The mass spectra of peaks 8 and 9 were similar to that of 6,10,13-trimethyltetradecyl isovalerate, but these peaks had different retention times, suggesting that they might be isomers. The valerate ester of 6,10,13-trimethyltetradecanol was synthesized, but its retention time (19.08 min) was slightly different than that of peak 8 (19.01 min) and peak 9 (19.13 min). Thus, these

two compounds appear to be isomers of 6,10,13-trimethyltetradecyl isovalerate, but the exact structures are unknown.

Peaks 3, 5, 7, 10, 11, and 12: These six components had similar mass spectra in the lower mass ranges to that of 6,10,13-trimethyltetradecyl isovalerate, but different fragment ions in the higher mass ranges. All of these peaks had a significant *m/z* 103 fragment ion, typical of isovalerate or valerate esters. For peak 3, an ion at *m/z* 255 and a molecular ion of *m/z* 312, as compared with the analogous ion of *m/z* 283 and molecular ion of *m/z* 340 of the 6,10,13-trimethyltetradecyl isovalerate, suggested that peak 3 might be the isovalerate or valerate ester of an alcohol with similar structure but two methylene units fewer than 6,10,13-trimethyltetradecanol. Similarly, for peaks 5 and 7, with significant ions at *m/z* 269, and molecular ions of *m/z* 326, the compounds are proposed to be isovalerate or valerate esters of an alcohol with one methylene fewer than 6,10,13-trimethyltetradecanol. For peaks 10 and 11, which gave ions with *m/z* 297 and 354, the compounds are proposed to be the isovalerate or valerate ester of an alcohol with one methylene unit more than 6,10,13-trimethyltetradecanol. Similarly, for peak 12, with ions of *m/z* 311 and 368, the compound was suggested to be the isovalerate or valerate ester of an alcohol with two methylene units more than 6,10,13-trimethyltetradecanol.

These isomers with different chain lengths were further grouped into three series of homologs according to the Kovats retention indices. Peaks 3 and 7 were homologs of peak 9 (IVb) with one or two methylene units less than peak 9 (Kovats indices are 2016, 2120, and 2217, respectively). Peaks 5 and 11 were homologs of peak 8 (IVb) with one methylene unit less and one unit more than peak 8 (Kovats indices are 2101, 2299, and 2205, respectively). Peaks 10 and 12 are homologs of IV with one or two more methylene units than IV (Kovats indices are 2263, 2367, and 2163, respectively).

Thus, the evidence suggests that these six compounds are isovalerate or valerate esters of homologs of 6,10,13-trimethyltetradecanol, with extra or fewer methylene units in the carbon backbone, but their exact structures remain to be fully elucidated.

DISCUSSION

Amount of 6,10,13-Trimethyltetradecyl Isovalerate Produced by Bugs Reared under Different Conditions. Production of SG secretions of some asopine hemipterans was stimulated by first starving and then feeding adult bugs after eclosion (Aldrich and Lusby, 1986; Aldrich et al., 1986). In contrast, in our study, we found that rearing density was the key factor affecting the production of the SG secretion, with isolated bugs producing more than two orders of magnitude more of the isovalerate ester than bugs reared in groups. The dramatic decrease in production of the isovalerate ester in the SG secretion from grouped bugs in

comparison to isolated bugs may be triggered by a variety of cues, such as physical contact with other bugs, or visual, olfactory, or acoustic signals. This remains to be explored further experimentally.

A comparison of the amount of isovalerate from bugs grouped first and then isolated with that from bugs isolated first and then grouped suggested that a period of isolation stimulated the production of SG secretion, even in bugs that were initially held in a group before being isolated. However, comparison of the amount of isovalerate from male bugs paired with either a male or a female indicated that the sex of the companion did not affect the production of SG secretion. Thus, even though SG secretion is only produced by male bugs, and they are the only sex to possess the setiferous patches on the ventral abdominal surface for release of the pheromone, the pheromone may be an aggregation pheromone for attracting both sexes, rather than being for sexual attraction alone. Possible roles for the SG secretion currently are being tested in bioassays in our laboratory.

6,10,13-Trimethyltetradecanol and the corresponding isovalerate have been found from the SG of several asopine stink bugs (Aldrich and Lusby, 1986; Aldrich et al., 1986), and the alcohol alone was demonstrated to be an aggregation pheromone of *S. anchorago* (Kochansky et al., 1989). Our results showing that isolated bugs produce much more of the SG secretion than grouped bugs support the hypothesis of the SG secretion being used for aggregation purposes.

Homologs of 6,10,13-Trimethyltetradecyl Isovalerate. Of the minor compounds found in the SG secretion, only 6,10,13-trimethyltetradecanol has been reported before (Aldrich and Lusby, 1986; Kochansky et al., 1989). All the homologs of the isovalerate, including the acetate, propionate, butyrate, valerate, and isovalerate esters with different alcohol chain lengths, have never been reported from natural sources. Although the amounts of the homologs were small (less than 1% relative to the major isovalerate), it is possible that they have a biological function.

There were actually two series of homologs of the major isovalerate ester. In the first series, the acid portion of the esters were variable, and the structures in this series were confirmed as acetate, propionate, and butyrate esters of 6,10,13-trimethyltetradecanol (peaks 2, 4, and 6). In the second series, the acid portion was constant (isovaleric or possibly valeric acid), whereas the alcohol portion appeared to have a variable chain length, although the structures of these peaks (peaks 3, 5, 7, 10, 11, and 12) have not yet been confirmed. It remains unclear whether this plethora of minor homologs of the main component represents imprecision in the biosynthesis of the major compound, or whether these trace components of the SG secretion indeed have a biological function.

In conclusion, we demonstrated that rearing conditions can have a profound effect on the production of stink bug SG secretions. The generality of this observation is being studied for other bugs. In particular, if it is indeed a fairly general phenomenon that a simple change in the rearing conditions can dramatically

increase pheromone production for a variety of bug species, it will make it easier to collect sufficient quantities of secretions for identification and bioassays. As a case in point to be noted, the method of collecting relatively large amounts of the SG secretion and the identification of the homologs of isovalerate in the resulting SG secretion has accelerated studies on the function of the SG secretion that are currently in progress in our laboratory.

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SEX PHEROMONE COMPONENTS OF THE SANDTHORN CARPENTERWORM, *Holcocerus hippophaecolus*

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Abstract—Extracts of female sex pheromone gland of the carpenterworm moth, *Holcocerus hippophaecolus* Hua, a pest of sandthorn, *Hippophae rhamnoides* L. were found to contain (*E*)-3-tetradecenyl acetate (E3-14:Ac), (*Z*)-3-tetradecenyl acetate (Z3-14:Ac), (*Z*)-7-tetradecenyl acetate (Z7-14:Ac), the corresponding alcohols, E3-14:OH, Z3-14:OH, Z7-14:OH, and (*E*)-9-tetradecenyl acetate (E9-14:Ac). Electroantennographic (EAG) analysis of these chemicals and their analogs demonstrated that Z7-14:Ac elicited the largest male EAG response, followed by E3-14:Ac. In field trials, traps baited with either Z7-14:Ac or E3-14:Ac alone caught no male moths, whereas a combination of these two components in a 1:1 ratio caught more males than control traps. Addition of Z7-14:OH and Z3-14:OH or the alcohols plus E9-14:Ac did not enhance trap catches. We conclude that the sex pheromone of *H. hippophaecolus* is composed of Z7-14:Ac and E3-14:Ac. Optimal ratios and doses of these two components, and the possible role of other minor components, remain to be determined.

Key Words—*Holcocerus hippophaecolus*, sex pheromone, (*E*)-3-tetradecenyl acetate, (*Z*)-7-tetradecenyl acetate, Lepidoptera, Cossidae.

INTRODUCTION

The carpenterworm, *Holcocerus hippophaecolus* Hua (Lepidoptera: Cossidae) is a destructive forest pest, widely distributed throughout northern and western regions of China, including Inner Mongolia Autonomous Region, Ningxia Autonomous Region, Shanxi, Shaanxi, Gansu, and Liaoning provinces. Its primary host is the sandthorn plant (*Hippophae rhamnoides* L.). Sandthorns have been planted widely

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in China (>1.4 million ha), often in single-species plantations, to prevent soil erosion and desertification (Tian and Tang, 1997). The fruits of sandthorn are processed for juice and traditional Chinese medicines. Currently, the sandthorn carpenterworm infests sandthorn plantations totaling 50,000 ha in area, often at high levels. In Liaoning Province, for example, infestation levels of sandthorns by *H. hippophaecolus* can reach 85% (Zhou, 2002; Luo et al., 2003). The damage is so severe and extensive that the sandthorn carpenterworm is considered a major threat to the continued existence of sandthorn plantations in China.

Larvae of *H. hippophaecolus* burrow in the root crowns of sandthorns, resulting in a high probability of plant mortality. Chemical insecticides are ineffective due to the cryptic nature of *H. hippophaecolus*. In addition, the indiscriminate use of insecticide can lower the quality of sandthorn. Thus, an alternative strategy for monitoring and controlling sandthorn carpenterworms is urgently needed.

Sex pheromones have been identified for three related Cossidae species: *Cossus cossus* L. (Capizzi et al., 1983), *Cossus mongolicus* Ersch (Qi et al., 1990), and *Holcocerus insularis* Staudinger (Zhang et al., 2001). Attractants for *H. hippophaecolus* have not been reported. In this paper, we report identification of the sex pheromone for *H. hippophaecolus*. Field trapping studies using blends of synthetic compounds were conducted to develop an efficient trap lure that can be used to monitor this important pest of sandthorns in China.

METHODS AND MATERIALS

Insects. Roots of sandthorn infested with larval *H. hippophaecolus* were collected from Jianping county, Liaoning Province, in the spring of 2003. The roots were kept at 25 ± 1°C and 20 ± 1°C during light and dark hours, respectively, at 65 ± 5% RH, under 16L:8D photoperiod. Pupae were collected from soil and kept under the same conditions until moth emergence. Upon emergence, male and female moths were held at room temperature under a 16L:8D photoperiod. The antennae of male moths were used for electroantennographic (EAG) analyses whereas the abdominal tips of females were used for pheromone extraction and identification.

Chemicals. Semiochemicals (>98% purity) used in analytical work, EAG analysis, and lures for field trials were supplied by Plant Research International (Wageningen, The Netherlands). Reagents and solvents were obtained from Fisher Chemicals (New Jersey, USA), and dimethyl disulfide (DMDS) was obtained from ACROS Organics (New Jersey, USA).

Pheromone Extracts. Observations of 1-d-old virgin female moths for 24 hr revealed that their calling period was generally 2.5–5.5 hr into the scotophase. During this period, 84% of females were calling. The pheromone glands of calling females were extruded by gently pressing the abdominal tips, and then excised with a small blade. The single excised gland was immersed in 10 μl hexane containing

delta13–14:OH (1 ng) as internal standard for 20 min at room temperature and then immediately analyzed by gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS). The remaining extract was transferred into a clean conical glass vial and kept at −20°C for additional analysis.

Derivatization with Dimethyl Disulfide. Pheromone extracts were reacted with DMDS (Leonhardt and DeVilbiss, 1985). A mixture of 50 μl DMDS and 5 μl iodine-diethyl ether solution (0.06%) was added to an extract containing 12 FE (female equivalents) and kept at 40°C overnight. After the addition of 10 μl hexane and 20 μl of aqueous sodium thiosulfate (5%), the mixture was stirred and the hexane layer was transferred into a small vial, and concentrated under nitrogen prior to analysis by GC–MS.

Chemical Analysis. GC analyses of sex pheromone gland extracts were performed on an HP 5890 II GC with a flame ionization detector (FID) and a splitless injector using nitrogen as carrier gas. The GC conditions were as follows: BP-20 column (50 m × 0.25 mm i.d., Scientific Glass Engineering Pty. Ltd., Australia); temperature program, 50°C for 1 min to 150°C at 25°C/min, then 4°C/min to 200°C, hold for 10 min, finally 5°C/min to 210°C and hold for 30 min. The GC–MS analyses of pheromone gland extracts were performed on an HP 6890 GC interfaced with an HP 5973 mass selective detector using a 60 m × 0.22 mm i.d. HP-INNOWAX column. The temperature program was the same as that used in the GC analyses. Analyses of the DMDS derivatives were carried out on the same GC–MS instrument with a DB-5MS column (30 m × 0.25 mm i.d., J&W Scientific, USA). The column temperature was 100°C for 1 min then programmed at 10°C/min to 230°C, and hold for 20 min.

Electroantennograms. Dissected antennae from 1- to 3-d-old males were used for EAG measurements. Previously described methods (Fang and Zhang, 2002) were used for determining EAG responses to a series of monounsaturated 14-carbon alcohols and acetates. An aliquot of hexane solution of each tested compound was deposited onto a filter paper strip (5 mm × 30 mm). After allowing the solvent to evaporate at room temperature, the filter paper was inserted into a Pasteur pipette. A stimulus was introduced as a short puff (2 ml air) into a purified, humidified air stream from the Pasteur pipette, through a stainless steel tube positioned 9 cm from the antennae. The duration of each puff was kept at about 0.1 sec. Solvent blank puffs (filter paper and hexane) served as the controls. To compensate for possible deterioration of the antennal preparation, a stimulation with a reference compound, (*E*)-5-tetradecenol (E5-14:OH) (1 μg), preceded each test stimulus puff. Relative response to a test compound was expressed as a percentage relative to the response to E5-14:OH.

Field Trials. Trapping tests were carried out in Jianping County, Liaoning Province from June 15 to July 7, 2003. Semiochemicals were dissolved in hexane and loaded onto gray rubber septa (The West Company, Phoenixville, PA) at a dose of 500 μg/septum. After the solvent had evaporated, 2 × 100 μl aliquots

of dichloromethane were added to each septum to help the chemical permeate into the septum. Sticky traps, similar to the Pherocon 1C trap (Trécé Inc., Salinas, CA), were constructed from two pieces of cardboard (42 × 28 cm). All traps were hung on branches of *Hippophae rhamnoides* at 1.5–1.8 m above ground level. Seven synthetic chemical blends and a blank control were tested in a completely randomized block design with five replicate blocks. Traps were spaced 50 m apart and the trap positions were rotated to minimize the effects of habitat heterogeneity. Captured mothes were recorded and removed daily.

Statistical Analysis. Differences among the EAG responses to different compounds were analyzed by one way ANOVA. Values for field traps were compared with the Bonferoni test. All data were analyzed with a statistical program for Windows 11.0 (SPSS Inc., 2001).

RESULTS

Analysis of Sex Pheromone Gland Extracts. Sex pheromone gland chemicals of female *H. hippophaecolus* were identified and quantified by GC–MS and GC. The GC–MS analyses of the gland extracts revealed that the mass spectra of peaks I, II, III, and IV were consistent with those of monounsaturated 14-carbon acetates with a comparatively strong fragment at m/z 194 (27–30% of the base peak, loss of acetic acid), and a small but distinct fragment at m/z 61 (1–8%, $CH_3COOH_2^+$) (Figure 1, Table 1). The mass spectra of peaks V, VI, and VII contained diagnostic ions at m/z 194 (10–12% of the base peak, loss of H_2O) and m/z 31 (12–14%, CH_2OH^+). This suggested that the compounds were monounsaturated 14-carbon primary alcohols. After the comparison of the relative intensities of five diagnostic ions $[(m_1/z)/(m_2/z)]$: 54/55, 67/68, 81/82, 81/95, and 95/96 with literature data on positional isomers of 14-carbon acetates and alcohols (Horiike et al., 1990, 1991; Zhang et al., 2001) we determined the identities of the seven compounds as follows: peak I and II were 3-tetradecenyl acetates (3-14:Ac), peak III was a 7-tetradecenyl acetate (7-14:Ac), peak IV was a 9-tetradecenyl acetate (9-14:Ac); peak V and VI were 3-tetradecenols (3-14:OH), and peak VII was a 7-tetradecenol (7-14:OH).

Double bond locations of the pheromone components were further confirmed by analyses of their DMDS derivatives. Analyses revealed the existence of DMDS adducts derived from 7-14:OH (diagnostic ions at m/z 145, 161, and 306 [M^+]); 3-14:OH (diagnostic ions at m/z 105, 201, and 306 [M^+]); 3-14:Ac (diagnostic ions at m/z 87, 147, 201, and 348 [M^+]); 7-14:Ac (diagnostic ions at m/z 143, 145, 203, and 348 [M^+]); and 9-14:Ac (diagnostic ions at m/z 117,171, 231, and 348 [M^+]). The DMDS derivatives of E3-14:Ac and Z3-14:Ac and the corresponding alcohols could not be separated efficiently by DB-5MS column in our GC–MS system.

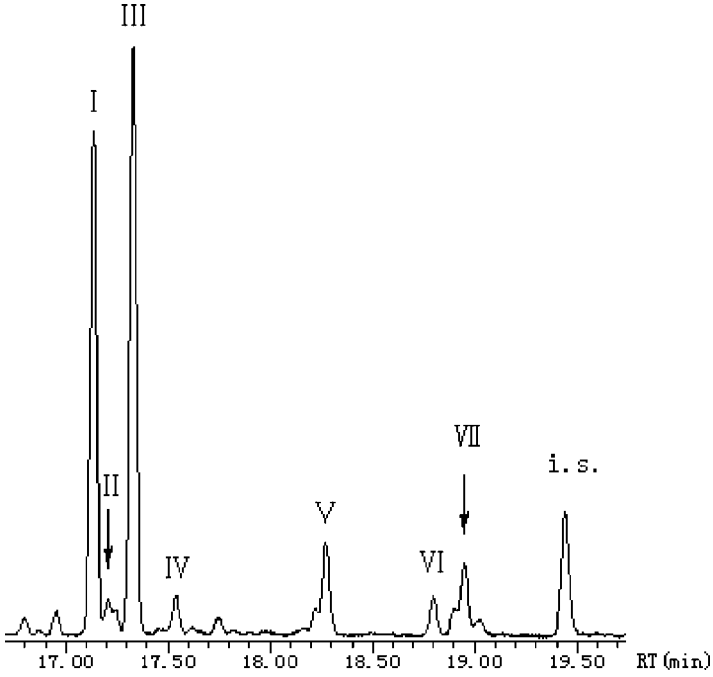

FIG. 1. Total ion chromatogram of GC–MS analysis of a single-gland extract from a female *H. hippophaecolus*. Identities of components are as follows: (I) E3-14:Ac; (II) Z3-14:Ac; (III) Z7-14:Ac; (IV) E9-14:Ac; (V) E3-14:OH; (VI) Z3-14:OH; and (VII) Z7-14:OH. Internal standard (i.s.): delta-13-14:OH (1 ng).

The configurations of monounsaturated 14-carbon compounds in the pheromone gland were first determined by a comparison of their retention times with those of standard compounds on a BP-20 GC column. However, the separation for Z7-14:Ac and E7-14:Ac was still poor, whereas the other components were well separated and were determined to be Z3-14:OH, E3-14:OH, Z3-14:Ac, E3-14:Ac, and E9-14:Ac. In GC–MS analyses, a more polar column, HP-INNOWAX was used. With this column, all possible isomers in female extracts were separated and the configurations of all monounsaturated 14-carbon compounds, including Z7-14:Ac, in the gland extracts were determined (Table 2). In the analyses of the single gland extracts, we found that the two gland components, E3-14:Ac and Z7-14:Ac were detected in calling females but not in noncalling ones. In calling females, the titer of Z7-14:Ac varied from 8.3 to 52 ng, whereas the titer of E3-14:Ac varied from 6.4 to 60.9 ng. Relative amounts of E3-14:Ac and Z7-14:Ac were equal, whereas for other components, the relative amounts were much lower than that of Z7-14:Ac (Table 2).

TABLE 1. MASS SPECTRAL DATA FOR CHEMICALS IN SEX PHEROMONE GLAND EXTRACTS

Component	Identity	Relative intensity of major ions (m/z)
I	3-14:Ac	194 (27) [M^+–60], 152 (10), 110 (23), 96 (66), 95 (37), 82 (71), 81 (57), 68 (67), 67 (65), 61 (1) [$CH_3COOH_2^+$], 55 (39), 54 (60), 43 (100) [$O{=}C^+CH_3$]
II	3-14:Ac	194 (27) [M^+–60], 152 (11), 110 (24), 96 (62), 95 (39), 82 (71), 81 (60), 68 (74), 67 (62), 61 (1) [$CH_3COOH_2^+$], 55 (39), 54 (57), 43 (100) [$O{=}CCH_3^+$]
III	7-14:Ac	194 (30) [M^+–60], 152 (3), 110 (31), 96 (85), 95 (69), 82 (100), 81 (89), 68 (46), 67 (92), 61 (8) [$CH_3COOH_2^+$], 55 (62), 54 (42), 43 (82) [$O{=}CCH_3^+$]
IV	9-14:Ac	194 (30) [M^+–60], 152 (3), 110 (37), 96 (84), 95 (65), 82 (100), 81 (92), 68 (45), 67 (78), 61 (10) [$CH_3COOH_2^+$], 55 (87), 54 (42), 43 (72) [$O{=}CCH_3^+$]
V	3-14:OH	194 (10) [M^+–H_2O], 166 (6) [M^+–C_2H_5OH], 109 (23), 96 (56), 95 (43), 82 (87), 81 (66), 68 (100), 67 (72), 55 (94), 54 (34), 31 (14) [CH_2OH^+]
VI	3-14:OH	194 (8) [M^+–H_2O], 166 (6) [M^+–C_2H_5OH], 109 (19), 96 (48), 95 (40), 82 (80), 81 (70), 68 (100), 67 (71), 55 (74), 54 (30), 31 (12) [CH_2OH^+]
VII	7-14:OH	194 (11) [M^+–H_2O], 166 (4) [M^+–C_2H_5OH], 109 (33), 96 (65), 95 (65), 82 (94), 81 (87), 68 (57), 67 (100), 55 (79), 54 (44), 31 (14) [CH_2OH^+]

TABLE 2. RETENTION TIMES AND RELATIVE QUANTITIES OF GLAND CHEMICALS AND KNOWN STANDARDS[a]

Retention time (min)				Mean (± SE) percentage relative to quantity of Z7-14:Ac ($N = 17$)
Synthetic compound		Component in female extracts		
17.135	E3-14:Ac	Peak I	17.135	102.5 ± 21.6
17.212	Z3-14:Ac	Peak II	17.211	17.6 ± 5.1
17.303	E7-14:Ac			
17.338	Z7-14:Ac	Peak III	17.336	100
17.445	E9-14:Ac	Peak IV	17.445	9.3 ± 5.6
17.541	Z9-14:Ac			
18.275	E3-14:OH	Peak V	18.277	28.1 ± 10.2
18.805	Z3-14:OH	Peak VI	18.801	21.2 ± 9.8
18.877	E7-14:OH			
18.953	Z7-14:OH	Peak VII	18.957	18.5 ± 7.7

[a] Temperature programs of GC column and determination of relative amounts of the gland components are described in "Methods and Materials."

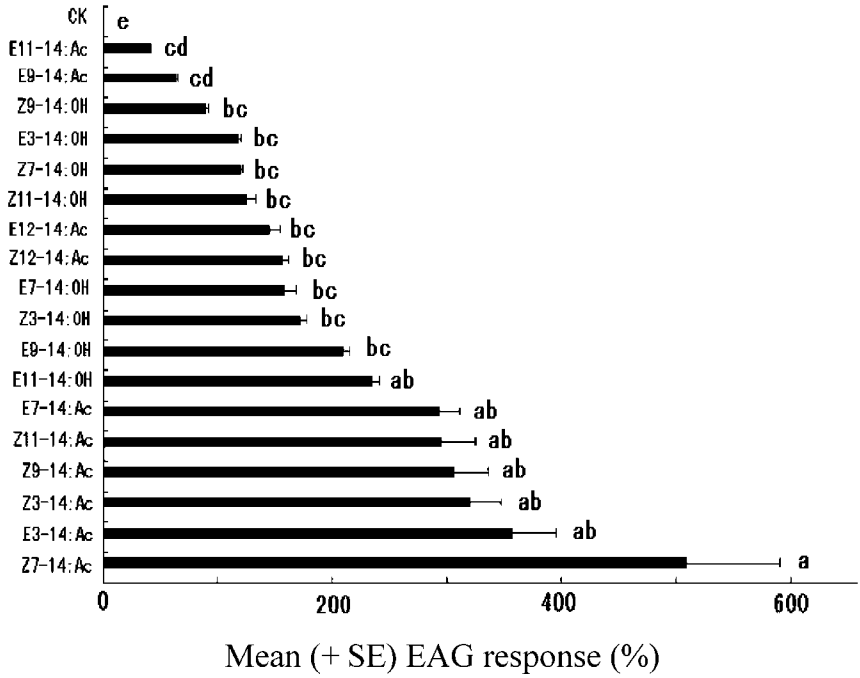

FIG. 2. EAG responses of male *H. hippophaecolus* (relative to responses to E5-14:OH) to a series of monounsaturated 14-carbon alcohols and acetates at doses of 10 μg ($N = 30$). Bars followed by different letters are significantly different at $P = 0.05$. Check control (ck). Response to standard application of 10 μg (*E*)-5-tetradecenol (E5-14:OH) was 4.56 $\pm$ 1.77 mV.

Electroantennographic Analyses. Male EAG responses to the gland components and their analogues varying in double bond positions and configurations are summarized in Figure 2. Results show that Z7-14:Ac elicited the strongest response (29.59 mV), followed by E3-14:Ac. Among tested compounds, the EAG response elicited from each acetate was higher than that from the corresponding alcohol, except for E11-14:Ac, which elicited a much lower EAG response than the corresponding alcohol, E11-14:OH.

Field Trapping. Traps baited with a single gland component, Z7-14:Ac or E3-14:Ac failed to attract male *H. hippophaecolus* males in the field. However, traps baited with the two gland components in a 1:1 ratio captured more males than control traps (Table 3). Catches were not affected by addition of Z7-14:OH and Z3-14:OH or the mixture of Z7-14:OH, Z3-14:OH, and E9-14:Ac.

TABLE 3. MEAN CATCHES OF MALE *H. hippophaecolus* IN TRAPS BAITED WITH VARIOUS SEX PHEROMONE GLAND CHEMICALS

	Composition of baits/μg[a]							
	Acetates					Alcohols		Mean ($\pm$ SE)
Treatment	Z7-14:Ac	E3-14:Ac	E7-14:Ac	Z3-14:Ac	E9-14:Ac	Z3-14:OH	Z7-14:OH	trap catch[b]
A	500							0
B		500						0
C	250	250						30.0 ± 4.8*
D		250	250					0
E	250			250				0
F	250	250				65	50	12.7 ± 4.1*
G	250	250			16	65	50	34.0 ± 7.5*
CK	0	0	0	0	0	0	0	0

Notes. Mean difference: C–F = 1.7700, Sig.: C–F = 0.091; mean difference: C–G = 0.1500, Sig.: C–G = 1.000; mean difference: C–A, C–B, C–D, C–E, C–CK = 2.1429, Sig.: C–A, C–B, C–D, C–E, C–CK = 0.0000; mean difference: F–A, F–B, F–D, F–E, F–CK = 0.8714, Sig.: F–A, F–B, F–D, F–E, F–CK = 0.004; mean difference: G–A, G–B, G–D, G–E, G–CK = 2.4286, Sig.: G–A, G–B, G–D, G–E, G–CK = 0.0000.

[a] The ratios of the components in baits were similar to that found in the pheromone gland.

[b] The mean difference is significant at the $P = 0.05$ level.

DISCUSSION

We have demonstrated that E3-14:Ac and Z7-14:Ac are the major components of the sex pheromone of *H. hippophaecolus*. The two compounds are produced by adult females, and the antennae of male *H. hippophaecolus* were highly sensitive to both compounds. In field trials, traps baited with the two compounds in a 1:1 ratio were attractive to adult males (Table 3).

Both compounds were required for attraction to occur. Sex pheromones and attractants identified to date for carpenterworms have been mono- or di-unsaturated straight chain acetates, most being 14-carbon acetates. Although Z7-14:Ac has been reported as a pheromone component for species in many families of Lepidoptera, E3-14:Ac has been reported only in *Symmetrischema tangolias* (Gyen) (Gelechiidae) (Griepink et al., 1995).

During the course of our studies, we found that *H. hippophaecolus* lacks *instrumenta suctoria*. Adults are, therefore, unable to feed during their short lifespan. They mate and lay eggs within a short period of time, with female moths mating only once during their adult life (Tian and Tang, 1997). These facts suggest that it may be possible to control this important pest by mass trapping through focused trap efforts or by mating disruption. However, development of a mass trapping or mating-disruption system will require identification of the optimum pheromone dosage on lures and determination of any other potential synergists. Currently, a wing trap baited with the synthetic compounds Z7-14:Ac and E3-14:Ac in a 1:1 ratio at 500 μg dosage can be used to monitor population levels of *H. hippophaecolus* within plantations of sandthorn in China.

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(7*R*,8*S*)-*cis*-7,8-EPOXY-2-METHYLOCTADEC-17-ENE: A NOVEL TRACE COMPONENT FROM THE SEX PHEROMONE GLAND OF GYPSY MOTH, *Lymantria dispar*

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Abstract—Considering the vast Eurasian distribution of gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae), the many subspecies, and their presence in different lymantriid communities, we tested the hypothesis that *L. dispar* populations in eastern Asia employ one or more pheromone components in addition to the previously known single component pheromone (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane [= (+)-disparlure]. Coupled gas chromatographic–electroantennographic detection (GC–EAD) analyses of pheromone gland extracts of female *L. dispar sensu lato* (including both AGM and NAGM) on four GC columns (DB-5, DB-23, DB-210, and SP-1000) revealed a new trace component that eluted just before (DB-5; DB-210) or after (DB-23, SP-1000) disparlure, and elicited strong antennal responses. Isolation of this compound by high-performance liquid chromatography and hydrogenation produced disparlure, suggesting that the new component had the molecular skeleton of disparlure, with one or more double bonds. Of all possible monounsaturated *cis*-7,8-epoxy-2-methyloctadecenes, only *cis*-7,8-epoxy-2-methyloctadec-17-ene co-chromatographed with the insect-produced compound on all GC columns and elicited comparable antennal responses. In field experiments in Honshu (Japan)

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with enantioselectively synthesized compounds, (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadec-17-ene (7*R*8*S*-epo-2me-17-ene-18Hy) was weakly attractive to male *L. dispar*, but was less effective as a trap bait than (+)-disparlure, and failed to enhance attractiveness of (+)-disparlure when tested in blends. The antipode, (7*S*,8*R*)-*cis*-7,8-epoxy-2-methyloctadec-17-ene, was not attractive, and when added to (+)-disparlure and/or 7*R*8*S*-epo-2me-17-ene-18Hy reduced attractiveness. Thus, the biological role of 7*R*8*S*-epo-2me-17-ene-18Hy remains unclear. It may enhance pheromone attractiveness or specificity in other *L. dispar* populations within their vast Eurasian distribution.

Key Words—Asian gypsy moth, North American gypsy moth, *Lymantria dispar*, nun moth, *Lymantria monacha*, (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadec-17-ene, (7*S*,8*R*)-*cis*-7,8-epoxy-2-methyloctadec-17-ene, (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane, (7*R*,8*S*)-*cis*-7,8-epoxy-octadecane, 2-methyl-(*Z*)-7-octadecene, (+)-disparlure, (+)-monachalure.

INTRODUCTION

Pioneering work by Bierl et al. (1970) identified *cis*-7,8-epoxy-2-methyloctadecane (= disparlure) as a sex pheromone component of the European gypsy moth (EGM), *Lymantria dispar* (Lepidoptera: Lymantriidae). Further studies (Klimetzek et al., 1976; Cardé et al., 1977; Miller et al., 1977; Plimmer et al., 1977) determined that only (+)-disparlure [(7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane] mediates attraction of male moths, whereas the presence of (–)-disparlure [(7*S*,8*R*)-*cis*-7,8-epoxy-2-methyloctadecane] reduced attractiveness of the lure. Pheromone reanalysis of European *L. dispar* revealed *cis*-7,8-epoxy-3-methylnonadecane (Gries et al., 1996), which enhances the specificity of the pheromone signal (Gries et al., unpublished).

The so-called "Asian gypsy moth" (AGM) in eastern Asia encompasses all those *L. dispar* forms with female moths capable of flight, unlike their North American gypsy moth (NAGM) and European gypsy moth (EGM) counterparts that cannot fly. The AGM populations have distinct morphological variations. In Japan alone, AGM populations have been divided into five subspecies, including *hokkaidoensis, japonica, obscura, tsushimensis*, and *postalba* (Inoue, 1957). Recent genetic analyses of these subspecies support the hypothesis that at least two of them are distinct and should be raised to species status (Bogdanowicz et al., 2000). Several species with wide distributions, such as *L. dispar,* have geographically specific pheromone blends. For example, populations of the congeneric nun moth, *Lymantria monacha,* in Bohemia (Europe) and Honshu (Japan) differ in both their pheromonal signal and diel periodicity of signaling (Gries et al., 2001). Conceivably, some *L. dispar* populations could utilize a pheromone signal in addition to, or other than, (+)-disparlure. Here, we report identification and field testing of (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadec-17-ene, a novel pheromone gland component from *L. dispar*.

METHODS AND MATERIALS

Experimental Insects

Female pupae of the NAGM were obtained from the laboratory colony maintained at USDA, Beneficial Insects Introduction Research Laboratory, Newark, DE. Additional NAGMs were field collected as late instar larvae at Welch Point, Elkton, Cecil Co., MD (N 39° 32′, W 75° 52′) and held in mesh cages in an outdoor insectary until eclosion of adult moths.

Egg masses of AGM were obtained from several locations in Asia, including Japan (Honshu, Okinawa), Korea (near Seoul and Cheju Island), and China (Hebei and Liaoning Provinces). The larvae were reared on standard gypsy moth diet (Bell et al., 1981) in the USDA Quarantine Facility, Newark, DE. Female pupae were held under a photoperiod of 14L:10D, 24–26°C, and 60–80% relative humidity, whereas male pupae were held at 15°C to retard development. Abdominal tips with pheromone glands of calling, 1- to 3-d-old virgin female moths were removed and extracted for 15–30 min in HPLC-grade hexane. Ampoules with the supernatant of pheromone extracts and male pupae were sent by courier to Simon Fraser University (SFU). Male pupae were held in SFU's Global Forest Quarantine Facility.

Analyses of Pheromone Extract

Aliquots of 1–3 female equivalents (FE) of combined pheromone gland extracts were analyzed by coupled gas chromatographic–electroantennographic detection (GC–EAD) (Arn et al., 1975) with procedures and equipment recently described in detail (Gries et al., 2002). Pheromone extract was fractionated by high-performance liquid chromatography (HPLC), employing a Waters LC 625 high-performance liquid chromatograph equipped with a Waters 486 variable wavelength UV–visible detector set at 210 nm , HP Chemstation software (Rev.A.07.01), and a reverse phase Nova Pak® C_{18} (3.9 mm × 300 mm) column (Waters) eluted with acetonitrile (1 ml/min). For HPLC fractionation, 200 FE of pheromone gland extracts were evaporated to dryness, 50 μl of acetonitrile were added, and the 50-μl extract was injected into the HPLC. Seventy-five 20-sec (200 μl) fractions of this pheromone extract (200 FE) were collected and analyzed individually without concentration by GC–EAD on a DB-5 column. Coupled GC–mass spectrometric (MS) analyses of pheromone extract and of synthetic standards employed a Varian Saturn 2000 ion trap GC–MS fitted with a DB-5 column (30 m × 0.25 mm ID; J&W Scientific, Folsom, CA).

General Methods and Instrumentation

Tetrahydrofuran (THF) and *N*-methylpyrrolidinone (NMP) were dried by standard methods. Oven-dried glassware was assembled hot under Ar flow, and

maintained under Ar; liquids were transferred by cannula under Ar pressure. Dry LiCl and $CuCl_2$ were weighed out in a dry box. Infrared spectra were recorded on Perkin-Elmer Paragon 1000 and 1600 FT–IR instruments. Optical rotations were measured on Jasco DIP-1000 and Perkin-Elmer 341 polarimeters. Nuclear magnetic resonance (NMR) spectroscopy of synthetic compounds was conducted on Bruker 300 (at 300 MHz for ^{1}H and 75 MHz for ^{13}C) and Varian AS500 (at 499.77 MHz for ^{1}H and 125.68 MHz for ^{13}C) spectrometers, with chemical shifts reported in ppm relative to TMS (^{1}H, δ 0.00) and $CDCl_3$ (^{13}C, δ 77.00). Elemental analyses were performed using a Carlo-Erba model 1106 elemental analyzer.

Syntheses

(*2′S,3′R*)-*8′-Methyl-cis-2′, 3′-Epoxy-1-Nonyl (1S)-10-Camphorsulfonate* (**2**) (Figure 1). (2′*S*, 3′*S*)-4′-Bromo-*cis*-2′,3′-epoxy-1-butyl (1*S*)-10-camphorsulfonate (**1**, >99% ee; Aldrich) (11.0 g, 28.9 mmol) was melted *in vacuo*, and dissolved in THF (30 ml). The solution was cannulated into a 300-ml 3-neck flask fitted with a low-temperature thermometer, magnetic stir bar, and a pressure-equalizing 125-ml dropping funnel. A 0.1 M solution of Li_2CuCl_4 in THF (3 ml , 0.3 mmol) was cannulated in, followed by NMP (20 ml, 207 mmol). The red-orange solution was cooled to −30° (dry ice–acetone bath), and freshly prepared 4-methylpentylmagnesium chloride (large excess, 1.0 M in THF) was cannulated into the dropping funnel. Dropwise addition of this solution to the flask rapidly changed the color to colorless, then faint red-brown. Precipitate began to form after addition of 20 ml, and gummy solid stopped the stir bar soon after. Addition was continued (hand swirling, pot temp. −28° to −23°) until 38 ml (1.3 equiv.) of the Grignard reagent had been added. The NMR assay of an aliquot showed ∼90% consumption of **1**. After addition of more Grignard reagent (8 ml) at −23°, the mixture was allowed to stand from −23° to −20°, then poured into a mixture of Et_2O (100 ml) and 1 N aq. HCl (100 ml). The separated organic layer was washed with H_2O (5 × 100 ml) and brine (25 ml), dried over anhydrous Na_2SO_4–$MgSO_4$, filtered, and concentrated to a pale yellow oil. This was further stripped of volatiles (Kugelrohr oven, 75° @ <0.1 mm) to leave 10.21 g of impure **2** containing unreacted **1** and appreciable amounts of ring-opened alcohol (NMR assay). Column chromatography of this material (silica gel, elution with 5–10% Et_2O–hexanes) yielded 7.66 g of 90+% pure **2** still containing ring-opened alcohol, plus >700 mg of unreacted **1**. The 90+% pure **2** was dissolved in MeOH (80 ml) and stirred for 70 min at 23° with 440 mg of K_2CO_3. The mixture was partitioned between Et_2O and H_2O; standard workup afforded **2** containing terminal epoxide, and no detectable ring-opened alcohol (NMR assay). Column chromatography of this material as before gave 7.00 g of 99% pure **2** (67% yield, based on unrecovered **1**). Purified **2**: $[\alpha]_D^{26}$ +23.0° (*c* 3.50, $CHCl_3$); IR (film) 2954, 1748, 1365, 1171, 968 cm^{-1}; ^{1}H NMR δ 4.54–4.49 (dd, J = 11.4, 4.0 Hz, 1H), 4.30–4.24 (dd, J = 11.4, 7.2 Hz, 1H),

3.66 (d, J = 15.1 Hz, 1H), 3.26 (m, 1H), 3.06 (m, 1H), 3.06 (d, J = 15.1 Hz, 1H), 2.51–2.35 (m, 2H), 2.14 (t, J = 4.5 Hz, 1H), 1.96 (d, J = 18.5 Hz, 1H), 1.12 (s, 3H), 0.89 (s, 3H), 0.87 (d, J = 6.7 Hz, 6H); ^{13}C NMR δ 214.27, 68.60, 57.82, 56.68, 53.32, 48.00, 47.09, 42.64, 42.42, 38.70, 27.89, 27.78, 27.03, 26.79(2), 24.76, 22.52(2), 19.65, 19.58. HRMS (ESI) calcd for $C_{20}H_{34}O_5SNa$ (M^+ + Na) 409.2019, found 409.2004.

*(2R,3R)-1-Iodo-8-Methyl-cis-2,3-Epoxynonane (**3**).* To a solution of **2** (7.00 g, 18.1 mmol) in reagent grade acetone (50 ml) in an Ar-swept flask were added a few mg of $NaHCO_3$, and a solution of NaI (5.5 g, 37 mmol) in acetone (80 ml). Precipitation occurred almost at once. The magnetically stirred mixture was heated in a 45° bath (reflux condenser, positive Ar pressure) for 41 hr (no remaining **2** by NMR analysis). The solid was collected by suction filtration, and washed thoroughly with reagent acetone; the yellow filtrate was concentrated at 40°–45°, and taken up in H_2O (100 ml) and 10% Et_2O–hexanes (150 ml). The organic phase was washed with dilute Na_2SO_3 and water, dried (Na_2SO_4–$MgSO_4$), filtered through silica gel (5 g), stripped of solvent, and pumped under vacuum to leave 4.71 g (92%) of colorless oil: $[\alpha]_D^{26}$ +73.1° (*c* 4.85, $CHCl_3$); IR (film) 2952, 1466, 1166 cm^{-1}; ^{1}H NMR δ 3.36–3.27 (m, 2H, H-1 and H-2), 3.08–3.05 (m, 1H, H-3), 3.05–2.96 (m, 1H, H-1), 1.62–1.16 (3 m's, 9H), 0.88 (d, J = 6.6 Hz, 6H); ^{13}C NMR δ 59.91, 56.74, 38.74, 27.83, 27.21, 27.05, 26.77, 22.57(2), 1.33. HRMS (ESI) calcd for $C_{10}H_{19}IONa$ (M^+ + Na) 305.0373, found 305.0387.

*(7R,8S)-cis-7,8-Epoxy-2-Methyloctadec-17-ene (**4**).* A solution of 1-nonen-9-yl-magnesium bromide [freshly prepared from 2.70 g (13.20 mmol) of 9-bromo-1-nonene with 2 equiv. of magnesium turnings] in 50 ml of THF was added dropwise into a mixture of iodoepoxide **3** (1.24 g, 4.40 mmol), CuI (0.167 g, 0.88 mmol), distilled HMPA (4.60 ml, 26.40 mmol), and dry THF (2 ml) at −23°C. After stirring for 25 min, the reaction mixture was quenched with aq. NH_4Cl solution. The products were extracted with hexane–ether (1:1), the extracts were washed (water, saturated aq. $Na_2S_2O_3$, brine), and dried (anhyd. $MgSO_4$), and solvents and low-boiling byproducts were removed *in vacuo*. Flash chromatography (50 g SiO_2, 0–4% ether in hexane) yielded 0.813 g (2.90 mmol) of pure **4** (66%). $[\alpha]_D^{21}$ +2.3° (*c* 0.48, $CHCl_3$); IR (film) 2930, 2856, 1641, 1466, 1385, 1366, 992, 909 cm^{-1}; ^{1}H NMR δ 5.81 (m, 1H), 4.99 (dd, J =17.1, 2.1 Hz, 1H), 4.93 (d, J = 10.3 Hz, 1H), 2.90 (m, 2H), 2.04 (td, J = 7.6, 6.8 Hz, 2H), 1.15–1.57 (4 m's, 23 H), 0.87 (d, J = 6.6 Hz, 6H); ^{13}C NMR δ 139.18, 114.10, 57.24, 38.88, 33.78, 29.59, 29.49, 29.36, 29.08, 28.91, 28.90, 27.88, 27.85, 27.80, 27.32, 26.84, 26.58, 22.61, 22.60; Anal. calcd. for $C_{19}H_{36}O$ (%): C, 81.36; H, 12.94, found: C, 80.96, H, 12.91.

*(2′R,3′S)-8′-Methyl-cis-2′,3′-Epoxy-1-Nonyl (1S)-10-Camphorsulfonate (**2′**).* This epoxide was prepared from (2′*R*,3′*R*)-4′-bromo-*cis*-2′,3′-epoxy-1-butyl (1*S*)-10-camphorsulfonate (**1′**, >99% ee; Aldrich) (11.0 g, 28.9 mmol) essentially as described for synthesis of **2**, except that the amounts of Li_2CuCl_4 and NMP were

doubled, and sufficient 4-methylpentylmagnesium chloride was added to consume all of **1′**. No ring-opened alcohol was detected. A single column chromatography of the crude product (silica gel, elution as for **2**) gave 6.10 g of ∼98% pure **2′** (54%). Purified **2′**: $[\alpha]_D^{26}$ +40.6° (*c* 4.65, $CHCl_3$); IR (film) 2954, 1748, 1365, 1172, 969 cm^{-1}; ^1H NMR δ 4.50–4.44 (dd, $J = 11.4, 4.2$ Hz, 1H), 4.34–4.28 (dd, $J = 11.7$, 7.1 Hz, 1H), 3.66 (d, $J = 15.1$ Hz, 1H), 3.27 (m, 1H), 3.49–3.42 (m, 2H), 3.11 (d, $J = 15.1$ Hz, 1H), 3.08 (m, 1H), 2.50–2.35 (m, 2H), 2.14 (t, $J = 4.3$ Hz, 1H), 2.12–2.03 (m, 1H), 1.96 (d, $J = 18.5$ Hz, 1H), 1.12 (s, 3H), 0.89 (s, 3H), 0.87 (d, $J = 6.6$ Hz, 6H); ^{13}C NMR δ 214.21, 68.46, 57.86, 56.69, 53.34, 47.95, 47.18, 42.64, 42.41, 38.69, 27.86, 27.77, 27.02, 26.79(2), 24.85, 22.52(2), 19.66, 19.59. HRMS (ESI) calcd, for $C_{20}H_{34}O_5SNa$ (M^++ Na) 409.2019, found 409.1999.

*(2S,3S)-1-Iodo-8-Methyl-cis-2,3-Epoxynonane (**3′**).* This iodomethyl epoxide, prepared from **2′** in 93% yield as described for enantiomer **3**, was obtained as a colorless oil: $[\alpha]_D^{25}$ –73.4° (*c* 4.80, $CHCl_3$); all other physical data were identical to those recorded for **3**.

*(7S,8R)-cis-7,8-Epoxy-2-Methyloctadec-17-ene (**4′**).* Compound **4′** was prepared from iodoepoxide 3′ under the same conditions as described for **4** with 58% yield. $[\alpha]_D^{21}$ –2.1° (*c* 0.71, $CHCl_3$); all spectral data were identical to those recorded for **4**.

Field Experiments

All field experiments employed a complete randomized block design with 10 blocks (replicates) each, and were conducted in forests (N 39° 52′, E 141° 23′ and N 39° 53′, E 141° 18′) near (<35 km) the city of Morioka (Iwate Prefecture, Japan). *Lymantria dispar* experiments 1–4 were set up in forests with mixed oak, birch, and maple, and the *L. monacha* experiment (Exp. 5) in a forest with Japanese larch, *Larix leptolepsis*. Delta-like traps were made from 2-l milk cartons (Gray et al., 1984), coated with Tanglefoot (The Tanglefoot Company, Grand Rapids, Michigan, PA), and suspended from trees 1.5 m above ground at 15 to 20-m spacing. They were baited with a gray sleeve stopper (West Pharmaceutical Services, Lionville, PA) impregnated with candidate pheromone components in HPLC-grade hexane.

Experiment 1 tested 7*R*8*S*-epo-2me-17-ene-18Hy (50 μg) and 7*S*8*R*-epo-2me-17-ene-18Hy (50 μg) singly and in combination. Experiment 2 compared the relative attractiveness of 7*R*8*S*-epo-2me-17ene-18Hy (50 μg) with that of (+)-disparlure (50 μg). Experiment 3 tested whether attractiveness of (+)-disparlure could be enhanced by addition of 7*R*8*S*-epo-2me-17ene-18Hy (0.5, 5, or 50 μg). Experiment 4 explored whether attractiveness of (+)-disparlure was affected by addition of either 7*R*8*S*-epo-2me-17ene-18Hy (0.5 μg), 7*S*8*R*-epo-2me-17ene-18Hy (0.5 μg), or both (0.5 μg each). Final experiment 5 investigated whether 7*R*8*S*-epo-2me-17ene-18Hy enhanced species specificity of the *L. dispar* pheromone by testing the *L. monacha* pheromone blend

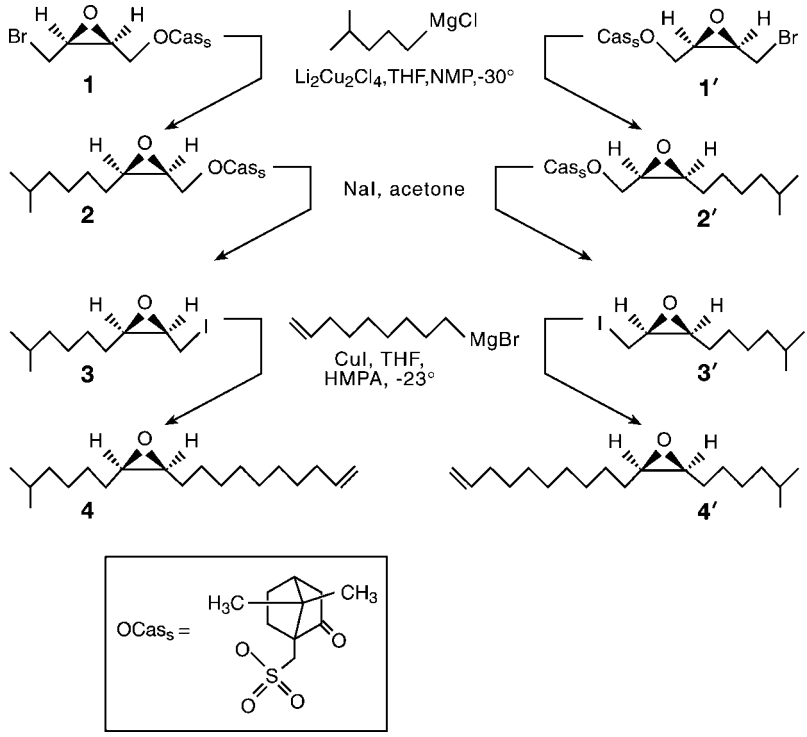

FIG. 1. Scheme for enantioselective syntheses of (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadec-17-ene (**4**), and (7*S*,8*R*)-*cis*-7,8-epoxy-octadec-17-ene (**4′**).

[(7*R*,8*S*)-*cis*-7,8-epoxy-octadecane = (+)-monachalure (50 μg); (+)-disparlure (50 μg); (*Z*)-2-methyl-7-octadecene (2me-*Z*7-18Hy) (5 μg)] vs. the *L. monacha* pheromone blend in combination with 7*R*8*S*-epo-2me-17-ene-18Hy.

Trap catch data were subjected to nonparametric analyses of variance (Friedman's test) followed by comparison of means by Scheffé test (Zar, 1984; SAS/STAT, 1988). In all analyses, $\alpha = 0.05$.

RESULTS AND DISCUSSION

In GC–EAD analyses of pheromone gland extracts of female *L. dispar* from various geographic locations, a previously unknown component elicited strong antennal responses (Figure 2). Even in concentrated pheromone gland extracts [>300 FE], the unknown occurred below detection threshold of the GC–MS, and needed to be identified without spectroscopic data.

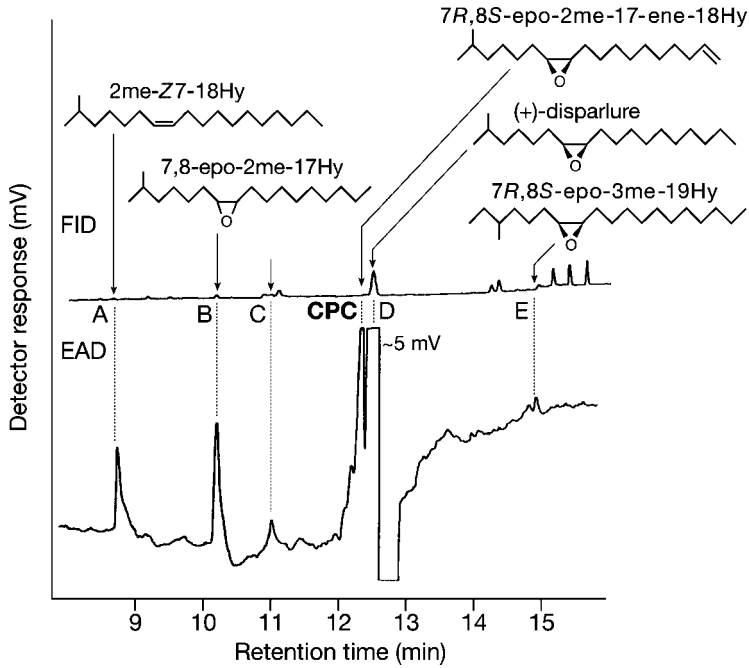

FIG. 2. Flame ionization detector (FID) and electroantennographic detector (EAD: male *L. dispar* antenna) responses to 1 FE of *L. dispar* pheromone gland extract. Chromatography: DB-5 column; 100°C (1 min), 20°C per min to 190°C (held for 8 min), then 25°C per min to 280°C. Except for (+)-disparlure, all components eliciting antennal responses occurred below detection threshold of the FID. Compound identities: **A** = (*Z*)-2-methyl-7-octadecene; **B** = *cis*-7,8-epoxy-2-methylheptadecane (absolute configuration not yet determined); **C** = unknown; **CPC** = candidate pheromone component = (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadec-17-ene; **D** = (+)-disparlure = (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane; **E** = (7*R*,8*S*)-*cis*-7,8-epoxy-3-methylnonadecane.

The GC–EAD analyses of pheromone gland extracts on four GC columns (DB-5, DB-23, DB-210, SP-1000) indicated that the unknown component had retention indices (RI) (Van den Dool and Kratz, 1963) of 2027 (DB-5), 2403 (DB-23), 2297 (DB-210), and 2304 (SP-1000) relative to straight-chain alkanes. Retention-indices assignments for the component on both DB-23 and SP-1000 columns remained tentative, because the compound eluted just after disparlure, and elicited only weak antennal responses, possibly due to an antennal refractory phase after a strong response to disparlure.

To confirm correct assignment of RIs for the compound on DB-23 and SP-1000 columns, seventy-five 20-sec (200 μl) HPLC fractions of pheromone extract (200 FE) were analyzed by GC–EAD on a DB-5 column. The fraction containing

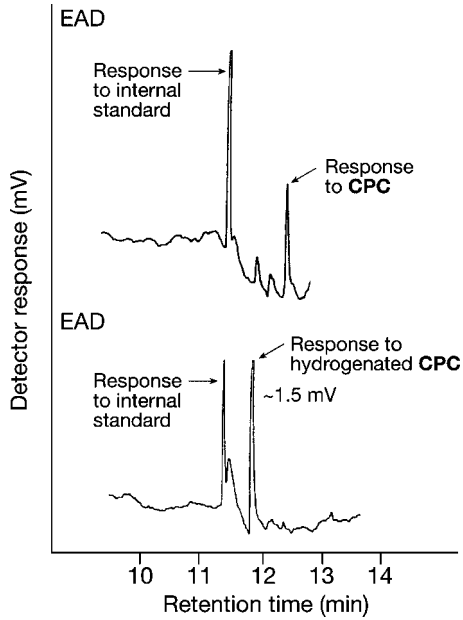

FIG. 3. Electroantennographic detector (EAD: male *Lymantia dispar* antenna) responses to the HPLC-isolated CPC before (top) and after (bottom) hydrogenation. Chromatography: DB-23 column; 100°C (1 min), 10°C per min to 200°C. Corresponding FID traces are omitted. Stronger response to hydrogenated CPC (bottom) may be explained by a more concentrated (×10) sample. (7*R*,8*S*)-*cis*-7,8-Epoxyoctadecane [(+)-monachalure (10 pg)] was used as an internal standard.

the unknown compound was further analyzed on all four columns, revealing antennal responses with the familiar RIs of 2027 (DB-5), 2403 (DB-23), 2297 (DB-210), and 2304 (SP-1000) (as mentioned earlier). Calculations of intercolumn RI differences (e.g., RI on DB-5 minus RI on DB-23; RI on SB-1000 minus RI on DB-23) suggested that the compound might be a monounsaturated epoxide. Hydrogenation and GC–EAD analyses of the fraction (Figure 3) exhibited a different antennal response with RIs on all columns consistent with those of disparlure. To confirm that the unknown compound had the molecular skeleton of disparlure, related 5,6-, 6,7-, 8,9-, and 9,10-epoxy-2-methyloctadecanes were synthesized (Table 1). Their GC characteristics were similar or identical to those of disparlure (Table 1), but their EAD activities were significantly lower.

To determine the double bond position in the unknown (= unsaturated disparlure), all possible *cis*-7,8-epoxy-2-methyloctadecenes were synthesized (Table 2). Of these, only *cis*-7,8-epoxy-2-methyloctadec-17-ene had GC- and EAD-characteristics entirely consistent with those of the novel compound.

TABLE 1. RETENTION INDICES RELATIVE TO STRAIGHT-CHAIN ALKANES OF VARIOUS SATURATED EPOXIDES SYNTHESIZED FOR THE IDENTIFICATION OF THE CANDIDATE PHEROMONE COMPONENT (CPC) IN FIGURE 2

	Type of column		
	DB-23	DB-5	DB-210
Synthetic chemical	*trans*/*cis*	*trans*/*cis*	*trans*/*cis*
5,6-Epoxy-2-methyloctadecane	2321/2343	2018/2030	2289/2309
6,7-Epoxy-2-methyloctadecane	2316/2340	2015/2030	2286/2307
7,8-Epoxy-2-methyloctadecane[a]	2315/2337	2015/2028	2287/2305
8,9-Epoxy-2-methyloctadecane	2315/2337	2015/2028	2287/2306
9,10-Epoxy-2-methyloctadecane	2315/2337	2015/2028	2287/2306

[a] Disparlure.

TABLE 2. RETENTION INDICES RELATIVE TO STRAIGHT-CHAIN ALKANES AND COMPARATIVE ANTENNAL RESPONSES ELICITED BY *cis*-7,8-EPOXY-2-METHYLOCTADECENES

	Retention indices			
Compound	DB-23	DB-5	DB-210	EAD-activity*
cis-7,8-Epoxy-2-methyl-Δ**1**-*ENE*-18Hy	2442	2050	2343	+
cis-7,8-Epoxy-2-methyl-Δ**2**-*ENE*-18Hy	2425	2056	2329	
cis-7,8-epoxy-2-methyl-***Z*3**-*ENE*-18Hy	2334	2009	2267	+
cis-7,8-epoxy-2-methyl-***Z*4**-*ENE*-18Hy	2374	2019	2300	+
cis-7,8-epoxy-2-methyl-***Z*10**-*ENE*-18Hy	2354	2004	2284	
cis-7,8-epoxy-2-methyl-***Z*11**-*ENE*-18Hy	2356	2006	2281	
cis-7,8-epoxy-2-methyl-***Z*12**-*ENE*- 18Hy	2363	2005	2286	+
cis-7,8-epoxy-2-methyl-***Z*13**-*ENE*-18Hy	2380	2014	2301	
cis-7,8-epoxy-2-methyl-***Z*14**-*ENE*-18Hy	2390	2020	2308	
cis-7,8-epoxy-2-methyl-***Z*15**-*ENE*-18Hy	2396	2025	2315	+
cis-7,8-epoxy-2-methyl-***Z*16**-*ENE*-18Hy	2431	2045	2338	
cis-7,8-epoxy-2-methyl-Δ**17**-*ENE*-18Hy	2402	2020	2315	++
cis-7,8-epoxy-2-methyl-***E*3**-*ENE*-18Hy	2332	2001	2263	
cis-7,8-epoxy-2-methyl-***E*4**-*ENE*-18Hy	2361	2021	2292	
cis-7,8-epoxy-2-methyl-***E*10**-*ENE*-18Hy	2362	2020	2292	
cis-7,8-epoxy-2-methyl-***E*11**-*ENE*-18Hy	2354	2014	2280	
cis-7,8-epoxy-2-methyl-***E*12**-*ENE*-18Hy	2354	2010	2285	
cis-7,8-epoxy-2-methyl-***E*13**-*ENE*-18Hy	2366	2017	2288	
cis-7,8-epoxy-2-methyl-***E*14**-*ENE*-18Hy	2364	2016	2289	
cis-7,8-epoxy-2-methyl-***E*15**-*ENE*-18Hy	2372	2020	2290	
cis-7,8-epoxy-2-methyl-***E*16**-*ENE*-18Hy	2392	2030	2307	

"+" indicates strong EAD-activity.
"++" indicates very strong EAD-activity.

Enantiomers of *cis*-7,8-epoxy-2-methyloctadec-17-ene were synthesized according to the schemes in Figure 1. In the conversion of **1** to **2**, only 1 mol% of the copper complex catalyst and a minimal amount of NMP was used. Possibly as a consequence, the reaction could not be pushed to completion with excess Grignard reagent, which apparently reacted preferentially with already formed **2** to open the

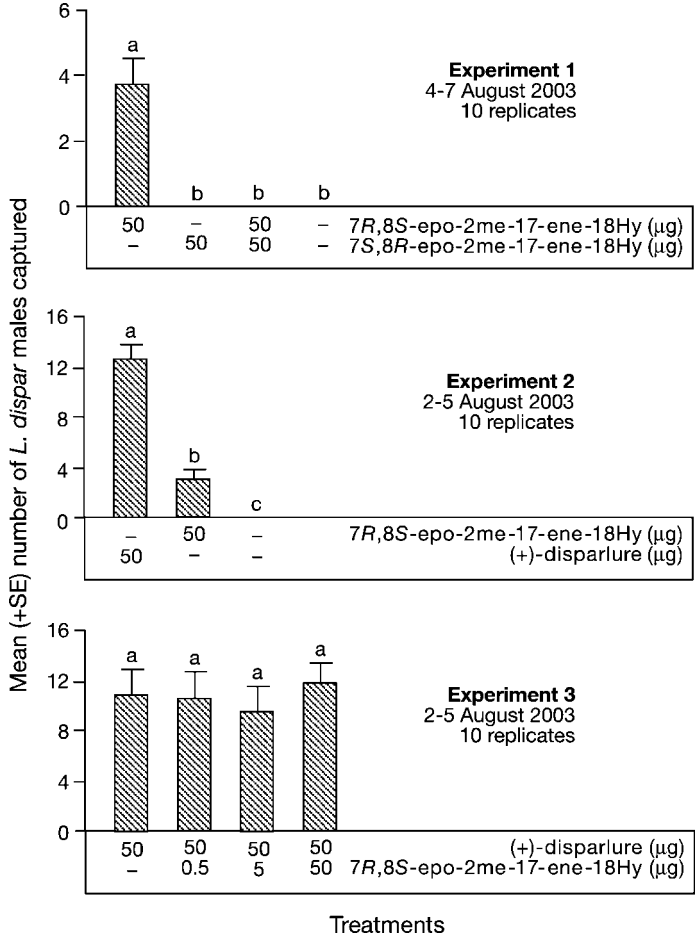

FIG. 4. Captures of male gypsy moths, *Lymantria dispar,* in experiments 1–3 in sticky traps baited with candidate pheromone components [(+)-disparlure = (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane; 7*R*8*S*-epo-2me-17-ene-18Hy = (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadec-17-ene; 7*S*,8*R*-epo-2me-17-ene-18Hy = (7*S*,8*R*)-*cis*-7,8-epoxy-2-methyloctadec-17-ene]; mixed forests near Morioka (Honshu, Japan); in each experiment, bars with different letter superscripts are significantly different, $\alpha = 0.05$.

epoxide ring at the bond distal to the camphorsulfonate. The ring-opened alcohol thus formed could not be separated from **2** by chromatography. However, treatment of the mixture with K_2CO_3 in methanol rapidly and selectively converted the alcohol to the terminal epoxide, which then was easily separated by chromatography. In the conversion of **1**′ to **2**′, with the relative amounts of catalyst and NMP doubled, coupling went to completion, and no ring-opened alcohol was detected.

The biological role of (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadec-17-ene (7*R*8*S*-epo-2me-17-ene-18Hy) is not yet clear. It is present in pheromone glands (Figure 2), but it is not known whether it is released by calling females. By itself it attracted male moths (Figure 4; Exps. 1, 2). However, it neither enhanced attractiveness of (+)-disparlure (Figure 4, Exp. 3; Figure 5, Exp. 4) nor did it affect

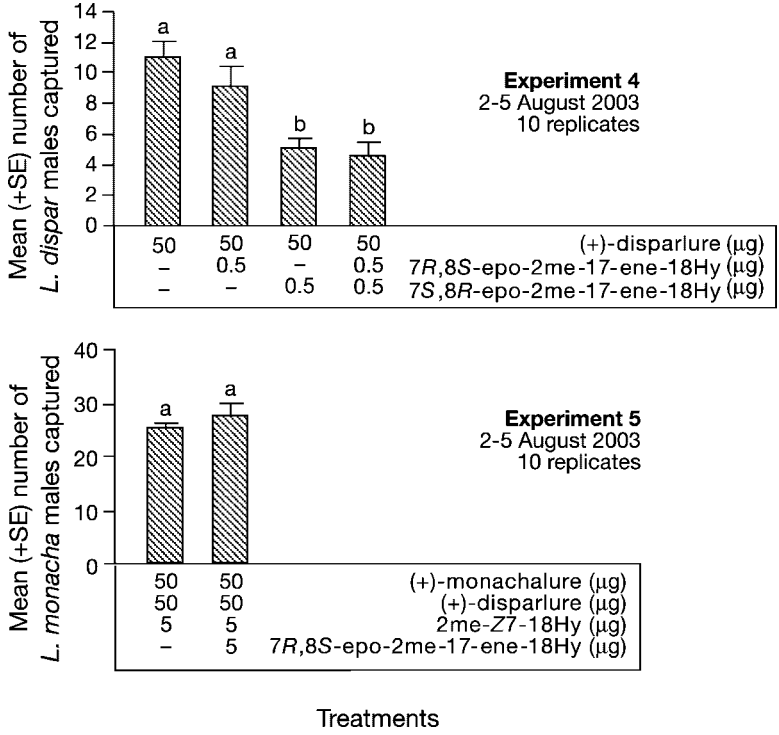

FIG. 5. Captures of male gypsy moths, *Lymantria dispar* (Exp. 4), and male nun moths, *Lymantria monacha* (Exp. 5), in sticky traps baited with candidate pheromonal and allomonal components [(+)-monachalure = (7*R*,8*S*)-*cis*-7,8-epoxy-octadecane; 2me-*Z*7-18Hy = (*Z*)-2-methyl-7-octadecene; other abbreviations as in caption of Figure 4]; Larch (*Larix* spp.) forest near Morioka (Honshu, Japan); in each experiment, bars with different letter superscripts are significantly different, $\alpha = 0.05$.

the specificity of the blend with regard to *L. monacha* (Figure 5; Exp. 5). A discernible effect of 7*R*8*S*-epo-2me-17-ene-18Hy may yet be discovered, if it were tested in a context other than long-range attraction of males (Cameron, 1981), or if it were dispensed from a more suitable release device. In field experiments with codling moths, *Cydia pomonella*, for example, secondary pheromone components failed to affect blend attractiveness when they were dispensed from rubber septa (El-Sayed et al., 1999a), but enhanced blend attractiveness when they were dispensed from a piezoelectric sprayer (El-Sayed et al., 1999b; El-Sayed and Trimble, 2002). It is also possible (although not likely) that the effects of 7*R*8*S*-epo-2me-17-ene-18Hy will only be manifested when this component is tested together with other components found in pheromone gland extracts, such as *cis*-7,8-epoxy-2-methylheptadecane or (7*R*,8*S*)-*cis*-7,8-epoxy-3-methylnonadecane (Figure 2; Gries et al., 1996; unpublished). Alternatively, 7*R*8*S*-epo-2me-17-ene-18Hy may be a trace component that provides insight into pheromone phylogeny, and may serve as a pheromone component or antagonist in *Lymantria* congeners.

Furthermore even though 7*R*8*S*-epo-2me-17-ene-18Hy had no detectable role in the *L. dispar* population of northern Honshu, it may be important in other *L. dispar* populations. In *L. monacha*, for example, (+) -disparlure is the most and least important pheromone component in populations in Bohemia (Europe) and Honshu (Japan), respectively (Gries et al., 2001). This contrasting role of (+)-disparlure may be attributed to reproductive character displacement caused by congeneric *L. fumida* on Honshu (Gries et al., 2001), which utilizes (+)-disparlure as its major pheromone component (Schaefer et al., 1999). Considering the wide distribution of *L. dispar*, and its presence in many different lymantriid communities, there may be some communities in which *L. dispar* maintains specificity of sexual communication by using 7*R*8*S*-epo-2me-17ene-18Hy in addition to, or as a substitute for, (+)-disparlure. Until such communities are found, synthetic (+)-disparlure as a single-component lure remains the best lure for detection of (Asian) *L. dispar.*

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ANOINTING CHEMICALS AND ECTOPARASITES: EFFECTS OF BENZOQUINONES FROM MILLIPEDES ON THE LONE STAR TICK, *Amblyomma americanum*

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Abstract—Many mammals and birds roll on or rub themselves with millipedes that discharge benzoquinones. Chemicals transferred from millipedes onto the integument of anointing animals are thought to deter ectoparasites. We tested the lone star tick, *Amblyomma americanum* (L.), for responses to three widespread components of millipede defensive secretions, 1,4-benzoquinone; 2-methyl-1,4-benzoquinone (toluquinone); and 2-methoxy-3-methyl-1,4-benzoquinone (MMB). In toxicity tests, ticks were confined for 1 hr in filter-paper packets treated with serial dilutions of each of the benzoquinones or the commercial acaricide permethrin. Ticks were least affected by toluquinone, and most affected by permethrin. Of the benzoquinones, only MMB showed repellent activity. Behavioral assays were more sensitive than mortality for measuring the effects of the benzoquinones. Latencies for ticks to right themselves and to climb were greater with all compounds, even at the lowest concentrations, than with controls. Ticks exposed to low concentrations of benzoquinones appeared to recover over time, whereas those exposed to high concentrations exhibited behavioral abnormalities 1–3 mo later. Our results indicate that benzoquinones appropriated via anointing may reduce the tick loads of free-ranging animals,

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although key questions remain on the amounts of these compounds available to and effectively appropriated by anointing animals.

Key Words—Benzoquinones, ectoparasites, ticks, anointing, repellent, toxicant.

INTRODUCTION

A number of vertebrates rub on, or roll in, selected plants, arthropods, and other scent-bearing materials. Chemicals topically appropriated by self-anointing are known or suspected to deter predators, ectoparasites, or microbial pathogens (summarized by Weldon, 2004). Millipedes are used for self-anointing by a variety of birds and mammals. Free-ranging birds in the New and Old Worlds have been observed rubbing these myriapods against their plumage (reviewed in Parkes et al., 2003). Among mammals known to self-anoint with millipedes are Malagasy lemurs (Overdorff, 1993; Birkinshaw, 1999) and Central and South American cebid monkeys (Baker, 1996; Valderrama et al., 2000; Zito et al., 2003). Most identified millipedes used in self-anointing belong to the orders Julida (Harper in Cramp, 1981; Harrup, 1992), Spirobolida (Clunie, 1976; Overdorff, 1993; Zito et al., 2003), or Spirostreptida (Overdorff, 1993; Birkinshaw, 1999; Valderrama et al., 2000). These taxa, which collectively occur throughout temperate and tropical regions worldwide, comprise the "quinone millipedes," so called because they characteristically secrete benzoquinones from their numerous paired segmental glands (Eisner et al., 1978).

Valderrama et al. (2000) reported that the spirostreptidan millipede *Orthoporus dorsovittatus* was used in self-anointing by free-ranging wedge-capped capuchin monkeys, *Cebus olivaceus*, in Venezuela. They hypothesized that mosquitoes are repelled by benzoquinones appropriated from this millipede. To evaluate this hypothesis, Weldon et al. (2003) presented female yellow fever mosquitoes, *Aedes aegypti*, with the two secretory compounds isolated from *O. dorsovittatus*, 2-methyl-1,4-benzoquinone (toluquinone) and 2-methoxy-3-methyl-1,4-benzoquinone (MMB), on nylon-reinforced silicone membranes placed over wells filled with human blood. Mosquitoes landed and fed less frequently, and flew more frequently (a possible indication of repellency), in the presence of membranes treated with benzoquinones than with controls.

Studies with a variety of other insects demonstrate that benzoquinones deter feeding (Loconti and Roth, 1953; Ogden, 1969; Mondal and Port, 1984), act as topical irritants (Ogden, 1969; Peschke and Eisner, 1987), or are toxic (Kanehisa, 1969). Some benzoquinones also have been shown to be noxious or toxic to vertebrates, and to inhibit microbial growth (De Rosa et al., 1994). However, apart from the recent findings with mosquitoes (Weldon et al., 2003), no studies have tested the effects of these compounds on the ectoparasites of tetrapods.

Ixodid (hard) ticks are important pests of a wide range of vertebrates, including mammals and birds (Sonenshine, 1993). Heavy infestations of these

ectoparasites can debilitate or kill even large mammals (Strickland et al., 1976), but tick-borne diseases caused by bacteria (e.g., rickettsiae, spirochetes), viruses, and protozoa may pose an even greater danger to wildlife (Strickland et al., 1976; Sonenshine, 1993). In the New World tropics and subtropics, wounds caused by tick bites can serve as entry sites for flesh-eating and potentially deadly screwworms, *Cochliomyia hominivorax* (Strickland et al., 1976). Ticks, therefore, exert significant selective pressure on host species.

We report here on the behavioral responses and morbidity of the lone star tick, *Amblyomma americanum* (L.), to three compounds that occur widely in the defensive secretions of millipedes, 1,4-benzoquinone; toluquinone; and MMB (Eisner et al., 1978) and, for comparative purposes, to permethrin, a commercially available synthetic pyrethroid used to repel and kill ticks (summarized by Sonenshine, 1993). *A. americanum* ranges from the south-central and southeastern United States northward along the Atlantic seaboard to New England (Keirans and Durden, 1998; Guglielmone et al., 2003). In order to complete its life cycle, *A. americanum* must find a host three times and remain attached for several days each time (Strickland et al., 1976). The genus *Amblyomma* is predominantly tropical and subtropical in distribution (Hoogstraal, 1973; Hoogstraal and Aeschlimann, 1982), and includes species known to parasitize the monkeys *C. capucinus* and *C. olivaceus* (Fairchild et al., 1966; Jones et al., 1972), which are known to self-anoint with benzoquinones. Various *Amblyomma* species feed on birds (Strickland et al., 1976).

METHODS AND MATERIALS

Subjects. Nymphs of *A. americanum* were obtained from a colony at the United States Department of Agriculture, Agricultural Research Service, Knipling-Bushland US Livestock Insects Laboratory, Kerrville, TX. They were maintained at 23–24°C, ≈99% RH, and on a photoperiod of 16:8 hr (L:D). At the time of testing, ticks had been in the nymphal stage 5–8 wk.

Chemicals. 1,4-Benzoquinone and toluquinone were obtained commercially (Sigma-Aldrich, St. Louis, MO). MMB was synthesized, as described by Weldon et al. (2003). Permethrin was obtained from FMC Corporation (Philadelphia, PA). Solutions of 7.8, 31.0, 125, and 500 mM in acetone were prepared for each compound; an additional solution of 1.9 mM was prepared for permethrin. This dilution series was chosen, on the basis of results from preliminary studies, to include threshold concentrations; the lower dilution of permethrin was included because this compound appeared to be more potent than the benzoquinones we used. Solutions were kept refrigerated (5°C) and removed only for bioassay.

Repellent Bioassay. Repellency tests were conducted on a plastic Petri dish (5.5 cm diam, 1.5 cm high) containing a flat layer of modeling clay 0.5 cm deep, and glued centrally within a larger (9 cm diam, 1.3 cm high) dish. Water (≈0.5 cm deep) was added to the space between the concentric walls of the two Petri dishes, creating a moat to contain ticks. A 2 ×10-cm rectangle was marked with a lead

pencil on a 3 × 11-cm rectangular piece of filter paper (Whatman No. 4), 1 cm from a long and short edge. The filter paper was placed into a glass Petri dish (15 cm diam, 2 cm high), and 165 μl of either solvent (acetone) or test solution were evenly applied by a pipettor to the 2 × 10-cm rectangular area. After drying for 10–15 min, the paper was rolled crosswise and taped together to form a short cylinder (≈3.2 cm diam, 3 cm high). The treated portion of the filter paper formed a continuous band around the inside surface of the cylinder. The untreated edge of the filter paper cylinder was pushed ≈3 mm into the central area of the modeling clay, allowing a ≈7 mm high band of untreated filter paper surmounted by the 2-cm high band of treated filter paper to project above the clay.

Three groups of 10 ticks were tested with acetone and a 500 mM solution of each compound. MMB also was tested as a 125 mM solution. A different filter paper cylinder was used for each group of ticks. Ten active and ostensibly healthy nymphs were placed into a Teflon-lined plastic test tube (9 cm deep). The ticks were dumped from the tube onto the clay in the center of the filter paper cylinder. The water moat confined ticks that climbed out of the filter paper cylinder. The locations of ticks were recorded at 15 min after they were released. Ticks on the clay or on the untreated lower portion of filter paper inside the cylinder were considered repelled.

Exposure Packets. The process by which ixodid ticks find attachment sites and begin to feed generally takes several hours. Thus, in the absence of strong repellency, ticks would have extended exposure to benzoquinones on pelage or plumage, which we attempted to simulate to evaluate its possible physiological consequences on ticks. A 5 × 6-cm rectangle of Whatman No. 4 filter paper was marked with a lead pencil with lines 0.5 cm from each side. The filter paper was placed into a glass Petri dish and 165 μl of solvent or one of the test solutions (7.8, 31.0, 125, and 500 mM, and for permethrin, 1.9 mM) were applied evenly with a pipettor to the 4 × 5-cm rectangle bordered by the pencil lines. After the filter paper had dried for 10–15 min, it was folded crosswise. A bulldog clip (5.3 cm wide) closed each of two margins of the folded filter paper. Ten active nymphs were placed into the cavity formed by the folded paper, and another bulldog clip was attached to the open end of the packet to enclose the ticks. The clips were affixed slightly more than 0.5 cm from the edges of the paper, so that the confined ticks could only contact the treated area of the filter paper. The packet holding the ticks was kept for 1 hr in a glass desiccator jar (≈2 l) containing water (below the shelf) to maintain high humidity (>95% RH). The jars were rinsed with water after each exposure. Five replicates of 10 ticks each were prepared for each concentration–compound combination.

Righting and Climbing Responses. The packet was removed from the desiccator, opened, and the 10 nymphs were removed singly with forceps. Each tick was placed on its dorsum on clay within an encircling cylinder of untreated filter paper similar to that used for the repellent test. As with the repellent test, the clay arena was situated in a moated Petri dish. To prevent ticks from using the forceps

as leverage to right themselves as they were released, they were grasped so that their legs did not contact the forceps. The number of ticks that were right-side-up, and the number of ticks that had climbed to the rim of the filter paper cylinder or beyond, were recorded at 0.5, 1, 5, 10, and 15 min after release. After 15 min, the ticks were placed into a plastic vial with holes in the cap and returned to a regime of 23–24°C, 99% RH and a photoperiod of 16:8 hr (L:D). We refer to these trials below as 0-hr trials because they immediately followed the 1-hr exposure to one of the solutions. To evaluate how long the exposure to the benzoquinones and permethrin affected the nymphs, the behavioral tests were repeated with the ticks that were used in the last three trials at 24 hr (referred to as 24-hr trials) and again 1–3 mo after exposure (1–3-mo trials). Only the ticks exposed to the acetone (control) and 125 mM solutions were used in the 1–3-mo trials.

Toxicity. At 24 hr after exposure in filter paper packets, the survivorship of ticks was determined by observing the ticks in a moated Petri dish. Ticks moving only in an uncoordinated manner and a distance of <5 mm were considered moribund. Ticks that failed to move after 5 min in the Petri dish, even when exhaled upon or prodded with forceps, were considered dead. The behavioral test was repeated 24 hr after exposure with ticks in the last three replicates. In the analysis, we combined dead and moribund ticks because ticks in neither category would pose a risk to hosts. Ticks that were alive 24 hr after exposure were again examined for toxic effects 1–3 mo later. The criteria for mortality used in the toxicity tests were applied to these ticks to determine whether they should be excluded from the trial.

Statistical Analysis. We used logistic regression (Proc Genmod in SAS, 1999) to analyze the repellency of the compounds at 500 mM at 15 min. Since MMB repelled all ticks, we subtracted 0.5 from one of the scores (changing 10/10 to 9.5/10) to obtain meaningful parameter estimates. We estimated four one-degree-of-freedom contrasts with the acetone control.

Data on the number of ticks that were dead or moribund (incapacitated) 24 hr following the 1-hr exposure were modeled as dose–response relationships by using the arcsine-transformed proportion of dead and moribund ticks as the dependent variable and the square root of concentration as the independent variable. The square-root transformation of concentration yielded a straight-line relationship for all compounds except permethrin. We modeled permethrin only at the lowest concentrations, and set the percent of incapacitated ticks to 100% for concentrations >7.8 mM. Since the bioactivity of the benzoquinone solutions appeared to deteriorate over time, and the first trial on each group of 10 ticks was conducted from 24 to 104 days after the solutions were prepared, we also included the age of solution in the model for incapacitated ticks and for the models of behavioral change (discussed subsequently). We used the log of age $-$ log (66) (66 days was the mean solution age) to reduce colinearity with the intercept term. We used the Proc Mixed routine in SAS (1999) for this analysis.

The behavioral data were not independent because each group of 10 ticks was observed five times within a concentration–compound combination on two different criteria and, for some groups, on three trials (0 hr, 24 hr, 1–3 mo following the initial 1-hr exposure). We found that the relationship between the arcsine-transformed proportion of ticks exhibiting the behavior (excluding incapacitated ticks) and the log of time was approximately linear. Therefore, for each of the six behavior-trial combinations (two behaviors, three trial times), we estimated a slope and an intercept (and their standard errors) for each concentration-compound combination. In effect, we modeled each group of 10 ticks with a straight line that relates the proportion (transformed scale) exhibiting the behavioral measure to observation time (transformed), yielding six lines (two behaviors, three trials) per group. For the righting behavior, we used all five observation time points to construct the lines, but for climbing behavior we used only the last four time points because the proportion for the first time point was zero for all groups.

We then created one degree-of-freedom contrasts (as t-values) between the control groups, which were modeled in the same way for each of the treatment groups. Each pair of contrasts compared the control intercept and control slope with the intercept and slope from one of the treatments. The magnitude and sign of the pair of contrasts indicate how the treatments differed from controls, with absolute values less than about two indicating no statistical difference. A positive value for an intercept contrast indicated that controls were more active at the earliest observation time. A positive value for a slope contrast indicated that, over the 15 min period, the proportion of ticks exhibiting the behavior in the treatment group increasingly lagged behind that of the control, i.e., differences between the treatment and control groups increased over time.

A multiple comparison adjustment was not required when comparing treatment effects within one of the three trials (0 hr, 24 hr, and 1–3 mo) and within one of the two behavioral measures. However, analyses of the two behaviors were correlated (ticks cannot climb if they have not first righted), as were analyses over the three trials (repeated measures of some ticks). Rather than present a recondite analysis with the data modeled jointly using a complicated covariance structure (to incorporate the time series structure and other potential dependencies), we present unadjusted t-values for the slopes and intercepts, which readers can interpret conservatively, and focus attention on their patterns.

Not all contrasts with controls could be made for two reasons. Because only live ticks were used to calculate these values for the 24-hr and 1–3-mo trials, there were some treatment combinations with no live ticks, and, thus, no possible estimate. In other cases, especially for higher concentrations, no living ticks exhibited the behavior for the entire 15 min observation period. In that case, a flat line of zero was estimated (with no variance), so a t-value based on a comparison with the control would be inappropriate. Model fitting was performed by using the R software (Ihaka and Gentleman, 1996), output was parsed, and t-values calculated using Perl programs. Other statistical comparisons were performed with standard t-tests.

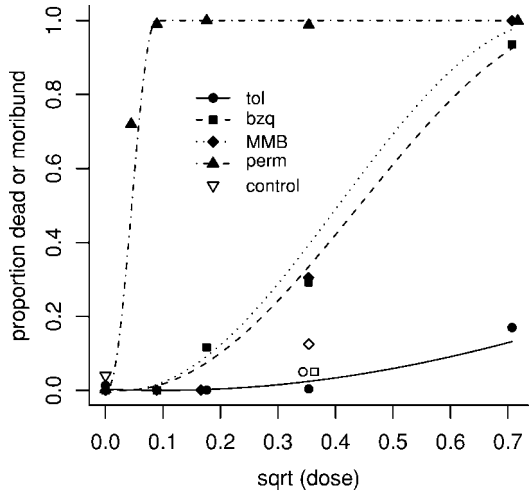

FIG. 1. Dose–response curves for incapacitation (morbidity and mortality) of *A. americanum* nymphs 24 hr after 1-hr exposure to benzoquinones or permethrin. The open symbols represent values for ticks tested 1–3 mo later. The compounds indicated are toluquinone (tol); 1,4-benzoquinone (bzq); 2-methoxy-3-methyl-1,4-benzoquinone (MMB); and permethrin (perm).

RESULTS

Repellency. Of the benzoquinones, only MMB repelled *A. americanum* nymphs ($\chi^2 = 17.56$, $P < 0.001$, 1 *df*). MMB repelled all nymphs at 500 mM (25.0 mM/cm^2), but none at the 125 mM (6.3 mM/cm^2) treatment. At 500 mM, permethrin repelled 63% of the nymphs, significantly more than the control ($\chi^2 = 14.94$, $P = 0.001$, 1 *df*).

Toxicity. Dose–response curves for incapacitation of *A. americanum* nymphs, adjusted to the mean solution age of 66 days and back-transformed to the original proportion scale, are shown (Figure 1). The model (on the transformed scale) is $t(p_i) = 0.982 + \beta_i \sqrt{\text{dose}} - 0.248 \log(d)$, where $t(p)$ gives the expected value of the arcsine-transformed proportion of incapacitated ticks, i indexes compounds, β_i estimates (standard errors) are 1.911 (0.189) for 1,4-benzoquinone, 2.084 (0.189) for MMB, 0.609 (0.189) for toluquinone, and 18.482 (1.482) for permethrin (only modeled for concentrations <7.8 mM), and d +66 is the number of days since the solution was prepared. The proportions of ticks incapacitated 1–3 mo after they were exposed to the 125 mM 1,4-benzoquinone treatment and acetone control also are depicted as open symbols in Figure 1.

Toluquinone was less toxic than either 1,4-benzoquinone or MMB ($P <$ 0.001, *t*-test, 84 *df*), with the latter two statistically indistinguishable ($P = 0.74$, *t*-test, 84 *df*). Only 2% of ticks exposed to 7.8 mM permethrin were alive and

active 24 hr after exposure. At this concentration, the other compounds had little or no effect. For 1,4-benzoquinone and MMB, the proportion of ticks incapacitated decreased 1–3 mo later, as some of the ticks moribund at 24 hr recovered. All ticks classified as moribund at 24 hr after exposure to permethrin died within 1–3 mo. The proportion incapacitated 1–3 mo later changed little for toluquinone.

Behavioral Responses—Righting. In general, slope differences between control and treatment groups were not as great as intercept differences (Figure 2). Thus, the proportion of ticks righting at the beginning of testing differed between the control and treatment groups, but righting rates following the initial observation were roughly the same for the remainder of the observations. In general, these contrasts mirrored the toxicity statistics, with larger t-values for higher doses within a compound and the smallest t-values for toluquinone, the least toxic compound. Many of the intercept t-values were quite large and all were >3 for the first trial (0 hr), even for toluquinone, indicating an effect of all compounds at all doses

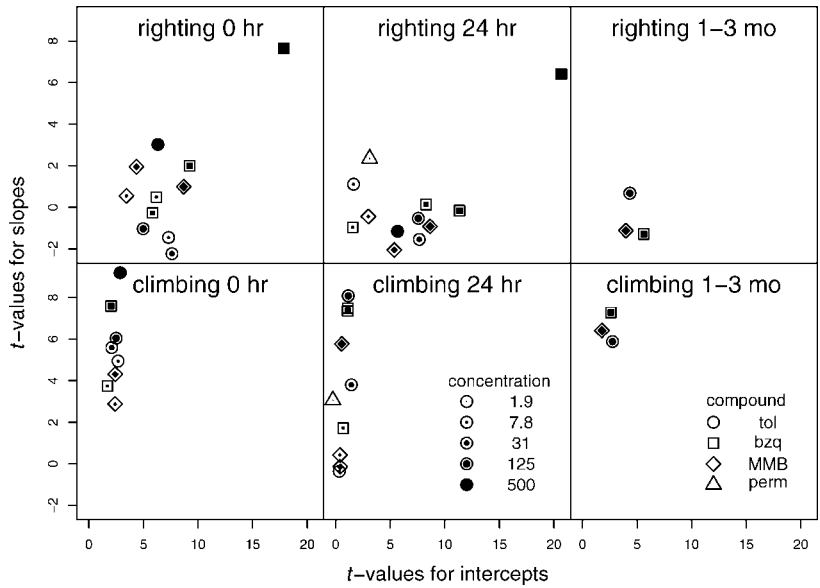

FIG. 2. Contrasts (as t-values) between treatment and control groups for the righting and climbing responses and for three trial times for the behavioral tests were calculated from regressing the arcsine-transformed proportion of ticks exhibiting the behavior on the log of time since the test started. Each point is associated with an intercept contrast (on abscissa) and slope contrast (ordinate). Increased filling of symbols indicates higher concentrations (mM). The greater the t-value differs from zero, the greater the behavioral difference between the treated and control group, with significant differences greater than about two. Not all treatment/dose combinations are given (see text).

on the righting behavior. One day later, some ticks appeared to have recovered at the lower concentrations, where the intercept *t*-values had moved toward 0 for all compounds, even permethrin. However, for higher concentrations, *t*-values for intercepts increased, indicating that the effect on ticks at these concentrations is more pronounced after 24 hr than immediately after exposure. There is evidence of continued recovery for the trials conducted 1–3 mo later: intercept *t*-values for compounds at these concentrations decreased relative to righting at 24 hr.

Behavioral Responses—Climbing. In contrast to righting, *t*-values were largest for slope contrasts (Figure 2). This is readily understood since intercepts, the estimates for 0 min, should be near 0 because all individuals, regardless of treatment, first had to right. However, intercept *t*-values did shrink from values close to 2.5 in the 0 hr trials to close to 0 in the 24-hr trials. Thus, earlier small, but consistent, differences in initial observations nearly disappeared 24 hr later.

In contrast to the intercept *t*-values, the slope *t*-values were large for almost all compounds at all concentrations. A clear concentration effect is evident, as the *t*-value order closely follows that of concentration. This indicates that the latency (or inability) to climb becomes more obvious over the 15-min observation period and depends on concentration. After 24 hr, there appears to be some return to control levels for low concentrations, as in the righting data. However, even after 1–3 mo, large lags in the climbing behavior of the ticks receiving the 125 mM concentration were evident. These individuals appeared to have suffered long-lasting effects from exposure to the compounds months earlier. With few exceptions, the differences between compounds were not as pronounced as differences between doses for both kinds of behavioral data. The highest concentrations generally are not depicted in Fig. 2 for either behavioral response because most ticks at these concentrations were incapacitated, and these contrasts could only be made for ticks that were not dead or moribund.

DISCUSSION

The phenomenon of anointing is unique among allelochemical interactions because organisms may respond in nature to chemicals from species with which they ordinarily do not interact (Weldon, 2004). Millipedes rarely and inconsequentially encounter tetrapod ectoparasites, but the responses of such ectoparasites to millipede chemicals may attain ecological relevance through the vehicle of avian and mammalian anointing. Our study is predicated on the assumption that ticks encounter benzoquinones deposited on the plumage or pelage of anointing animals.

The preprandial behaviors of ixodid ticks differ from those of mosquitoes and most other biting flies in ways that dispose ticks to receive greater exposure to anointing chemicals. Ixodids are highly selective in identifying feeding sites

(Waladde and Rice, 1982), wandering extensively on the skin before locating a suitable site of attachment (Kemp et al., 1982). Ticks crawling through the pelage or plumage of a host would be surrounded by anointed surfaces, encountering chemicals by contact or, in the case of volatiles, as fumigants. After penetration of their host's integument, many ticks secrete a hardened encasement around their feeding appendage, the hypostome, and proceed to engorge over a few days. Thus, the 1-hr confinement of ticks in treated filter paper packets during our bioassays was not excessively protracted in simulating chemical exposure to an anointed host.

A repellent, by definition, elicits movement away from a chemical source (Dethier et al., 1960). Our repellency bioassay allowed ticks to either crawl away from a treated area or fall off a vertical surface to avoid contact with repellent treatments. Only MMB at the highest dose we used (500 mM) prevented ticks from crawling across treated filter papers; the next lower dose (125 mM) of this compound had no detectable effect. These results are worth noting in light of numerous reports indicating that insects are irritated or repelled by benzoquinones (e.g., Ogden, 1969; Peschke and Eisner, 1987). Nonetheless, MMB was significantly more effective in preventing *A. americanum* from climbing out of treated filter paper cylinders than was permethrin, a synthetic pyrethroid marketed as a tick repellent and acaricide. Several ticks dropped from the permethrin-treated filter paper, whereas the ticks tended not to crawl on the MMB-treated filter paper. The repellency of permethrin may be due as much to its fast-acting toxicity requiring contact with treated surfaces as to actual avoidance (summarized in Sonenshine, 1993).

The morbidity/mortality of ticks in response to the compounds we used was greatest for permethrin, which was effective even at 1.9 mM. Morbidity/mortality in response to benzoquinones was manifest only at intermediate and high doses (125 and 500 mM), but ticks exposed even to the most dilute concentrations of these chemicals exhibited persistent impairments in righting and climbing abilities. The clearest behavioral difference in response to the three benzoquinones we tested, and among the doses of each, was manifest with respect to climbing. Climbing ability, an integral part of host acquisition by many ixodid ticks (Lees and Milne, 1951; Sonenshine, 1993), was found impaired upon retesting ticks more than 1 mo after exposure in our tests. Climbing inhibition would seriously impair a tick's ability to contact a suitable host, and may be a manifestation of a more basic behavioral or physiological impairment. The toxicities of toluquinone and other benzoquinones also have been demonstrated with insects (Kanehisa, 1969). Mosquitoes (*Ae. aegypti*) exposed to toluquinone and MMB on feeding membranes sometimes became immobilized and died, especially with MMB (Weldon et al., 2003).

The benzoquinones were not as toxic to ticks as permethrin. However, ticks exposed for 1 hr to benzoquinones even at low concentrations behaved abnormally, and intermediate concentrations clearly were toxic. Other than MMB, these

compounds do not seem to repel ticks, suggesting that, for protection against ticks, anointing animals must apply sufficient quantities of compound to disorient or physically disable potential tick parasites. Tests of climbing produced a clearer pattern than righting for detecting the behavioral effects of the compounds and their concentration effects. The climbing test also better showed concentrations at which effects were temporary and at which they were long-lasting. Both measures demonstrated that even low concentrations adversely affected these two behaviors important for successful host attachment.

The toxicities of benzoquinones demonstrated in our study of *A. americanum* indicate the potential for protection against ticks afforded by anointing with these compounds. Key questions remain, however, on the quantities of benzoquinones available to and effectively appropriated by free-ranging animals. Estimates of the quantities of benzoquinones produced by millipedes range up to 350 mg per individual for some tropical species (Fairhurst, 1993). These compounds may be appropriated from other sources, as well. Laughingthrushes (*Garrulax* spp.) from Fiume, Italy (currently Rijeka, Croatia), for example, are reported to anoint with tenebrionid beetles of the genus *Blaps* (Callegari, 1955), which may defensively discharge benzoquinones (Blum, 1981).

Because attachment and commencement of feeding in ixodid ticks takes hours and completion of feeding is a matter of several days, the exposure that ticks receive to chemicals applied to the integument of their hosts is protracted. Repeated anointment may be necessary to maintain the efficacy of topically applied compounds due to their volatility and/or degradation. We detected a reduced efficacy of the benzoquinones used in our tests over time, even though solutions were stored in cold, light-free conditions; this prompted us to include a time covariate for solution age when modeling the data. Quantitative studies of the transfer and retention of anointing chemicals, and of their efficacy in deterring ectoparasites on hosts, are needed.

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ANTENNAL AND BEHAVIORAL RESPONSES OF GRAPEVINE MOTH *Lobesia botrana* FEMALES TO VOLATILES FROM GRAPEVINE

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Abstract—Grapevine moth *Lobesia botrana* is the economically most important insect of grapevine *Vitis vinifera* in Europe. Flower buds, flowers, and green berries of Chardonnay grapevine are known to attract *L. botrana* for oviposition. The volatile compounds collected from these phenological stages were studied by gas chromatography—mass spectrometry, and the antennal response of *L. botrana* females to these headspace collections was recorded by gas chromatography—electroantennography. The compounds found in all phenological stages, which consistently elicited a strong antennal response, were pentadecane, nonanal, and α-farnesene. In a wind tunnel, gravid *L. botrana* females flew upwind to green grapes, as well as to headspace collections from these berries released by a piezoelectric sprayer release device. However, no females landed at the source of headspace volatiles, possibly due to inappropriate concentrations or biased ratios of compounds in the headspace extracts.

Key Words—*Lobesia botrana*, host plant volatiles, kairomone, upwind attraction, wind tunnel, GC–EAD, electrophysiology, headspace collection, chemical analysis.

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INTRODUCTION

Larvae of grapevine moth *Lobesia botrana* (Denis and Schiffermüller) (Lepidoptera, Tortricidae) feed on all developmental stages of grapevine *Vitis vinifera*, and occur in up to four generations throughout the growing season. Fungi, especially grey mold *Botrytis cinerea*, develop rapidly on the damaged grapes, causing entire clusters to rot. Grapevine moth is the most damaging insect throughout the European wine-growing area (4 million ha) as well as adjacent Mediterranean countries in North Africa and Asia Minor (Bovey, 1966; Fermaud and Le Menn, 1989; Gabel and Roehrich, 1995).

The female sex pheromone of *L. botrana* and male attraction to synthetic pheromone have been studied in detail since the seventies (Roelofs et al., 1973; Arn et al., 1988; El-Sayed et al., 1999). Today, pheromone-based disruption of mating is used on ca. 50,000 ha in Europe. However, the mating-disruption technique is not efficient at high population densities (Arn and Louis, 1996; Louis and Schirra, 2001; Varner et al., 2001). One important drawback is that only male behavior is affected, and not the behavior of gravid females. The efficacy of pheromonal methods may accordingly be enhanced by kairomonal chemicals affecting also female host-finding and egg-laying behavior, such as plant volatile compounds (Light et al., 2001; Bengtsson et al., 2001).

Volatiles from grape have been widely studied, especially with respect to aromas of flowers and ripe fruit (Schreier et al., 1976; Welch et al., 1982; Buchbauer et al., 1994, 1995; Rosillo et al., 1999; Darriet et al., 2002). Floral volatiles of cv. Müller-Thurgau have been shown to modify oviposition behavior in grapevine moth females (Thiéry and Gabel, 2000).

We demonstrate here that upwind flight attraction of female grapevine moth *L. botrana* is mediated by grape volatiles. As a first step towards the identification of host plant signals that mediate female reproductive behavior, we have used insect antennae to screen headspace collections from grape leaves, flower clusters, and berries for bioactive compounds.

METHODS AND MATERIALS

Headspace Collections. Headspace collections were made from branches with leaves (3–4 wk after bloom), flower clusters before bloom, clusters during early bloom (10–30% blossoms open), clusters at full bloom (100% blossoms open), and grape berries (3–4 wk after bloom) of the cv. Chardonnay. Five samples per phenological stage were analyzed by coupled gas chromatography and mass spectrometry (GC–MS), and six samples per developmental stage were analyzed by coupled GC and electroantennographic detection (GC–EAD).

Freshly cut branches or grape clusters (ca. 200 g) were placed in to a 2000-ml glass jar. The cut end of the clusters was in a 10-ml vial with water. Charcoal-filtered

air was pulled through the jar at 150 ml/min and over a Porapak Q cartridge containing 50 mg of adsorbent (Sigma-Aldrich). Collections were done for 22 hr in a climatic chamber at 25 ± 2°C and 30 lux. Volatiles were desorbed by eluting the cartridge with 500 μl hexane (redistilled; LabScan, Malmö, Sweden), and 500 ng heptyl acetate (99.8% chemical purity) were added as an internal standard. Sample volumes were reduced to 50 μl at room temperature in vials with an elongated tip (5 cm × 2 mm ID; Klimetzek et al., 1989). Samples were sealed in glass vials and stored at −18°C.

Solid Phase Microextraction. A manual solid phase microextraction (SPME) holder (Supelco, Bellefonte, PA) with a fiber coated with 100 μm of polydimethylsiloxane was used. The fiber was conditioned for 30 min in a GC injection port at 250°C. Meanwhile, flower clusters (early bloom) were placed in to a 2000-ml glass jar. Cluster stems were provided with water to maintain the physiological water content. The fiber was then placed into the jar above the plant material for 22 hr. After sampling, the fiber was immediately desorbed for two min in a GC injection port.

Gas Chromatography and Mass Spectrometry. Grape volatiles were identified with an Hewlett-Packard 5970B MS with electron impact ionization (70 eV), which was interfaced to an HP 5890 gas chromatograph, equipped with an HP-INNOWax column (30 m × 0.2 mm) with an oven temperature program of 5 min at 50°C, 10°C/min to 60°C, 1 min at 60°C, 2.5°C/min to 200°C, 30 min at 200°C.

Gas Chromatography and Electroantennographic Detection (GC-EAD). An HP 5890 GC, using both an HP-INNOWax (30 m × 0.2 mm) and a DB-Wax (30 m × 0.25 mm; J&W Scientific, Folsom, CA) capillary column, programmed from 60°C at 8°C/min to 220°C (10-min hold), was interfaced with an electroantennogram apparatus (Arn et al., 1975). The outlet of the GC column was split in a 1:1 ratio between the flame ionization detector (FID) and a cut antenna of a *L. botrana* female. The antenna was mounted in a holder, and the cut ends rested in two wells containing Beadle–Ephrussi Ringer solution. Compounds eluting from the capillary column were delivered to the antenna through a glass tube (12 cm × 8 mm) by a charcoal-filtered and humidified airstream. The antennal signal and the FID signal were amplified and recorded simultaneously using Syntech (Hilversum, The Netherlands) software.

Chemicals. The identity of most compounds in volatile collections was verified by comparison with purified, authentic plant compounds, and synthetic compounds. Compounds were purchased from Carlo Erba Reagenti (Milan, Italy), Sigma-Aldrich, Bedoukian Research (Danbury, CT), Firmenich (Geneva, Switzerland), Fluka, and Shin-Etsu Chemical Co. (Tokyo, Japan), or obtained as gifts from Hans Alborn (Gainesville, FL), Anna-Karin Borg-Karlson and Ilme Liblikas (Stockholm, Sweden), and Wittko Francke (Hamburg, Germany) (see Table 1). Compounds, which did not elicit antennal responses, and for which no standards were available, were tentatively identified using the Wiley mass spectral database.

TABLE 1. VOLATILE COMPOUNDS IN HEADSPACE VOLATILES FROM GRAPEVINE (CV. CHARDONNAY) AT DIFFERENT PHENOLOGICAL STAGES[a]

Compound	Source of standards[b]	Leaves[c] PQ[h]	Flower buds[d] PQ	Early bloom[e] PQ	Early bloom[e] SPME[i]	Full bloom[f] PQ	Green berries[g] PQ
Acids							
acetic acid	SA	1	1				2
hexanoic acid	SA	1					<1
ethylhexanoic acid	SA	20					4
Hydrocarbons							
tetradecane	F			1	1		
1-tetradecene*	W	3		1	2		
pentadecane	F	**2**	**2**	**23**	**15**	**11**	**9**
1-pentadecene	F	**1**		**4**	**5**	**6**	
heptadecane	F	**1**		**4**	**1**		**9**
1-heptadecene	F			**2**	**1**		**16**
octadecane	F	**3**		**3**	**2**		
nonadecane	F	**3**		**3**	**2**		
1-nonadecene	F			**4**	**3**		
eicosane	F					7	
heneicosane	F			**3**	**1**	**7**	
Alcohols							
ethanol	CE						2
1-butanol	F						4
(Z)-3-hexen-1-ol	SA						**12**
(*E*)-3-hexen-1-ol	SA						10
3-methylbutanol	F						3
1-hexadecanol	F			**1**	**1**	**4**	
1-heptadecanol	F			**1**	**1**	**2**	
1-octadecanol	F			**1**	**1**	**5**	
Aldehydes							
hexanal	F	2		2	2		2
(*E*)-2-hexenal	SA	3					4
(Z)-3-hexenal	SA	**1**				**29**	
(*E*,*E*)-2,4-hexadienal*	W						1
nonanal	SA	**2**	**4**	**6**	**5**	**3**	**2**
(*E*)-2-nonenal*	W	1				3	1
(*E*,*Z*)-2,6-nonadienal	SA					1	
Ketones							
2-undecanone	F			**3**	**3**	**4**	
2-dodecanone	F			**2**	**2**	**3**	
2-tridecanone	F			**3**	**2**	**5**	
2-tetradecanone	F					3	
Esters							
methyl acetate*	W					2	12
ethyl acetate	CE						2
butyl acetate	CE						3
(Z)-3-hexenyl acetate	SA					**24**	14
methyl hexadecanoate*	W		<1	2	1	2	18

TABLE 1. CONTINUED

Compound	Source of standards[b]	Leaves[c] PQ[h]	Flower buds[d] PQ	Early bloom[e] PQ	Early bloom[e] SPME[i]	Full bloom[f] PQ	Green berries[g] PQ
Aromatics							
1-methylethyl benzene*	W			1	1	1	
propyl benzene*	W			<1	<1	2	1
methyl benzoate	F						19
benzyl alcohol	CE	2	2	<1	3	2	3
benzaldehyde	SA						1
methyl salicylate	SA	**32**		**<1**	**2**	**5**	
Irregular terpenoids							
$\beta-$ionone	F	1					
4,8-dimethyl-1,(*E*)3,7-nonatriene	WF	**37**				**10**	
Monoterpenes							
limonene	SA		**10**	**3**	**1**	**1**	
(*Z*)-ß-ocimene	F					4	
(*E*)-ß-ocimene	HA	**27**	**16**	**1**	**tr**	**0,5**	
$\alpha-$phellandrene	SA					1	
$\alpha-$terpineol	SA	13				4	
geraniol	SA	2					
linalool	SA						12
Sesquiterpenes							
$\beta-$caryophyllene	FI		**1**	**5**	**4**	**4**	
humulene	SA			2	1	3	
germacrene-D	AB	42					
methyl farnesoate*	W		1			4	
(*E*)-β-farnesene	B			**49**	**36**	**42**	
(*Z*,*E*)-α-farnesene	AB	**5**				**3**	**1**
(*E*,*E*)-α-farnesene	FI	**100**	**100**	**100**	**100**	**100**	**100**

Notes: Compounds in bold face type elicited responses from antennae of female grapevine moths in GC-EAD experiments (see Table 2). Identifications of asterisked compounds are tentative only.

[a] Amounts relative to (*E*,*E*)-α-farnesene. The average amount of (*E*,*E*)-α-farnesene collected from 200 g of clusters in full bloom and from 200 g of green berries was 0.25 ± 0.1 and 0.28 ± 0.15 μg/h, respectively ($N = 5$).

[b] Standards were obtained from Hans Alborn, Gainesville, FL (HA), Bedoukian Research Inc., Danbury, CT (B), Anna-Karin Borg-Karlson, Stockholm, Sweden (AB), Carlo Erba Reagenti, Milan, Italy (CE), Firmenich, Geneva, Switzerland (FI), Fluka (F), Wittko Francke, Hamburg, Germany (WF), Ilme Liblikas, Stockholm, Sweden (IL), and Sigma-Aldrich (SA). Compounds which were not available were tentatively identified according to the Wiley mass spectra library (W).

[c] Branches with leaves ($N = 5$).

[d] Clusters with flower buds ($N = 5$).

[e] Clusters with 10–30% flowers open ($N = 5$).

[f] Clusters with all flowers open ($N = 5$).

[g] Green berries, 5 wk after flowering, during the second generation of grapevine moth ($N = 5$).

[h] Porapak Q air filters.

[i] Solid phase microextraction, using clusters at early bloom.

Insects. A laboratory culture of grapevine moth was maintained on a semi-synthetic diet (Mani et al., 1978). Insects were collected in vineyards in Trento, Italy. Larvae and pupae were reared under a 16L:8D photoperiod and 22°C. Pupae were separated by sex and emerged were adults provided with sugar-water solution. Virgin females used for GC–EAD recordings were 2–3-days old.

Females used in wind tunnel bioassays were 2–4-days old. After eclosion, females were mated in 33 × 33 × 33-cm^3 Plexiglass cages. Their mating status was verified by placing them into glass tubes (12.5 cm × 2.5 cm^2); females laying eggs were used for wind tunnel experiments.

Wind Tunnel Bioassay. The Plexiglass wind tunnel had a flight section of 63 × 90 × 200 cm^3. Air was blown by a horizontal fan (Fischbach, Neunkirchen, Germany) at 30 cm/sec through activated charcoal filters (Camfil, Trosa, Sweden) into the tunnel (Arn et al., 1986). The flight section was lit diffusely from the side at 10 lux, and the room was kept at 23 ± 2°C and 40–60% RH.

Female moths were placed singly in to glass tubes (12.5 cm × 2.5 cm^2). The females were tested for 15 min, using batches of 10–16 females per test stimulus, during the first 2 hr of the scotophase. The glass tubes were positioned at the downwind end of the tunnel, 30 cm from the floor and ca. 180 cm from the source. The tubes were baked at 375°C for 8 hr before use.

Plant material and headspace collections were applied as odor sources, at the upwind end of the wind tunnel. Unripe grape clusters (ca. 150 g, berries 5–10-mm diam) or green branches (shoots with up to 10 green leaves) were kept in a 1-l Plexiglass cylinder covered with gauze. Thus, the plant material did not provide visual cues. Grapes were freshly picked and stored at 4°C and 100% RH for up to 2 days.

A headspace extract (see above) from green berries was reduced to ca. 50 μl and then diluted with ethanol (redistilled; LabScan, Malmö, Sweden) to contain the most abundant compound (*E*,*E*)-α-farnesene at 1 ng/μl. This extract was released from a sprayer, which allowed volatilization of all compounds at a known and constant rate, regardless of their vapor pressures (Gödde et al., 1999). The solution was fed into a glass capillary with an elongated tip, which was vibrated at ultrasonic frequency (ca. 100 kHz) by means of a piezo-ceramic disc. The solution volatilized within a few cm of the outlet of the capillary. A motor-driven syringe ensured a constant delivery of the solution at 10 μl/min, resulting in a release rate of 10 ng/min of (*E*,*E*)-α-farnesene. The sprayer was rinsed with 2 ml of redistilled ethanol before each treatment.

The numbers of females flying upwind over 150 cm and landing at the source was compared using Fisher's exact test ($P < 0.05$).

RESULTS AND DISCUSSION

Identification of Grapevine Volatiles. Compounds identified from different developmental stages of grapevine, from nonflowering buds to green berries, are

shown in Table 1. Compounds eliciting reproducible responses from antennae were identified according to their mass spectra and retention times, in comparison with synthetic or authentic standards. The most abundant compound in headspace collections from all phenological stages was (*E*,*E*)-α-farnesene. Comparison of volatiles recovered from clusters at early bloom by two sampling techniques, SPME and Porapak Q air filters, showed only quantitative differences (Table 1).

Grapevine Volatiles Eliciting an Antennal Response. The headspace collections used for identification of grape volatiles by GC–MS were also analyzed by GC–EAD. Antennae of grapevine moth females responded to 27 compounds (Table 1). These include the most abundant compounds, such as α-farnesene, (*E*)-β-farnesene, and (*Z*)-3-hexenyl acetate, but the antennae also responded to compounds that were present in much smaller amounts, for example β-caryophyllene, limonene, and heneicosane. Many alcohols, ketones, and hydrocarbons with straight carbon chains elicited antennal responses. In contrast, only a few cyclic compounds such as methyl salicylate, limonene, and caryophyllene elicited antennal responses (Table 2).

Further GC–EAD analysis, using synthetic or authentic compounds at a constant amount of 10 ng, was done to quantify the antennal responses. The most active compounds were 4,8-dimethyl-1,(*E*)3,7-nonatriene and α-farnesene (Table 2).

Changes in Headspace Composition During Phenological Development. Headspace collections from shoots with leaves included typical green odorants such as hexanoic acid, hexanal, (*E*)-2-hexenal, and (*Z*)-3-hexenal (Table 1; Hashizume and Samuta, 1997). Collections from flower buds contained only few volatiles. However, several of these elicited an antennal response, which is in agreement with a study by Gabel and Roehrich (1995), who reported that extracts of flower buds stimulated egg-laying in grapevine moth females. The seasonal flight period of grapevine moth starts before the onset of flowering and females oviposit on the flower buds.

Hydrocarbons were characteristic for partly flowering clusters, and many disappeared during full flowering. Flower clusters at full bloom contained, in comparison, a group of terpenoids (Table 1; Buchbauer et al., 1994, 1995). Green grapes contained fewer terpenoids, whereas several alcohols, aldehydes, esters, and aromatic compounds emerged. The relative abundance and the number of esters further increased in collections from mature grapes (data not shown). This increase in ester production associated with grape ripening is known (Hardy, 1970).

Wind Tunnel Bioassay. Mated *L. botrana* females flew upwind to clusters of green grapevine berries in the wind tunnel (Table 3). Female moths became activated and started to orient upwind between 2 and 11 min after they were exposed to grape odor. Twenty-five percent ($N = 140$) flew upwind over 150 cm, and 17.9% of the test females landed at the cage containing grape berries. The upwind flight pattern was similar to male flights towards female sex pheromone (El-Sayed et al., 1999). Fewer females landed at shoots without grapes, and females did not fly

TABLE 2. ANTENNAL RESPONSES OF FEMALE GRAPEVINE MOTHS TO VOLATILE COMPOUNDS IDENTIFIED FROM GRAPEVINE (CV. CHARDONNAY) AS DETERMINED BY GC-EAD

Compound	Antennal response ± SD (mV × 10^4ng)[a]	Relative response (%) vs. (*E*,*E*)-α-farnesene
Hydrocarbons		
pentadecane[b,c,d,e,f]	2.03 ± 0.91	12
1-pentadecene[b,d,e]	0.39 ± 0.04	2
heptadecane[b,d,f]	5.48 ± 0.93	33
1-heptadecene[d,f]	1.08 ± 0.12	6
octadecane[b,d]	1.99 ± 0.61	12
nonadecane[b,d]	6.51 ± 1.52	39
1-nonadecene[d]	0.95 ± 0.28	6
heneicosane[d,e]	7.16 ± 1.71	43
Alcohols		
(*Z*)-3-hexenol[f]	2.22 ± 1.16	13
1-hexadecanol[d,e]	2.23 ± 1.91	13
1-heptadecanol[d,e]	4.76 ± 2.21	28
1-octadecanol[d,e]	6.59 ± 2.14	39
Aldehydes		
(*Z*)-3-hexenal[b,e]	4.76 ± 0.32	28
nonanal[b,c,d,e,f]	4.98 ± 4.18	30
Ketones		
2-undecanone[d,e]	1,86 ± 1,04	11
2-dodecanone[d,e]	6.30 ± 5,03	38
2-tridecanone[d,e]	1.03 ± 0.67	6
Esters		
(*Z*)-3-hexenyl acetate[e,f]	2.50 ± 1,27	15
Aromatic compounds		
methyl salicylate[b,d,e]	5.37 ± 3.12	32
Terpenoids		
(*E*)-4,8-dimethyl-1,3,7-nonatriene[b,e,]	66.02 ± 39.14	393
limonene[c,d,e]	5.61 ± 4.55	33
(E)-β-ocimene[b,c,e]	3,25 ± 2.55	19
linalool[f]	3,33 ± 1.68	20
β-caryophyllene[c,d,e]	7.03 ± 4.50	42
(E)-β-farnesene[d,e]	2.95 ± 1.07	18
(*E*,*E*)-α-farnesene[b,c,d,e,f]	16.79 ± 3.53	100

[a] Mean antennal response, using 10 ng of standard compounds ($N = 6$).
[b] Compound found in volatile collections from leaves.
[c] Compound found in collections from cluster with flower buds.
[d] Compound found in collections from clusters with 10–30% open flowers ($N = 6$).
[e] Compound found in collections from clusters in full bloom ($N = 6$).
[f] Compound found in collections from immature berries ($N = 6$).

TABLE 3. ATTRACTION OF MATED FEMALE GRAPEVINE MOTHS IN A WIND TUNNEL TO GRAPEVINE (CV. CHARDONNAY) BRANCH WITH GREEN LEAVES, GREEN BERRIES, OR TO EXTRACTS OF HEADSPACE VOLATILES COLLECTED FROM BERRIES AND RELEASED FROM A SPRAYER[a]

Treatment	Amount	Number of test females	% Oriented flight[b]	% Landing[c]
Branch[d]	ca. 100 g	46	13.0 a	6.5 ab
Green berries[e]	ca. 150 g	140	25.0 a	17.9 a
Sprayed volatiles[f]	10 ng/min α-farnesene	48	12.5 a	0 b
Blank	-	50	0 b	0 b

[a] Within columns, means followed by different letters are significantly different (Fisher's exact test; $P < 0.05$).
[b] Females flying upwind over 150 cm.
[c] Females landing at the odor source, 180 cm from the release cage.
[d] Shoot with green leaves.
[e] Immature fruit (diam 5–10 mm).
[f] Headspace collection from green grapevine berries on Porapak Q filter (see Table 1), containing 1 ng/μl (*E*,*E*)-α-farnesene.

upwind at all without a plant odor stimulus (Table 3). Attraction of females to grapes by flight, over a distance, demonstrates a role for plant volatile compounds in host-finding in *L. botrana*.

Volatiles from freshly picked grape berries eliciting upwind attraction were collected on Porapak Q, and the resulting extract was diluted to contain (*E*,*E*)-α-farnesene at 1 ng/μl and was then released from a sprayer at a rate of 10 μl/min into the wind tunnel. A significant number of grapevine moth females flew upwind towards this sprayed volatiles extract over at least 150 cm, but the females did not land at the source (Table 3).

The average amount of α-farnesene collected from 200 g of green berries was 0.28 ± 0.15 μg/hr (Table 1). In comparison, the release rate of the volatiles extract in the wind tunnel experiment was set to 10 ng/min or 0.6 μg/hr α-farnesene. Headspace collections on Porapak filters contain at least part of the odor cues responsible for female attraction to grape, as evidenced by the significant response to sprayed extracts (Table 3). However, both release rate and composition of the blend were probably suboptimal.

The composition of the bouquet emitted from plant material is likely to be different from the blend of compounds extracted from Porapak air filters. A distorted composition of this blend may be due to differences in the compounds' affinity to the glass jar holding the grapes, to filter material and solvent, as well as to their volatility, which affects the bleeding of compound through the air filter. No single volatile eliciting antennal activity (Table 2) is unique for grapevine; a blend of compounds is expected to mediate attraction of gravid *L. botrana* females to grapevine.

Future studies will include calibration of the headspace collection system for the compounds eliciting antennal responses (Table 2), in order to enable a reconstruction of the blend of compounds released from grapes. The focus of continued behavioral studies will be on compounds that were present throughout the season, in all phenological stages (Tables 1). First-generation females of *L. botrana* oviposit on flower buds and partly flowering clusters, whereas the second and third generation oviposits on berries (Bovey, 1966; Thiéry and Gabel, 2000). Availability of a laboratory bioassay (Table 3) will allow identification of the compounds eliciting attraction and egg-laying in gravid *L. botrana* females.

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ISOLATION OF THE DITERPENOIDS, *ENT*-KAURAN-16α-OL AND *ENT*-ATISAN-16α-OL, FROM SUNFLOWERS, AS OVIPOSITION STIMULANTS FOR THE BANDED SUNFLOWER MOTH, *Cochylis hospes*

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Abstract—Two diterpenoid alcohols, *ent*-kauran-16α-ol (**1**) and *ent*-atisan-16α-ol (**2**), were isolated from pre bloom (R3-R4 stage) sunflower heads as oviposition stimulants for the banded sunflower moth, *Cochylis hospes*. Fractionation of a sunflower head extract, by normal-phase flash column chromatography, resulted in an early eluting fraction exhibiting significant activity in an egg-laying bioassay. Compounds **1** and **2**, along with *ent*-trachyloban-19-oic acid (**3**) and *ent*-kaur-16-en-19-oic acid (**4**), were isolated as the major components of this fraction and identified by their NMR and mass spectra. The purified compounds were individually tested for ovipositional activity in dose-response bioassays. In these bioassays, compounds **1** and **2** gave linear dose responses, with increasing numbers of eggs laid as the dosage of either increased. Compounds **3** and **4** failed to stimulate significant egg-laying at any of the dosages tested. A factorial design bioassay, using compounds **1** and **2**, showed that **1** was relatively more stimulatory than **2**, and that there was no synergistic effect on oviposition when the two compounds were combined.

Key Words—Diterpenoid, *ent*-kauran-16α-ol, *ent*-atisan-16α-ol, mass spectrum, HPLC, NMR, Lepidoptera, Cochylidae, *Helianthus annuus*, oviposition stimulant.

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INTRODUCTION

The banded sunflower moth (BSFM), *Cochylis hospes* Walshingham (*Lepidoptera: Cochylidae*), is an important pest of cultivated sunflower (Charlet et al., 1997) in the northern Great Plains of North America. The larvae are restricted to feeding on sunflower (*Helianthus spp.*), and have been found on nine wild sunflower species, along with cultivated sunflower, *Helianthus annuus* (Charlet et al., 1997). BSFM females oviposit preferentially on pre bloom sunflower heads (R2-R4 stage), rather than early bud or post bloom (R5-R6) heads, with the majority of eggs being laid on the outer whorl of involucral bracts (Charlet et al., 1997). Experiments by Barker (1997) indicated that extracts of macerated sunflower bracts stimulated BSFM oviposition. Further experiments (Foster et al., 2003) showed that extracts made by dipping pre bloom heads (R3) in hexane, dichloromethane, or methanol, contained compounds that stimulated BSFM oviposition. Preliminary fractionations of hexane and dichloromethane extracts, using silica gel solid phase extraction tubes, resulted in the concentration of ovipositional activity in several fractions (Foster et al., 2003).

This paper describes the continuation of this work, with the isolation and identification of two diterpenoid alcohols, *ent*-kauran-16α-ol (**1**) and *ent*-atisan-16α-ol (**2**), that are oviposition stimulants for BSFM (Figure 1). Compounds **1**

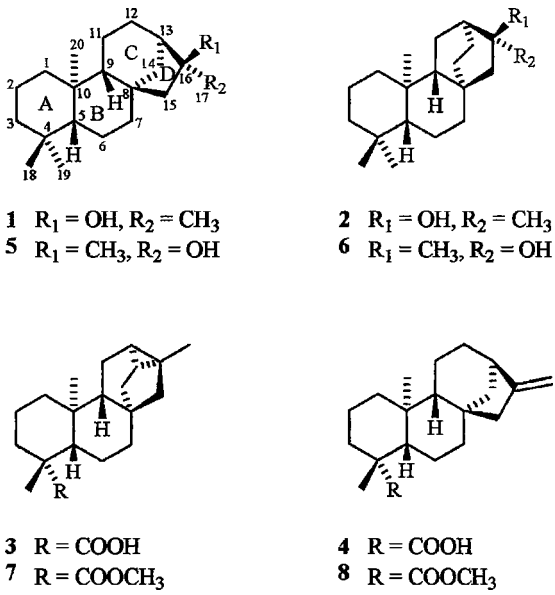

FIG. 1. Diterpenoid alcohols (1 and 2) and acids (3 and 4) isolated from sunflower heads, 16beta-epimers 5 and 6, and acid methyl esters 7 and 8.

and **2** were isolated by fractionation of dichloromethane extracts of sunflower heads. The diterpenoid acids *ent*-trachyloban-19-oic acid (**3**) and *ent*-kaur-16-en-19-oic acid (**4**) (Figure 1) were also isolated and found to be nonstimulatory at the concentrations tested.

METHODS AND MATERIALS

General Fractionation and Characterization Procedures. Flash column chromatography used either silica gel (60 Å, 130–270 mesh, Sigma-Aldrich, Milwaukee, WI) or, for argentation chromatography, silica gel impregnated with 10% $AgNO_3$ (200 mesh, Sigma-Aldrich). High performance liquid chromatography (HPLC) employed a Shimadzu LC-6A pump and SPD-10A UV detector, set to 220 nm, with a Luna column (5 μm normal-phase silica, 250 × 4.6 mm; Phenomonex, Torrance, CA). Solvents used were either Malinckdrodt nanograde (for column chromatography), Burdick and Jackson HPLC grade (for HPLC), or redistilled AR grade. Solvents were evaporated from fractions by rotary evaporator at 40°C for larger volumes, or by a gentle stream of nitrogen for smaller volumes. Diazomethane was prepared from *N*-methyl-*N*-nitro-*N*-nitroso-guanidine (Sigma-Aldrich) and 0.5 M $KOH_{(aq)}$ (Attygalle, 1998). Acetyl chloride was obtained from Sigma-Aldrich, and bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% TMCS from Pierce (Rockford, IL). Coupled gas chromatography-mass spectrometry (GC–MS) was performed on an Hewlett-Packard 5890 Series II Plus gas chromatograph interfaced with an Hewlett-Packard 5972 quadrupole mass spectrometer operated at 70 eV ionizing energy. Zebron ZB-1 or ZB-5 capillary columns (25 m, 0.25 mm i.d., 0.25-μm-film thickness, Phenomonex) were used for analyses, with GC temperature programs of 80°C (1-min hold) to 290°C at 15°C/min, and 80°C (2 min hold) to 360°C at 15°C/min, respectively. The ZB-5 column was only used for analysis of BSTFA-derivatized samples. Splitless injection was used, helium being the carrier gas, at a linear velocity of 30 cm/sec. Mass spectra were compared with those in the Wiley Registry of Mass Spectral Data (7th Ed., John Wiley and Sons, NY). Nuclear magnetic resonance (NMR) spectra were obtained on Varian Inova instruments, either an 11.7 T spectrometer (499.67 MHz for ^{1}H and 125.65 MHz for ^{13}C) or a 9.4 T instrument (399.94 MHz for ^{1}H and 100.57 MHz for ^{13}C). All spectra were recorded in $CDCl_3$ (Sigma-Aldrich). ^{1}H chemical shifts were referenced to residual $CHCl_3$ at 7.26 ppm and ^{13}C shifts to the solvent signal at 77.0 ppm.

Extraction and Isolation. Sunflower plants (*H. annuus* cv. 'RHA 274') were grown in a greenhouse at 20–30°C between September 2002 and February 2003. Two extracts were prepared from pre bloom heads (R3-R4 stage); extract A, 42 heads (mean diam 2 cm) and extract B, 149 heads (mean diam 3.6 cm). Heads were dipped into dichloromethane for 60 sec, by holding the stalk and avoiding

extraction of the cut end, similar to the method for extracting the epicuticular waxes of wheat leaf, previously described (Morris et al., 2000). Celite (1 g for extract A, 6 g for extract B) was added to the crude extracts, to aid later transfer to the chromatography column, and the solvent removed by rotary evaporation. Rotary evaporation was stopped 5 min after apparent dryness.

Extract A (0.16-g-dry weight) was fractionated by normal-phase flash column chromatography on 50 g of silica gel. Fractionation used a stepped solvent gradient (pentane 200 ml, fraction 1; pentane/diethyl ether (9:1) 2 × 100 ml, fractions 2 and 3; pentane/diethyl ether (4:1) 2 × 100 ml, fractions 4 and 5; pentane/diethyl ether (1:1) 2 × 100 ml, fractions 6 and 7; diethyl ether 2 × 100 ml, fractions 8 and 9; ethanol 100 ml, fraction 10). All of these fractions were tested in the bioassay.

Fraction 5 (11.4 mg) was active in the bioassay. Preliminary GC–MS analysis showed it contained compounds **1**–**4** as the major components. These were isolated as follows, identified, and tested individually in the bioassay.

Compounds **1** and **2** were isolated from extract B (dry weight 1.1 g). This was fractionated on 50 g of normal-phase silica gel using a similar solvent gradient to that for extract A (above). Three fractions containing **1** and **2** were combined (dry weight 76 mg) and fractionated on 7 g of normal-phase silica gel using the following solvent steps: hexane 20 ml, fraction 1; hexane/diethyl ether (9:1) 20 ml, fraction 2; hexane/diethyl ether (4:1) 10 × 10 ml, fractions 3-12. Fraction 6 (4.6 mg) contained **1**, while fractions 7 and 8, both containing **2**, were combined (14 mg) and further fractionated on 5 g normal-phase silica gel, using the same solvent gradient, to improve separation of **2** from tailing C_{20} to C_{34} aliphatic alcohols. Compounds **1** and **2** were purified by HPLC using isocratic *i*-PrOH/hexane (2:98) at 1 ml/min. Compound **1** (1.1 mg) eluted at 6.3 min and **2** (1.4 mg) at 6.8 min under these conditions.

Compounds **3** and **4** were isolated from fractions 4 (12.3 mg) and 5 from the 50 g silica gel fractionation of extract A. Further fractionation of fraction 5 on silica gel separated acids **3** and **4** from alcohols **1** and **2**. Fraction 4 from the first column and fractions from the second column containing **3** and **4** were combined (14 mg) and fractionated by argentation chromatography on silica gel/10% $AgNO_3$ (50 g), using pentane/diethyl ether 9:1, 4:1, 7:3, and 1:1 solvent steps, with **3** (2.4 mg) and **4** (4.8 mg) eluting in the 4:1 and 1:1 fractions, respectively. Compounds **3** and **4** were further purified by normal-phase HPLC, using an isocratic mobile phase of *i*-PrOH/hexane (1:99) at 1 ml/min flow rate. Both **3** (1.9 mg) and **4** (2.7 mg) eluted at 6.2 min.

Insect Culture. BSFM were obtained from a laboratory colony established in 1988 and now maintained at the USDA-ARS Northern Crop Science Laboratory, Fargo, ND. The colony was established from the larvae collected in North Dakota and reared on a semi-synthetic diet (Barker, 1988). Annual addition of wild insects from North Dakota, use of R2 stage sunflower heads for oviposition in the rearing cycle, and regular tests for selectivity toward sunflower are carried out to ensure

that the colony does not lose specificity to the host plant (Barker, 1997). Adult BSFM were obtained <24 hr after emergence and kept as mixed groups (20–30 insects) in 28 ml clear plastic containers, with a moist cotton wick, for 24 or 48 hr to allow mating. Containers were placed in to a controlled temperature room (26 ± 1°C, 30% relative humidity, 16L: 8D photoperiod), that was also used for the bioassay.

Bioassay. The bioassay was a modification of what was carried out previously (Foster et al., 2003). Fractions, or purified compounds **1**–**4** (Figure 1), were applied as solutions, using a 500 μl syringe, to scored chromatography paper attached over the mouth of vials (45-mm height × 15-mm diam) with rubber bands. Parallel grooves were scored in the paper using a scalpel, approximately 1.5-mm apart and 2-mm deep, with 7 grooves per paper cap, similar to the method of Kanno and Harris (2000). Without scoring, very few eggs were laid on the treated caps, indicating the importance of physical stimulation for oviposition (also observed previously, Foster et al., 2003). Solutions were applied over approximately a 10 min period; each application was confined to the area of scored paper (approximately 1 cm^2) on the top surface of the cap.

From the first flash column fractionation of extract A, fractions 3–10 were taken to dryness (for weighing) and then redissolved in 1.5 ml of diethyl ether. Fractions 1 and 2 were reduced in volume to 1.5 ml, without being taken to dryness, to avoid loss of volatile monoterpenes, which were significant components of these fractions as indicated by GC–MS. A treatment was 80 μl [2.2 head equivalents (HE)] of a fraction applied to a paper cap, with 80 μl of diethyl ether for solvent controls. A HE was the mean weight of fraction per head. Vials were attached to the base of the bioassay container (17 cm diam × 14 cm high, Rubbermaid, Wooster, OH) with mounting putty (Manco Inc., Avon, OH), and evenly spaced around the perimeter, approximately 3 cm from the wall. A replicate consisted of one treatment of each fraction plus a control, placed in the same container. A 10-cm diam circle was removed from the lid of the arena and replaced with fine nylon mesh. The arena floor was covered with moist vermiculite.

For dose-response bioassays, compounds were each dissolved in hexane, and the appropriate amounts were applied to paper caps to give 50, 10, and 2 μg dosages; 100 μl (for testing **1** and **2**) and 50 μl (for **3** and **4**) of hexane were used for the solvent controls, volumes equal to the maximum amount of solution applied for each bioassay. Based on the weight of these compounds recovered after purification, 50 μg is approximately 7 HE for compound **1**, 5 HE for **2**, and 1 HE for **3** and **4**. Replicates consisted of one treatment of each dosage of a compound, plus a control placed in the same container. The factorial-design bioassay used 25 μg dosages of **1** or **2**, or 50 μg of a 1:1 mixture of **1** and **2**, each applied in 100 μl of hexane; 100 μl of hexane were used for the solvent control. For these experiments, the vials were placed, as mentioned above, in 14-cm diam × 11-cm high containers.

Insects were sexed and placed in the bioassay containers 2–3 hr before the start of scotophase. Between 25 and 35 females (approximately half 24–48-hr old and half 48–72-hr old), and 5–8 males were used for each replicate. After 4 nights, the number of eggs on the top surface of the paper caps was counted. Eggs laid around the rubber band holding the paper to the vial were excluded, even though the majority of the eggs were laid there, since the physical stimulation of the rubber band varied between treatments. Consequently, a relatively low number of eggs were counted per female moth.

Statistical Analysis. Data from the bioassay of fractions were not normally distributed (Shapiro–Wilk–Whitney test), even after log-transformation. Therefore these data were analyzed by the nonparametric Wilcoxon–Mann–Whitney test, using Statxact software (Cytel Corporation, version 5.0.3), comparing each treatment with the control. Data from the dose response bioassays were normal (Shapiro–Wilk–Whitney test), and were analyzed by Student's t-test or ANOVA, using JMPin software (SAS Institute, version 4.0.4). Results with a linear trend were analyzed by linear regression. Results of the factorial design bioassay were analyzed by JMPin, with the two compounds as the main effects.

RESULTS

Bioassay of Fractions from Sunflower Head Extract. Ten fractions, obtained from silica gel fractionation of extract A (42 sunflower heads), were tested in the egg-laying bioassay. Statistical analysis (Wilcoxon–Mann–Whitney test) showed that 4 fractions had a significantly ($P < 0.01$) greater number of eggs laid on them than the control (Figure 2). These were in two groups; fraction 5 eluting with pentane/diethyl ether (4:1), and polar fractions 8–10 (eluting with diethyl ether or ethanol).

Preliminary GC–MS analyses of fractions 1–6, showed that the major classes of compounds were monoterpenes (mostly α-pinene) and germecrenoid sesquiterpenes (in fractions 1 and 2), and even numbered C_{20} to C_{34} aliphatic aldehydes and alcohols (in fractions 3 and 6, respectively). Fraction 3 (53 mg) and fraction 6 (17 mg) made up 43% of the weight of the crude extract. The major components of fractions 4 and 5 were two diterpenoid acids, which reacted with ethereal diazomethane to give methyl esters (Attygalle, 1998). Fraction 5 also contained two diterpenoid alcohols, which reacted with acetyl chloride to give acetates along with dehydration products (St. Pyrek, 1984). Minor compounds in fraction 5 consisted of mono- and sesquiterpenoids, comprising <5% of the GC–MS total ion current. Further work described in this paper is restricted to the characterization of bioactive compounds from fraction 5. Since the alcohols differentiated active fraction 5 from inactive fraction 4, these were isolated and characterized first.

*Isolation and Identification of Compounds **1–4**.* Comparison of the mass spectra of the two alcohols with spectra in the Wiley library indicated that they

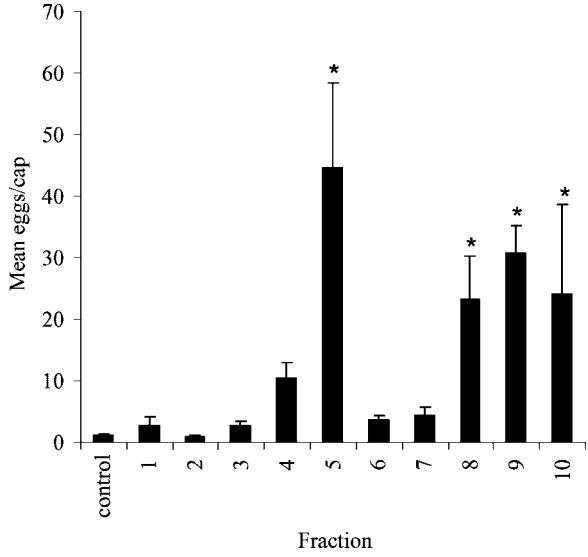

FIG. 2. Bioassay of fractions (2.2 HE/fraction) from a normal-phase flash column fractionation on silica gel, of extract A (42 sunflower heads). Mean number of eggs laid by *C. hospes* on paper caps, each treated with a fraction or solvent only as a control. Means were calculated from 5 replicates. Fractions with asterisks are significantly different from the control (Wilcoxon–Mann–Whitney test, $P < 0.01$). Bars are standard errors of means.

had kaurane and atisane skeletons. Their spectra were similar to literature data (Kalinovsky et al., 1970; St. Pyrek, 1984), for *ent*-kauran-16α-ol (**1**) or *ent*-kauran-16β-ol (**5**) (diagnostic ions at *m/z* (rel.int.): 290 (10) $[MH]^+$, 272 (100) $[M\text{-}H_2O]^+$, 257 (64) $[M\text{-}CH_3\text{-}H_2O]^+$, 232 (46), 217 (33), and *ent*-atisan-16α-ol (**2**) or *ent*-atisan-16β-ol (**6**) (ions at *m/z* (rel. int.) 290 (0.4), 272 (22), 257 (100), all previously isolated from sunflower heads (St. Pyrek, 1984). The two alcohols were isolated from extract B (149 heads), yielding 1.1 mg of **1** (or **5**) and 1.4 mg of **2** (or **6**) after normal-phase HPLC, which were pure (>99%) by GC–MS analysis of BSTFA-derivatized subsamples. The ^{1}H NMR spectra showed no contamination of **1** with **2** and vice versa, based on the methyl signals. These signals had shifts similar to the published values for **1** and **2** (St. Pyrek, 1984). Comparison of the ^{13}C NMR spectra of the alcohols with published data (Table 1) allowed their identification as the α-epimers, *ent*-kauran-16α-ol (**1**) and *ent*-atisan-16α-ol (**2**) (St. Pyrek, 1984). In particular, a signal at 32.37 ppm for **1** was similar to the literature value of 32.52 ppm for C-17 of *ent*-kauran-16α-ol (**1**), but differed from C-17 for *ent*-kauran-16β-ol (**5**) (24.52 ppm) (St. Pyrek, 1984). The signal for H-17 of isolated **1** (1.32 ppm) and was fount to be closer to the published for **1** (1.31 ppm) than **5** (1.36 ppm) (St. Pyrek, 1984). Similarly, the signals for C-17 and H-17 of

TABLE 1. COMPARISON OF ^{13}C NMR CHEMICAL SHIFTS OF *ent*-KAURAN-16α-OL (**1**) AND *ent*-ATISAN-16α-OL (**2**) ISOLATED FROM SUNFLOWER HEAD EXTRACTS, WITH PUBLISHED VALUES

	ent-kauran-16α-ol (**1**), δ (ppm), $CDCl_3$		*ent*-atisan-16α-ol (**2**) δ (ppm), CDC	
Carbon	Measured (125 MHz)[a]	Published[b]	Measured (100 MHz)[a]	Published[b]
1	38.71	38.96	39.63	39.83
2	18.61	18.81	18.70	18.91
3	42.12[c]	42.37	42.20	42.42
4	[d]	33.39	33.06	33.15
5	56.20	56.47	56.40	56.61
6	20.02	20.22	18.16	18.32
7	40.38	40.66	37.97	38.18
8	44.42	44.61	33.83	33.98
9	57.16	57.49	51.29	51.44
10	[d]	39.59	37.68	37.83
11	18.94	19.10	24.09	24.22
12	26.73	26.91	39.31	39.59
13	46.90	47.25	23.24	23.39
14	42.07[c]	42.37	27.27	27.57
15	57.68	58.08	57.74	58.08
16	[d]	77.69	72.13	72.03
17	32.37	32.52	30.42	30.52
18	33.60	33.69	33.40	33.49
19	21.57	21.74	21.68	21.78
20	17.62	17.74	13.87	14.03

[a] Referenced to residual $CHCl_3$ at 77.00 ppm.
[b] From St. Pyrek (1984). Referenced to internal TMS.
[c] Assignments may be interchanged.
[d] Unresolved from baseline or solvent peak due to small sample size.

2 matched those for the α-epimer of *ent*-atisan-16-ol (Table 1) (St. Pyrek, 1984). The most obvious differences in the ^{13}C NMR spectrum of 2 compared with that of **1** was the large upfield shifts of signals assigned to carbons 8, 13, and 14 in **1**, as expected with the differences in structure of rings C and D. Compounds **1** and **2** were assumed to have the same *enantio* absolute configuration as the kauranols and atisanols previously isolated from *H. annuus* (St. Pyrek, 1984).

A subsample of fraction 4 from extract A (42 heads), containing the two diterpenoid acids also present in fraction 5, was methylated by treatment with ethereal diazomethane (Attygalle, 1998). The mass spectra of the resulting methyl esters were similar to those previously published for methyl-trachyloban-19-oate (**7**) and methyl-kaur-16-en-19-oate (**8**) (Figure 1) (St. Pyrek, 1970; Mitscher et al., 1983), indicating that the acids were probably *ent*-trachyloban-19-oic acid (**3**) and *ent*-kaur-16-en-19-oic acid (**4**). The acids from fractions 4 and 5 were separated by

flash column chromatography on silica gel/$AgNO_3$, then purified by normal-phase HPLC to give 1.9 mg of **3** and 2.7 mg of **4**. Both GC–MS and ^{1}H NMR indicated that each acid was clean and without cross contamination. The mass spectrum of **3** contained diagnostic ions at *m/z* (rel. int.): 302 (99) $[M]^+$, 287 (51) $[M\text{-}CH_3]^+$, 246 (100), 231 (55), with the *m/z* 246 ion (loss of 56 amu) indicating a trachylobane derivative (St. Pyrek, 1970). This was similar to data for *ent*-trachyloban-19-oic acid from Mitscher et al. (1983). The mass spectrum of **4** (ions at *m/z* (rel. int.): 302 (58) $[M]^+$, 287 (44) $[M\text{-}CH_3]^+$, 259 (51), 243 (43), 241 (43), 213 (31)) was similar to that for *ent*-kaur-16-en-19-oic acid in the Wiley library and in Mitscher et al. (1983). The ^{1}H NMR shifts for the methyl protons of **3** ($CDCl_3$ 100 MHz):δ 0.88, 1.13, 1.21) matched those of Beale et al. (1983), but were downfield by 0.7 ppm from values in Mitscher et al. (1983), while the methyl and H-17 shifts for **4** ($CDCl_3$ 100 MHz):δ 0.95, 1.24, 4.77 (d, $J = 23$ Hz) were identical to those of Mitscher et al. (1983). Comparison of the ^{13}C NMR spectra of **3**[3] and **4**[4] with published values (Ohno et al., 1979; Beale et al., 1983; Faulkner et al., 1985; Wu et al., 1996; Leong and Harrison, 1997), especially the shifts for rings C and D carbons, and the presence of signals for C-18 (equatorial), rather than C-19 (axial) methyl carbons, agreed with the assignment of **3** as *ent*-trachyloban-19-oic acid and **4** as *ent*-kaur-16-en-19-oic acid.

Bioassay of Compounds 1–4. The HPLC purified samples of compounds **1–4** were tested separately in dose response bioassays. Alcohols **1** and **2** had similar dose-response profiles (Figures 3 and 4). A linear regression of dose vs. number of eggs for **1** showed a linear fit ($F = 58.1$; $df = 1, 22$; $R^2 = 0.73$; $P < 0.001$) with a significant ($P < 0.001$) slope of 0.80 ± 0.10, indicating that there was an increase in the number of eggs laid with increasing dosage of **1**. For compound **2**, a linear regression of dose vs. number of eggs laid on treatments showed a linear fit ($F = 41.1$; $df = 1, 22$; $R^2 = 0.65$; $P < 0.001$), and a highly significant ($P < 0.001$) slope of 0.67 ± 0.10. For the dose-response bioassays of acids **3** and **4**, ANOVA showed no significant difference between the treatments (for **3** $F = 1.01$; $df = 3, 20$; $P = 0.41$, and for **4** $F = 0.80$; $df = 3, 20$; $P = 0.51$) (data not shown). In the factorial-design bioassay, testing both **1** and **2**, the main effect of **1** was significant ($F = 27.9$, $df = 1$, $P < 0.001$), but there was no significant effect for **2** ($F = 1.35$, $df = 1$, $P = 0.26$), or any significant interaction between the two compounds ($F = 0.26$, $df = 1$, $P = 0.62$) (Figure 5), indicating a lack of any synergistic effect of combining the two alcohols. This also showed that compound **1** was relatively more stimulatory to female BSFM than compound **2**.

[3] ^{13}C NMR spectrum measured for *ent*-trachyloban-19-oic acid (**3**)($CDCl_3$ 100 MHz): δ 12.47, 18.68, 19.70, 20.53, 21.73, 22.39, 24.25, 28.83, 33.11, 37.93, 38.87, 39.19, 39.42, 40.73, 43.55, 50.35, 52.74, 56.92, 182.41.

[4] ^{13}C NMR spectrum measured for*ent*-kaur-16-en-19-oic acid (**4**) ($CDCl_3$ 100 MHz): δ 15.60, 18.42, 19.08, 21.83, 28.94, 33.02, 37.87, 39.65, 39.69, 40.70, 41.27, 43.66, 43.85, 44.22, 48.96, 55.11, 57.02, 102.99, 155.91, 182.95.

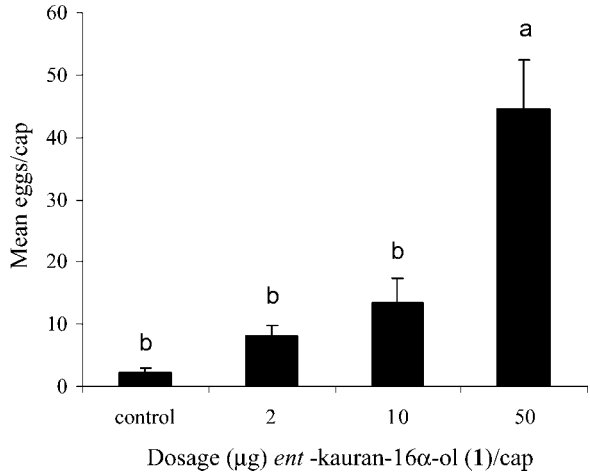

FIG. 3. Dose-response bioassay of *ent*-kauran-16α-ol (**1**). Mean number of eggs laid by *C. hospes* on paper caps treated with **1**, or solvent only for the control. Means were calculated from 6 replicates, bars are standard errors of means. Means with different letters were significantly different at $P < 0.05$ when compared by Student's *t*-test. A linear regression analysis of the data is described in the text.

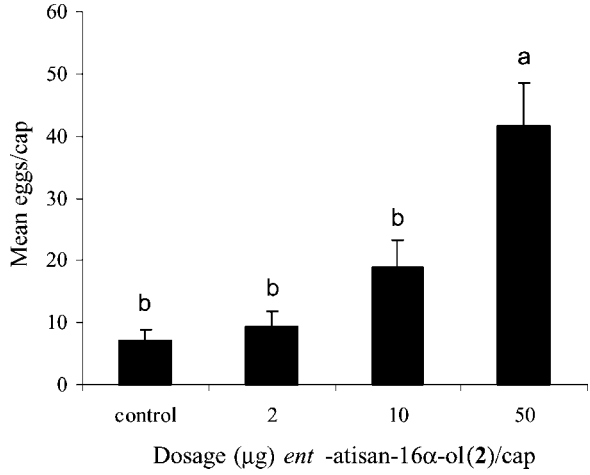

FIG. 4. Dose-response bioassay of *ent*-atisan-16α-ol (**2**). Mean number of eggs laid by *C. hospes* on paper caps treated with **2**, or solvent only for the control. Means were calculated from 6 replicates, bars are standard errors of means. Means with different letters were significantly different at $P < 0.05$ when compared by Student's *t*-test. A linear regression analysis of the data is described in the text.

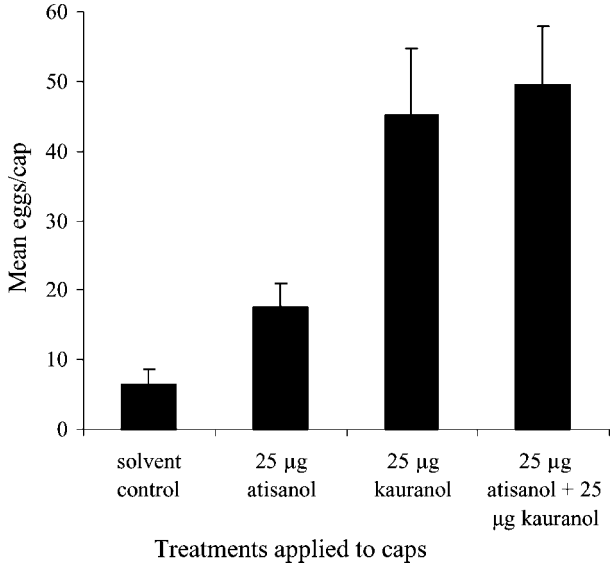

FIG. 5. Mean number of eggs laid by *C. hospes* in a factorial-design bioassay using paper caps treated with 25 μg or 0 μg of *ent*-kauran-16α-ol (**1**) and 25 μg or 0 μg of *ent*-atisan-16α-ol (**2**). Means were calculated from 8 replicates, bars are the standard errors of means.

DISCUSSION

In this study, we have isolated two diterpenoids from sunflower heads, *ent*-kauran-16α-ol (**1**) and *ent*-atisan-16α-ol (**2**), that stimulated oviposition by BSFM, when applied to an artificial substrate. Due to the unavailability of synthetic samples of these compounds, and the difficulty of total syntheses of atis-16-enes and kaur-16-enes (Toyota et al., 1998, 2000), samples for bioassay were obtained by fractionation of a sunflower head extract. The final HPLC separation yielded samples that were >99% pure by GC–MS analyses of their TMS ethers, and had no apparent signals for other compounds in their ^{1}H NMR spectra. This degree of purity reduces the likelihood that the ovipositional activity of the samples were due to unidentified minor components from the extract.

Although both *ent*-kauran-16α-ol (**1**) and *ent*-atisan-16α-ol (**2**) stimulated oviposition by BSFM when tested alone (Figures 3 and 4), only **1** was significantly active when these compounds were tested together (Figure 7). No synergistic effect of the addition of **2** to **1** was observed. *ent*-Trachyloban-19-oic (**3**) and *ent*-kaur-16-en-19-oic (**4**) acids were inactive at the concentrations tested. These results suggest a structure-activity relationship, with the alcohol group of **1** and **2**, absent

in **3** and **4**, being important for activity, along with the differences in the structures of rings C and D of **1** and **2** affecting activity.

Alcohols **1** and **2** have previously been isolated from macerated heads of *H. annuus* (St. Pyrek, 1984). Alcohol **1** has also been isolated from the leaves and stems of several other *Helianthus* spp. (Herz et al., 1982, 1983; Watanabe et al., 1982), and both **1** and **2** were found by GC–MS analysis to be widespread in the genus *Helianthus* (St. Pyrek, 1984). Therefore, it seems likely that these compounds are oviposition stimulants for BSFM on a number of *Helianthus* species in the host range of BSFM.

In preliminary experiments (Foster et al., 2003), extracts of both sunflower leaves and heads stimulated oviposition by BSFM, and it appeared that physical stimulation of the overlapping involucral bracts of heads resulted in eggs being laid on heads rather than leaves (Charlet et al., 1997). It is also possible that the concentrations of compounds **1** and **2** and/or other unidentified stimulatory compounds (in fractions 8–10, Figure 2) differ on leaves and heads, and could influence the preferential oviposition by BSFM on heads. While alcohols **1** and **2** are nonvolatile, and, therefore, require contact to stimulate oviposition, earlier work (Foster et al., 2003) showed that unidentified volatile compounds from sunflower head extracts were also behaviorally active for female BSFM and that these could act as attractants. Overall, it appears that BSFM use a combination of volatile, involatile chemical, and tactile stimuli in their oviposition behavior.

Several insects have been found to be stimulated by leaf waxes; cabbage leaf wax (inactive alone) had a synergistic effect on oviposition stimulation of the diamondback moth (*Plutella xylostella*), when combined with sinigrin (a glucosinolate) (Spencer, 1996), while Hessian flies were stimulated by C_{22} to C_{30} *n*-aldehydes from wheat leaf wax (Morris et al., 2000), and *H. zea* by long-chain alkanes from tomato (Breeden et al., 1996). Although our dichloromethane extract contained approximately 43% by weight leaf waxes, mainly C_{20} to C_{34} even-numbered *n*-aldehydes and lesser amounts of the corresponding alcohols, it appears that these do not stimulate oviposition by BSFM, since fractions 3 and 6 from the first silica gel fractionation, containing these compounds, were inactive in the egg-laying bioassay (Figure 2).

At present, further work is in progress to isolate and identify bioactive compounds in polar fractions (fractions 8–10 in Figure 2) from sunflower head extracts. Identification of these, along with compounds **1** and **2**, responsible for the chemical oviposition stimulation of BSFM on sunflowers, could be useful for studying differences in host preference between cultivars, and behavioral aspects of BSFM oviposition.

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EVIDENCE FOR AN ISOBUTYLAMIDE ASSOCIATED WITH HOST-PLANT RESISTANCE TO WESTERN FLOWER THRIPS, *Frankliniella occidentalis*, IN CHRYSANTHEMUM

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Abstract—Bioassay-directed fractionation of extracts of chrysanthemum leaves using a choice test permitted isolation of a fraction that exhibited repellent activity against the western flower thrips (WFT). Analysis of this fraction from cultivars exhibiting varying degrees of host-plant resistance to WFT by high performance liquid chromatography revealed a distinctive peak, the height of which correlated with the degree of resistance of those cultivars to WFT. The peak was attributed to a novel unsaturated isobutylamide, *N*-isobutyl-(*E*, *E*, *E*, *Z*)-2,4,10,12-tetradecatetraen-8-ynamide.

Key Words—Chrysanthemum, resistance, host-plant resistance, repellent, thrips, western flower thrips, *Frankliniella occidentalis*, isobutylamide.

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INTRODUCTION

The western flower thrips (WFT), *Frankliniella occidentalis* (Pergande), has been the major insect pest of greenhouse floriculture in Canada since the late 1980s (Broadbent et al., 1987). In addition to feeding on leaves and flowers, thrips can vector diseases such as tomato spotted wilt virus, which affects a wide range of plants (Broadbent et al., 1990). Control of WFT has become difficult due to resistance to conventional insecticides (Immaraju et al., 1992; Broadbent and Pree, 1997).

Host-plant resistance to WFT, particularly in chrysanthemum, is recognized as having potential to reduce reliance on pesticides for control of this pest (Van Dijken, 1992; De Jager et al., 1993). A study of chrysanthemum cultivars revealed a wide variation in degree of host-plant resistance or susceptibility to WFT (Broadbent et al., 1990). Variation in pollen production (De Jager and Butôt, 1993), difference in color of blooms (Van Dijken et al., 1993), and leaf pubescence and toughness (De Jager et al., 1995a) were found to have little impact on this interaction. Secondary metabolites of chrysanthemum have been suggested to be the primary factor in host-plant resistance (De Jager et al., 1995b, 1996).

In this study, we used bioassay-directed fractionation of chrysanthemum leaf extracts to isolate a fraction which exhibited thrips-repellent activity. We analyzed this fraction from extracts of chrysanthemum cultivars exhibiting varying degrees of host-plant resistance against WFT to obtain their metabolite profiles by using high performance liquid chromatography (HPLC). A distinctive peak was identified whose concentration in chrysanthemum cultivars was correlated with the degree of resistance of those cultivars to WFT.

METHODS AND MATERIALS

Chrysanthemum Plants. Rooted cuttings were potted and grown in the greenhouse at the Southern Crop Protection and Food Research Centre in Vineland Station, Ontario. Cultivars used in the study included, in order of increasing host-plant resistance (Broadbent et al., 1990), Florida Marble, Amber, Bright Yellow May Shoesmith, Golden Polaris, Polaris, May Shoesmith, Super White, and Super Yellow, all supplied by Yoder Canada, Leamington, Ont.

Extraction and Analysis. The 7th fully expanded whole leaf from the top of a chrysanthemum plant (~40 days after transplanting) was extracted for 15 min with 10 ml solvent/g fresh leaf. Four solvents, hexane, dichloromethane, acetone, and water, were evaluated for repellency of extracts. Extracts were evaporated and redissolved in acetone before being applied to leaf disks for evaluation using the choice bioassay.

For HPLC analysis, a 0.5-ml aliquot of the extract was filtered (Acrodisk GHP, 0.45 μm, Gelman Sciences) and evaporated to dryness under a stream of nitrogen. This was re-dissolved in 0.5 ml of acetonitrile for analysis on a

Sphereclone ODS-2 column (15 cm × 4.6 mm, 5 μm, Phenomenex, Torrance, CA) using an acetonitrile/water gradient (10–25% acetonitrile over 37.5 min). The HPLC was an Agilent Technologies Model 1100 equipped with a diode array detector monitored at 254 nm. Three replicate samples of each cultivar were analyzed. The mean peak height of the identified compound was correlated with the resistance rank for each cultivar, the latter based on a visual rating of feeding damage by WFT (Broadbent et al., 1990).

To investigate the distribution of the identified compound within a plant, samples consisting of two adjacent leaves, beginning with the first fully expanded leaf at the top of the plant, were removed from plants of Florida Marble and Super Yellow and extracted and analyzed by HPLC. To investigate the influence of the age of the plant on the concentration of the identified compound, leaves from whole 20- and 42-day-old plants of Florida Marble and Super Yellow were removed, extracted, and analyzed by HPLC.

For TLC fractionation studies, fresh leaves of five chrysanthemum cultivars with varying degrees of resistance against thrips (Broadbent et al., 1990), Super White (resistant), Super Yellow (resistant), Polaris (intermediate), Florida Marble (susceptible), and Amber (susceptible), were collected, extracted, and partitioned as described in Tsao et al. (2003). The extract was then fractionated by TLC (2-mm layer, Silica gel GF, Analtech, Newark, DE) using hexane/ethyl acetate (1:1). The fraction of interest (Fraction 2) was collected and extracted with hexane–ethyl acetate (1:1). Fraction 2 of Super Yellow extract was further separated into 6 subfractions by TLC using hexane–ethyl acetate–dichloromethane (3:2:1).

Bioassays. To evaluate the repellent activity of extracts, or fractions of extracts, two leaf disks (1.5-cm diam.) of green pepper (California Wonder bell peppers, *Capsicum annuum*) were used, one treated with a solution containing the extract or fraction in acetone–water (1:1) at a rate of 0.3 mg extracted material/ml, the other with solvent only (control). The pair of disks was placed in a glass Petri dish (5-cm diam.). Ten adult female WFT were collected in a vial from flowering potted WFT-susceptible chrysanthemum plants grown in the greenhouse at Vineland Station and starved for 4 hr before being transferred to the Petri dish by hitting the bottom of the inverted open vial. The dish was then sealed with Dura Seal^{TM} film (Diversified Biotech, Boston, MA) and placed in a growth chamber (12:12 LD, 20°C). The number of thrips located on each leaf disk was recorded after 24 hr. Each experiment was replicated 10 times. A Student's t-test (two-sample assuming equal variances) was performed to determine if the means for the two disks were equal.

RESULTS AND DISCUSSION

Extraction Solvent Selection. Bioassay results showed that extracts obtained with solvents of high and intermediate polarity, i.e., water, acetone, and

dichloromethane, had significant repellent activity compared to the corresponding control. The mean number of thrips on the treated disk was significantly lower ($P < 0.05$) than the number on the control disk for all but the hexane extract. Acetone extracts exhibited the greatest degree of repellence; acetone was, therefore, chosen as the most appropriate solvent for extracting the active compounds.

Thin Layer Chromatography. The TLC profiles of susceptible, intermediate, and resistant cultivars exhibited similar pigment patterns at the top ($R_f > 0.65$), and bottom ($R_f < 0.3$) regions of the TLC, whereas differences were observed in the region $R_f = 0.3$–0.65 (Fraction 2), which became the focus of further characterization.

Median R_f values for the 6 TLC subfractions of Fraction 2 were 0.37, 0.44, 0.54, 0.60, 0.66, and 0.71. Leaf disks treated with the subfraction at $R_f = 0.44$ (subfraction 2-2) from Super Yellow extract were found to be those least preferred by the adult female WFT. This fraction was the focus of subsequent studies.

Chemical Profiling by HPLC. The TLC subfraction 2-2 exhibited a cluster of peaks at $R_t = 30$–35 min when analyzed by HPLC. Figure 1 illustrates chromatograms of Florida Marble, a WFT-susceptible cultivar, and Super Yellow, a WFT-resistant cultivar. The height of a peak at 31.7 min in chromatograms of leaf extracts of eight chrysanthemum cultivars showed a strong correlation ($r = -0.8286$, $P < 0.01$) with the mean cultivar rank reported by Broadbent

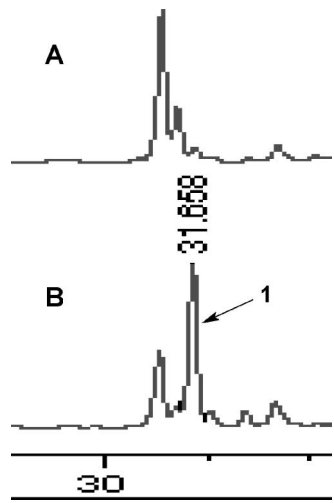

FIG. 1. HPLC chromatograms (monitored at 254 nm) of TLC subfraction 2-2 of leaf extracts from two chrysanthemum cultivars. A: Florida Marble (susceptible cultivar); B: Super Yellow (resistant cultivar).

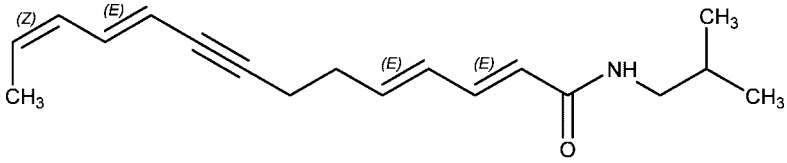

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FIG. 2. Structure of *N*-isobutyl-(*E, E, E, Z*)- 2,4,10,12-tetradecatetraen-8-ynamide.

et al. (1990). Structure identification by spectroscopic methods (Tsao et al., 2003) attributed this peak to *N*-isobutyl-(*E*, *E*, *E*, *Z*)- 2,4,10,12-tetradecatetraen-8-ynamide (**1**, Figure 2), a novel compound. Figure 3 illustrates the relation between the peak height of **1** and mean cultivar rank using an exponential curve ($r^2 = 0.802$).

Distribution of **1** *in Plants*. The concentration of **1** in mature vegetative chrysanthemums was highest in the first pair of fully opened leaves, and decreased toward the bottom of the plants in both Super Yellow and Florida Marble (Figure 4). Figure 4 also illustrates that the difference in marker concentration within cultivars was significantly less than between susceptible and resistant cultivars. The highest concentration of **1** in Florida Marble was lower than the lowest concentration of **1** in Super Yellow. These observations, combined with greenhouse observations that more thrips damage is found on leaves positioned lower on the plants, provide additional support for the association of the concentration of **1** with host-plant resistance against thrips. Choice bioassay tests between leaf disks of upper (2nd)

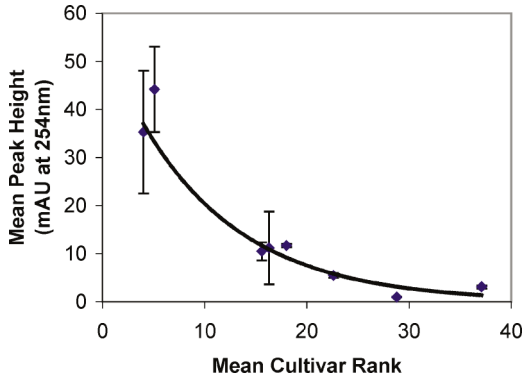

FIG. 3. Mean cultivar rank of eight chrysanthemum cultivars as determined by Broadbent et al. (1990) vs. mean peak height of compound **1** in each cultivar reported in milliabsorbance units (mAU) (mean of three replicates, error bars indicate standard deviation). The higher the cultivar rank, the more susceptible the cultivar is to damage by WFT.

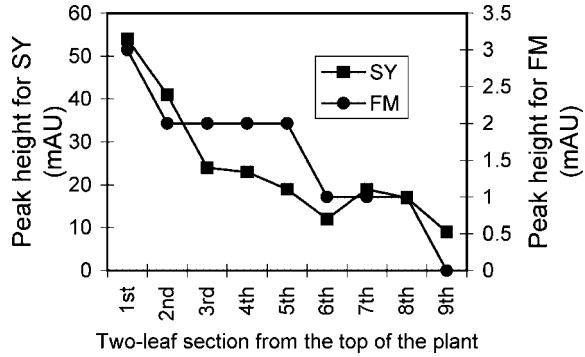

FIG. 4. Peak height of compound **1** in two-leaf sections from the top of Super Yellow (SY, left axis) and Florida Marble (FM, right axis) plants. mAU = milliabsorbance units

and lower (6th) leaves of the same plants of four cultivars confirmed that thrips preferred the lower leaves (Figure 5).

Concentration of **1** *in Chrysanthemums of Different Maturity.* The peak heights of **1** in leaf extracts of 20-day-old Super Yellow and Florida Marble plants (18.0 and 1.0 milliabsorbance units (mAU), respectively) were not appreciably different from those in leaf extracts of 42-day-old plants of the same cultivars (18.0 and 1.5 mAU, respectively). This suggests that the concentration of **1** may not be influenced by the age of the plant.

Other unsaturated isobutylamides have been shown to have insecticidal properties (Jacobson, 1971; Miyakado et al., 1989). The concentration of **1** in the leaves of chrysanthemum cultivars may be useful as a predictor of the degree of thrips-resistance exhibited by those cultivars. This information would be useful in breeding programs for identification of resistant parent and progeny lines.

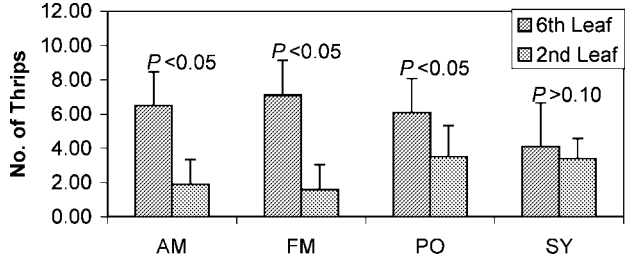

FIG. 5. Number of thrips (mean of 10 replicates) observed on leaf disks from lower (6th) and upper (2nd) leaves of Amber (AM), Florida Marble (FM), Polaris (PO) and Super Yellow (SY) plants.

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COMPOSITION AND INSECT ATTRACTING ACTIVITY OF THE ESSENTIAL OIL OF *Rosmarinus officinalis*

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Abstract—The essential oil and a number of extracts of *Rosmarinus officinalis* L. in solvents of increasing polarity were isolated, and their components identified and tested as pest control agents. Ethanol and acetone extracts attract grape berry moth *Lobesia botrana*. However, none of the extracts had a significant effect on western flower thrips *Frankliniella occidentalis*, which is attracted by 1,8-cineole, a major essential oil component.

Key Words—*Rosmarinus officinalis*, essential oil, *Lobesia botrana*, *Frankliniella occidentalis*, 1,8-cineole, insect attractants.

INTRODUCTION

Rosemary *(Rosmarinus officinalis* L.) is an evergreen shrub that grows in the Mediterranean region and is widely known for its culinary and folklore medicinal uses. The plant, belonging to Labiatae family, grows to a height up to 2 m, and has linear leathery leaves that are sharply pointed, deep green with revolute margins, and are whitish beneath. Its flowers are pale to mid-blue, 10–12 mm long, and borne in small lateral clusters (Blamey and Grey-Wilson, 1998).

The essential oil composition of rosemary has been investigated and reported in literature of several areas, mainly in the Mediterranean region. Studies in the Balkan and Northern Mediterranean regions include analyses of Greek, Yugoslavian, Bulgarian, Hungarian Portuguese, Spanish, French, and Italian oil.

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Reports on South-Eastern Mediterranean regions include Egyptian, Tunisian, and Turkish oil, whereas studies from other areas include USA, Cuban, and Argentinean oil (Lawrence, 1979, 1989, 1993, 1995). The consistency of the oils varies depending of the origin of the sample; major oil components include 1,8-cineole (eucalyptol), α-pinene, camphor, borneol, myrcene, and *p*-cymene.

Plant volatiles play an essential role in communication between host plants and herbivorous insects, and affect their behavior by stimulating host orientation, landing, oviposition, feeding, sexual maturity, as well as by attracting parasitoids of pests searching for potential insect hosts (Finch, 1980; Ahmad, 1983; Harborne, 1987; Mitchell et al., 1990; Lewis et al., 1994). During the last 10 years, plant allelochemicals, especially volatile compounds, have received increasing attention as important adjuncts to sex-pheromone technology. Identification of plant odors that affect host locating, feeding, deterring, and reproductive behavior, could complement other control methods that aim at the control of the establishment, development, and dispersal of crop insect pests and their parasitoids (Visser, 1986; Mitchell and Tingle, 1996; Hanson and Christensen, 1999).

Plant produced semiochemicals provide a variety of opportunities to develop several alternative strategies, e.g., push-pull (Bennison et al., 2002), and to control crop pests. Plants that attract parasitoids can be located strategically in and around cultivated crops as an "incubating" pool to increase the population dynamic of biological agents. Khan et al. (1997) reported that the parasitization of *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) and *Busseola fusca* Füller (Lepidoptera: Noctuidae) increased significantly in maize crops surrounded by Sudan grass as compared to the monoculture. The combination of trap and repellent plants has been also used successfully in a push–pull strategy. Several plants with such properties have been identified (Khan et al., 2001). Among them, Napier grass, *Pennisetum purpureum* Schumach, and Sudan grass, *Sorghum vulgare sudanense* Stapf, appeared to be most promising (Khan et al., 2001). Moreover, application of selected chemicals can mask pest attractants emitted by plants and confuse orientation to breeding areas (Thiéry and Visser, 1986).

The grape berry moth *Lobesia botrana* Den. and Schiff. (Lepidoptera: Tortricidae) is the most serious pest of grape berries causing heavy crop losses of up to 80% in Crete and Greece. It is a polyphagus insect that develops–two to four generations in vineyards per year, depending on the climate conditions. The 2nd and 3rd generations are destructive to grapes (Stellwaag, 1928; Bovey, 1966; Stoeva, 1982; Roditakis, 1987; Roditakis and Roditakis, 2003). Rosemary is a favorable autumn host of *L. botrana* in Greece, on which it develops a fourth generation (Roditakis, 1987; Roditakis and Roditakis, 2003). The role of volatiles emitted from this host plant in the orientation, host finding, egg laying, feeding, etc. of the insect is unknown. Gabel et al. (1992, 1994) reported that tansy flower extracts, a nonhost plant, and certain components of its extracts were attractive to *L. botrana*.

Western flower thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) is also one of the most serious pests of vegetables grown either in greenhouse or as outdoor crops in Crete (Roditakis, 1991; Roditakis et al., 2001). As *F. occidentalis* is resistant to most registered insecticides and a vector of tomato-spotted wilt virus, it can cause heavy crop losses in several vegetable fruits even in very low populations (Cho et al., 1989; Immaraju et al., 1992). Consequently, alternative control methods are needed.

Both insects have a wide range of host plants (Roditakis, 1987; Mantel and Van De Vrie, 1998; Roditakis and Roditakis, 2003). Studies on pest–host plant relations, based mainly on volatiles emitted from host plants (semiochemicals), can lead to new, effective, natural means of insect control. Pheromones have been used successfully for sex confusion control methods or to monitor the grape berry moth (Charmillot and Pasquier, 2000). Promising results have been obtained using neroly spec, the commercial essential oil, in mass trapping methods of *F. occidentalis* in greenhouse cucumber crops (Roditakis et al., 2002).

The aim of this study was to examine a pest control strategy using natural compounds from host plants of grape berry moth and western flower thrips. The essential oil and a number of increasingly polar extracts of *Rosmarinus officinalis* L. were isolated, and their components identified, and tested as biological agents in pest control.

METHODS AND MATERIALS

Chemical Analysis. The essential oil and extracts were analyzed by GC–MS on a Shimadzu GC-17 A gas chromatograph coupled with a Shimadzu GCMS-QP 5050 mass selective detector. A Supelco SBP-5 fused silica capillary column of a 30 m $\times$ 0.25 mm i.d. (0.25-μm film thickness) was used for the analysis. The carrier gas was helium (He) at flow rate of 0.9 ml/min, the injector temperature was 250°C, the detector temperature was 230°C, and the column was temperature programmed as follows: 50°C for 2 min, the temperature increased to 150°C at a rate of 10°C/min, increased to 290°C at a rate of 4°C/min, and then held constant at 290°C for 20 min. This oven program resulted in the best component resolution with a total analysis time of 67 min. The mass unit conditions were: ionization energy 70 eV, ion source 195°C, with 0.5 scans/sec from 35 to 450 m/z. Chromatographs and mass spectra were recorded using the CLASS 5000 program. Components were identified on the basis of their mass spectra using the NIST 64 and NIST 120 GC-MS libraries and the comparison of their retention times with those of reference compounds.

The essential oil from the leaves of the plant was received as follows: powdered leaves of *R. officinalis* (60 g, dw) were hydrodistilled to a total of 1.5 l of distillate (three fractions of 500 ml); 50 g NaCl were added to each fraction, which consequently was extracted four times with 40 ml of diethyl ether. The organic

phases were combined, dried over anhydrous magnesium sulfate, filtered, and the solvent was removed *in vacuo* to a volume of 1 ml. The remainder of the solvent was removed by a stream of nitrogen.

Leaves of *R. officinalis* were extracted as follows: powdered leaves (10 g) were extracted successively using a Soxhlet extractor, with hexane, dichloromethane, acetone, and ethanol. The extracts were filtered, the filtrate was evaporated to a volume of 1 ml, and the rest of the solvent was removed under a stream of nitrogen. Samples from the essential oil and the four extracts were diluted in methylene chloride to a concentration of 1 mg/ml and analyzed by GC-MS as described earlier.

Plants. Rosmarinus officinalis L. was collected from the island of Crete in Heraklion on September 1999 (coll. N. Roditakis). The plant was identified by Dr. Z. Kypriotakis, Technical University of Crete, and voucher specimens deposited at the Plant Protection Institute of National Agricultural Research Foundation (Heraklion Crete, Greece). Samples were dried in a dark, air conditioned room at $24 \pm 1^{\circ}$C. Although some volatiles might have been lost during the drying process, dry samples were used instead of fresh material for comparison with literature data.

Insects and Bioassays. Two types of olfactometers were used in the bioassays: (1) a modified "star" four arm Plexiglas olfactometer consisting of two plates ($105 \times 105 \times 6$ mm^3) with an air pump system directing a weak air stream from each of four arms towards the center, suitable for thrips olfactometer tests (Peterson, 1970; Pow et al., 1999), and (2) a Y-tube olfactometer designed for grape berry moth tests. The latter consisted of two cylindrical glasses (8 cm diam $\times$ 10 cm high) connected with a Y-tube (0.5 cm diam, 7 cm arm length). An electric pump was used to pump air from the Y-joint. The tested material was put onto cylindrical glasses. Air speed was regulated by a flow meter (GPE 314-271 UK) at 4.8 m/min. Laboratory tests on *L. botrana* were performed on the Y-tube olfactometer in a rearing conditioning room ($25 \pm 1^{\circ}$C, $50 \pm 5\%$ RH, and 3000 lux light intensity). Three quantities of neat extracts (20, 40, and 60 μl) were compared with the respective extraction solvent. Control experiments using air vs. air and solvent vs. solvent indicated that each arm of the Y-tube was equally visited.

Newly emerged virgin female adults of grape berry moth (3–4), from a laboratory colony grown on artificial media (Tzanakakis and Savopoulou, 1972) for 9 generations per year, were placed into the test tube, and their movement to the arms was recorded at 1, 2, and 24 hr at $25 \pm 1^{\circ}$C, $50 \pm 5\%$ RH, and 3000 lux. A total of 30–34 adults were tested per treatment.

Data Processing and Statistics. The choice test was statistically evaluated. Ten adults of western flower thrips were tested in each assay. Certain volatile compounds of plant extracts were also chosen for further tests on *L. botrana* and *F. occidentalis* under laboratory conditions ($25 \pm 1^{\circ}$C, $60 \pm 5\%$ RH). In the case of *L. botrana*, 1,8-cineole, camphor, and 1-borneol, major components of rosemary extracts, were tested. The attractiveness of linalool, and 1,8-cineole, components

of extracts of *R. officinallis,* were tested on *F. occidentalis*, and compared with *p*-anisaldehyde, a known component of floral scents attractive to *F. occidentalis* (Harborne, 1987; Teylon et al., 1993), and salicylaldehyde, previously tested at 1% concentration in paraffin oil under working greenhouse conditions by Roditakis and Lykouressis (1996).

RESULTS AND DISCUSSION

Results of the component analyses by GC–MS are listed in Table 1. The essential oil contains a group of major components, listed in decreasing abundance, and includes camphor, eucalyptol (1,8-cineole), and borneol, followed by α-terpineol, 4-terpineol, cuminal, cuminol, *trans*-carveol, and τ-cadinol. Major components in the essential oil appear also in most solvent extracts and in the same order of abundance. α-Pinene, mentioned as a major component in most literature reports, was not present in any of the analyzed samples, and β-pinene appeared as a trace component in the essential oil. α-Terpineol was identified as the major (5.67%) component in contrast to most literature reports. However, its presence in relatively large amounts (>5%) has been reported before (Boutekedjiret et al., 1999). Its large abundance combined with the absence of α-pinene could be used as a means of identification of the plants growing in the area.

L. botrana is attracted by certain extracts of tansy flowers *Tanacetun vulgare* L., even though it is not a host plant (Gabel et al., 1992, 1994). Analysis of extracts by GC–EAG methodology showed that a number of monoterpenes including *p*-cymene, *d*-limonene, α-thujene, α-thujone, a blend of β-thujone, and thujyl alcohol elicited responses in 70% of females (Gabel et al., 1992) Hurtrel and Thiéry (1999) reported that photoperiod, age, and mating plays a role on flight activity of grape berry moth. During a 24 hr ligh/dark period, its activity increased close to the end of the photophase. One-day old females did not fly as often as older ones, and mated females showed better response to plant odors from tansy flowers.

In our olfactometer tests, we used 1-day-old females for quick tests since they have to make a double choice (volatile vs. solvent), running (and not flying) a 2–3 cm glass tube up to the arena in 24 hr (8 hr:16 hr D:L).

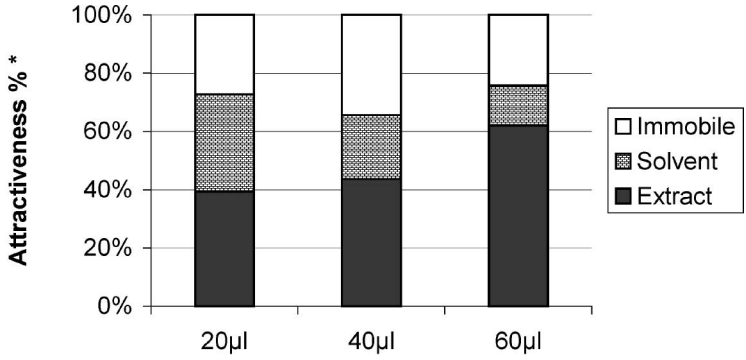
Adults did not respond during the first 2 hr of their exposure to volatiles. However, there was a distinct response close to the end of the photophase. A small percentage (20–40%) of adults tested remained immobile in the test tube for unknown reasons during the whole experimental period, while the rest made a choice between solvent and extract (Figures 1 and 2).

Given that *R. officinalis* is a good autumn host plant (Roditakis, 1987; Roditakis and Roditakis, 2003) for *L. botrana*, its response to three different quantities (20, 40, and 60 μl) of plant extracts was tested. The response depended on the type of extract as well as its quantity (Figures 1 and 2). Increasing quantities of acetone extract from 20–60 μl caused a linear response, while the corresponding

TABLE 1. COMPOSITION OF THE ESSENTIAL OIL AND EXTRACTS FROM *Rosmarinus officinalis* L.

	Component	Rt	Essential oil	Hexane extract	CH_2Cl_2 extract	Acetone extract	Ethanol extract
1	4,4-Dimethyl-2-buten-4-olide[a]	7.34	0.09				
2	Benzaldehyde	7.55	0.08				
3	1-Octen-3-ol	7.88	0.13				
4	$\beta-$Pinene	7.93	0.03	1.15		0.22	
5	2,3-Dehydro-1,8-cineole	8.15	0.26				
6	*p*-Cymene	8.46	0.10	2.02	1.61	0.89	1.08
7	1,8-Cineole	8.80	12.89	27.39	35.67	19.96	30.57
8	*o*-Cresol	9.40	0.04				
9	*cis*-Linalool oxide	9.66	0.42				
10	*cis*-Ocimene-8-oxo	9.84	0.63				
11	6-Camphenone	10.12	0.43				
12	Linalool	10.26	0.29	1.85			
13	*trans*-Pinocarveol	10.49	1.49		0.96	0.78	0.65
14	Camphor	11.01	22.24	30.23	33.45	27.76	32.71
15	Borneol	11.39	7.37	5.45	3.75	4.55	4.39
16	4-Terpineol	11.54	3.58				
17	$\alpha-$Terpineol	11.70	5.67		1.80	2.57	3.67
18	Myrtenal	11.86	1.85	0.86	0.68	3.24	0.62
19	Eucarvone	12.06	1.79	2.04			
20	*trans*-Carveol	12.17	2.36			0.82	
21	*p*-Cumenol	12.33	0.51				
22	Cuminal	12.56	3.39	2.11	1.80	1.93	
23	Carvone	12.79	1.29	0.78	0.69	0.88	
24	3[10]-Caren-2-ol	13.18	1.16				
25	*p*-Cymen-7-ol	13.38	2.51	1.01	1.64	2.20	0.92
26	Thymol	13.50	0.95				
27	Verbenone	13.82	0.86				
28	Thymoquinone	13.93	1.44	2.36	3.88	2.21	
29	$\alpha-$Terpinyl acetate	14.33	0.50				
30	Isoeugenol	14.54	0.51				
31	Geranyl acetate	14.89	0.29				
32	Coumarin	16.26	1.55	1.08	2.53	4.35	8.97
33	$\gamma-$Muurolene	17.91	0.85	1.33	1.05	1.52	1.52
34	Caryophyllene oxide	19.56	1.85	1.92	1.26	1.39	1.28
35	Cubenol	20.22	0.50	0.23			
36	*t*-Cadinol	20.79	2.25	2.08	1.61	2.06	1.59
37	$\alpha-$Bisabolol oxide	21.12	1.56	1.28		1.00	
38	$\alpha-$Bisabolol	21.69	0.67	0.46	0.56		
39	Megastigmatrienone	23.28	0.57				
Total % in the mixture			85.21	85.63	92.94	78.33	87.97

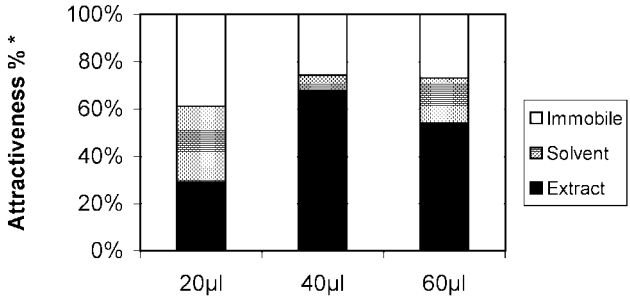
[a] Tentative identification. MS data: m/z: 112 (19.8), 110 (5.7), 97 (100), 81 (2.2), 69 (96.5), 67 (16.4), 59 (15.7), 54 (19.2).



* Immobile: Non responding insects. Solvent: Insects attracted by the solvent odor. Extract: Insects attracted by the odor of each dose of extract.

FIG. 1. Attractiveness of increasing doses of acetone extract of *Rosmarinus officinalis* L. on grape berry moth *Lobesia botrana*.

ethanol extract quantities peaked at 40 μl. Insects were attracted by rosemary acetone extracts at 60 μl, as well as ethanol extracts at 40 μl quantities. It appeared that these quantities of extracts had the optimum concentration of active attractants. Chi square analysis on the number of one-day-old females of *L. botrana* attracted by different quantities of extracts vs. those attracted by the corresponding extraction solvent (Table 2) showed that 60 μl of acetone extract and 40 and 60 μl of ethanol extracts of *R. officinalis* exhibited significant attractiveness. The response of insects to hexane extracts was minimal; further experiments were not



* Immobile: Non responding insects. Solvent: Insects attracted by the solvent odor. Extract: Insects attracted by the odor of each dose of extract.

FIG. 2. Attractiveness of increasing doses of ethanol extract of *Rosmarinus officinalis* L. on grape berry moth *Lobesia botrana*.

TABLE 2. CHI SQUARE ANALYSIS OF NUMBER OF 1-DAY-OLD *L. Botrana* FEMALES ATTRACTED BY DIFFERENT QUANTITIES OF ACETONE OR ETHANOL EXTRACTS OF *Rosmarinus officinalis* VS. THOSE ATTRACTED BY THE RESPECTIVE SOLVENTS

Treatments	Number of insects		$\chi^2(P = 0.05)$	
	Attracted by extract	Attracted by solvent		
1. Acetone extract				
20 μl	13	11	0.72	NS[a]
40 μl	14	7		
20 μl	13	11	3.99	SS[b]
60 μl	18	4		
2. Ethanol extract				
20 μl	9	10	6.55	SS
40 μl	21	2		
20 μl	9	10	2.75	SS
60 μl	14	5		

[a] *NS*: Non significant.
[b] *SS*: Statistically significant.

performed due to the limited availability of sample. Traces of methylene chloride in the extracts were fatal to insects.

Among selected components, 20 μl samples of 1,8-cineole (1% in paraffin oil) showed distinct activity (Table 3), borneol was neutral, and camphor was repellent. All three of these are known components of flowers in many plants (Harborne, 1987). Single compounds are less attractive than complete blends. Gabel and Thiéry (1995) reported that odorant traps with a complete floral blend of tansy flowers, and two floral blends reduced to eight and six monoterpenes, respectively, attracted many more *L. botrana* females than a binary blend consisting of α- and β-thujone. Although camphor and borneol were not attractive, we believe that they should be studied further at several doses or in blends before proper conclusions can be drawn.

TABLE 3. ATTRACTIVENESS OF 1,8-CINEOLE, CAMPHOR, AND BORNEOL ON GRAPE BERRY MOTH *Lobesia botrana*

Compound[a]	Number of tested females	Number of attracted females	Percentage of attracted females
1,8-cineole	16	12	75
Camphor	59	36	61
Borneol	38	16	23

[a] 20 μl dose, 1% in paraffin oil.

Analysis of rosemary extracts (Table 1) showed that, with the exception of *p*-cymene and α-terpineol, other components were different than tansy flowers' (Gabel et al., 1992). The attractive blend of *L. botrana* used by Gabel et al. (1992) had only two components (*p*-cymene and α-terpineol), similar to those of rosemary extracts. Analysis of volatiles in grape leaves, grapes, and immature grapes (Schreier et al., 1976) indicated that only 1-octene-3-ol, α-terpineol, and (±)-linalool were similar to the components listed in Table 1. The only component present in the above-mentioned host and nonhost plants, which is listed in Table 1, is α-terpineol. Studies on antenal lobe neuronal responses of *L. botràna* to α-terpineol showed significant differences between male and female insects and between mated and unmated females. The number of responding neurons was higher in mated females (Masante-Roca et al., 2002). α-Terpineol could be a promising attractant, but has to be tested further.

β-Thujone and thujyl alcohol, the main components of a nonhost plant that elicited the most frequent responses to antenal lobe neurons of grape berry moth (Masante-Roca et al., 2002), and also the main components in an attractive blend used by Gabel et al. (1992), are not present in its hosts, vines, and rosemary. Several functional hypotheses could be considered to provide an interpretation for their absence. (a) *L. botrana* may search for a more or less accurate "chemical image" from host and nonhost plants, or (b) the female adapts its behavior to different chemical compositions.

Rosemary essential oil at 0.1% and 1% concentrations in paraffin oil decreased feeding damage of onion thrips *Thrips tabaci*, while 1,8-cineole reduced oviposition rate by 30% in these concentrations (Koshier and Sedy, 2002). The attractiveness of these odors varies with the thrips species. Anisaldehyde attracted *F. occidentalis* but not *T. tabaci*. Salicylaldehyde in certain concentrations acted as repellent to *F. occidentalis*, but highly attractive to *T. tabaci* (Kögel and Koshier, 2002).

On the basis of these data, we tested the response of *F. occidentalis* to *R. officinalis* extracts as well as to several other compounds of plant origin. None of the extracts caused a significant effect. Also, no responses were positively correlated with an increase in concentration of single compounds (Table 4). Linalool at 2.0%, anisaldehyde at 0.5%, salicylaldehyde at 0.5%, and 1,8-cineole at 1% induced a positive response in 70–80% of the adults tested.

Floral volatiles, e.g., *p*-anisaldehyde, benzaldehyde, and ethyl nicotinate, are very good attractants to western flower thrips and could be used for accurate monitoring (Teylon et al., 1993). Temperature and wind strength affect the response of insects to volatiles. Colored traps with volatile chemicals may be useful for control trapping in a protected environment such as a greenhouse. Roditakis et al. (2002) reported that the commercial essential oil, neroly spec, at 1% concentration in paraffin oil is promising for control of *F. occidentalis* in greenhouse cucumbers. Traps with volatiles may not kill so many thrips, but they could be used in combination with other methods as part of an integrated pest management program. The use of

TABLE 4. ATTRACTIVENESS % OF 1,8-CINEOLE, LINALOOL, *p*-ANISALDEHYDE AND SALICYL ALDEHYDE (IN PARAFFIN OIL) ON WESTERN FLOWER THRIPS *Frankliniella occidentalis*

Concentration % v/v of tested compounds[a]	Effect (%) of tested compounds on *Frankliniella occidentalis*			
	1,8-cineole	Linalool	*p*-Anisaldehyde	Salicylaldehyde
0.125	NT[b]	NT	NT	70
0.25	NT	NT	50	40
0.50	50	0	70	20
1.00	80	50	40	0
2.00	60	80	NT	NT

[a] μl of each solution in parafin oil was used in each assay.
[b] Not tested.

1,8-cineole, *p*-anisaldehyde, linalool, and salicylaldehyde in appropriate concentrations could be used either for monitoring purposes or for mass trapping of *F. occidentalis* on greenhouse crops as alternative means to chemical control of pests.

In conclusion, initial tests, employing extracts from *R. officinalis* as well as selected compounds, indicate the ability of these agents to attract insect pests. The effect of specific components from the acetone and ethanol extracts on *L. botrana* is currently under investigation.

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FORAGING IN CHEMICALLY DIVERSE ENVIRONMENTS: ENERGY, PROTEIN, AND ALTERNATIVE FOODS INFLUENCE INGESTION OF PLANT SECONDARY METABOLITES BY LAMBS

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Abstract—Interactions among nutrients and plant secondary metabolites (PSM) may influence how herbivores mix their diets and use food resources. We determined intake of a food containing a mix of terpenoids identified in sagebrush (*Artemisia tridentata*) when present in isoenergetic diets of increasing concentrations of protein (6, 9, 15, or 21% CP) or in isonitrogenous diets of increasing concentrations of energy (2.17, 2.55, 3.30, or 3.53 Mcal/kg). Lambs were offered choices between those diets with or without terpenes or between diets with terpenes and alfalfa hay. Intake of the diets with terpenes was lowest with the lowest concentrations of protein (6%) and energy (2.17 Mcal/kg) in the diets, and highest with diets of 15% CP and 3.53 Mcal/kg. In contrast, when terpenes were absent from the diets, lambs consumed similar amounts of all four diets with different concentrations of protein, and more of the diets with intermediate amounts of energy. When given a choice between the diet with or without terpenes, lambs preferred the diet without terpenes. When lambs were offered choices between terpene-containing diets and alfalfa, energy and protein concentrations influenced the amount of terpenes animals ingested. Energy densities higher than alfalfa, and protein concentrations higher than 6%, increased intake of the terpene-containing diet. Thus, the nutritional environment interacted with terpenes to influence preference such that lambs offered diets of higher energy or protein concentration ate more terpenes when forced, but not when offered alternative food without terpenes. The nutrients supplied by a plant and its neighbors likely influence how much PSM an animal can ingest, which in turn may affect the dynamics of plant communities, and the distribution of herbivores in a landscape. We discuss implications

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of these findings for traditional views of grazing refuges and varied diets in herbivores.

Key Words—Terpenes, refuges, nutrient–toxin interactions, choice, preference, foraging, energy, protein, sagebrush.

INTRODUCTION

Herbivores can change ecosystems by removing specific kinds and amounts of tissues from plants (Hobbs, 1996), but the mechanisms that explain "which" and "how much" tissues are eaten are not well understood. Plants produce diverse mixtures of biochemicals that provide herbivores with nutrients for survival and reproduction and secondary metabolites that can both cause toxicity and yield health benefits (Cheeke and Shull, 1985; Engel, 2002). Despite the diversity of chemicals ingested in a meal, with few exceptions our understanding of multiple interactions among nutrients and plant secondary metabolites (PSM) is rudimentary (Simpson and Raubenheimer, 2001; Villalba et al., 2002a). We have relied on univariate approaches to studying the isolated effects of PSMs or nutrients on food selection.

Plant secondary metabolites satiate herbivores (Garcia, 1989; Provenza, 1996), and the degree to which a PSM limits food intake is influenced by the kinds and amounts of nutrients herbivores ingest (Villalba et al., 2002a). Many PSM are lipophilic compounds that must be transformed into hydrophilic substances before excretion (Cheeke and Schull, 1985), and these processes deplete the body of amino acids and glucose (Illius and Jessop, 1995, 1996). Thus, ingesting appropriate amounts of nutrients should increase the threshold of PSM satiation and enable animals to ingest more of foods that contain PSM, although the willingness of herbivores to consume PSM as a function of the concentration of nutrients containing PSM is not known.

Outcomes of interactions among nutrients and PSM may help explain the dietary choices of herbivores. Browsing by black-tailed deer causes serious damage to young Douglas-fir trees, which contain an array of volatile terpenes that can inhibit rumen microbial fermentation (Connolly et al., 1980). Browsing begins shortly after bud burst (Crouch and Radwan, 1981), when concentrations of nitrogen and fermentability of the new growth is higher than in the old growth (Oh et al., 1970). Nitrogen fertilization increases protein content and fermentability of seedlings, which increases acceptability to deer (Oh et al., 1970; Crouch and Radwan, 1981). Thus, the amount of a chemically defended species an herbivore will eat is influenced by concentration of nutrients and PSMs, likely in relation to those in neighboring plants. Because concentrations of nutrients and toxins covary in the field, controlled experiments are needed to disentangle the influence of PSM and nutrients on foraging behavior.

We hypothesized intake and preference for PSMs are influenced by the nutritional context where herbivores experience PSM. We further hypothesized that the willingness of herbivores to ingest PSM is influenced by the nutritional composition of the food containing the PSM relative to the nutritional composition and availability of alternative foods. Based on these hypotheses, we predicted that intake of PSM would increase with an increase in the concentration of either energy or protein in the food, and that preference for PSM would increase with the increased capacity—due to energy and protein—to consume higher amounts of PSM, and/or with the decrease in nutritive value of alternative foods. To assess the effects of PSM on food intake and preference, we exposed lambs to isoenergetic foods with increasing concentrations of protein and to isonitrogenous foods with increasing concentrations of energy, with or without the addition of a mix of terpenoids in sagebrush (*Artemisia tridentata*). We then determined preference for terpene- and nonterpene containing foods.

METHODS AND MATERIALS

We determined intake and preference of lambs for terpenes in isoenergetic and isonitrogenous foods that differed across gradients of protein and energy, respectively. For each gradient, we used a different set of 32 lambs (commercial crossbreds, 4–5 mo age). Throughout each experiment, lambs were individually penned and had free access to mineral blocks and fresh water.

Ingestion of Terpenes across Gradients of Protein and Energy. Lambs were weighed and randomly assigned to four diets (8 lambs/diet) that differed in concentrations of energy or protein. Diets contained different proportions of the same ingredients (Table 1), ground to 1–5 mm particle size.

To modify the nutritional state of the animals and familiarize them with the food ingredients, for the first 10 d, Groups 1, 2, 3, and 4 were offered control diets 1, 2, 3, and 4, respectively, without the addition of terpenes from 0900 to 1300 hr. At 1300 hr, refusals were collected, intake was determined and no other food was offered until the next day.

For the next 10 d, we used the same procedures, but terpenes were added to each diet at the same relative concentrations found in *Artemisia tridentata* (Personius et al., 1987; Launchbaugh, unpublished results): 1.82% camphor, 1.1% 1,8-cineole, 0.12% methacrolein, 0.06% *p*-cymene, dissolved in vegetable oil and then mixed with the rest of the food ingredients on a daily basis (Table 1). These terpenes adversely influence intake and preference (Bray et al., 1991; Villalba et al., 2002a).

Preference Tests Immediately after Exposure. We then determined preference of lambs for food with or without terpenes, or for food with terpenes vs. alfalfa hay. From 0900 to 1300 hr the day after exposure to the terpene-containing diets,

TABLE 1. INGREDIENTS, DIGESTIBLE ENERGY (DE) AND CRUDE PROTEIN (CP) IN FOODS (AS-FED BASIS)

Ingredient (g/100g)	Group 1	Group 2	Group 3	Group 4
	Protein gradient			
Control diets				
Beet pulp	48.0	39.0	14.0	1.0
Grape pomace	42.5	41.0	31.0	31.5
Alfalfa hay	5.0	11.0	39.0	41.5
Soybean meal	0.5	5.0	12.0	22.0
Vegetable oil	4.0	4.0	4.0	4.0
Terpene diets				
Beet pulp	48.6	40.0	15.0	2.0
Grape pomace	37.8	35.9	25.9	26.9
Alfalfa hay	6.0	12.0	40.0	42.0
Soybean meal	0.5	5.0	12.0	22.0
Vegetable oil	4.0	4.0	4.0	4.0
Terpenes	3.1	3.1	3.1	3.1
DE (Mcal/kg)[a]	2.55	2.55	2.55	2.55
CP (%)[b]	5.5	8.7	15.1	20.7
CP/DE ratio	21.0	30.0	50.0	65.0
	Energy gradient			
Control diets				
Beet pulp	5.0	14.0	65.0	81.0
Grape pomace	64.0	31.0	9.0	2.0
Alfalfa hay	2.0	39.0	12.0	4.0
Soybean meal	25.0	12.0	10.0	9.0
Vegetable oil	4.0	4.0	4.0	4.0
Terpene diets				
Beet pulp	6.8	15.0	66.5	83.0
Grape pomace	59.6	25.9	4.9	0.1
Alfalfa hay	1.5	40.0	11.5	0.1
Soybean meal	25.0	12.0	10.0	9.7
Vegetable oil	4.0	4.0	4.0	4.0
Terpenes	3.1	3.1	3.1	3.1
DE (Mcal/kg)[a]	2.17	2.55	3.30	3.53
CP (%)[b]	14.7	16.1	13.6	13.8
CP/DE ratio	59.0	50.0	39.0	36.0

[a] Calculated values of DE are based on values obtained from NRC (1985).
[b] Nitrogen was determined by Kjeldahl method (AOAC, 1975); CP calculated as $N \times 6.25$.

each group of lambs received simultaneously the two diets they received during exposure (control diet and diet with terpenes) for 2 d. Intake was determined and no other food was offered until the next day. For the next 2 d, preference tests were conducted as described earlier, but alfalfa hay (2.50 Mcal/kg; 16% CP) replaced the

control diet for each group of lambs. After preference tests, lambs were weighed, and daily weight gains (g/d) were estimated ([final BW–initial BW]/number of days elapsed between initial and final determination of BW).

Preference Tests 20 Days after Exposure. First, we put all lambs on a similar plane of nutrition after the initial preference tests by offering them *ad libitum* alfalfa pellets and 300 g of barley grain/lamb/day for 20 d. Then, we conducted preference tests as previously described.

Ingestion of Terpenes across a Gradient of Protein Concentrations. Lambs consumed terpenes in isoenergetic diets (2.55 Mcal/kg) with increasing concentrations of protein. Four groups of lambs were randomly assigned to four treatment diets, across a gradient of protein concentrations (Group 1 = 6%; Group 2 = 9%; Group 3 = 15%; Group 4 = 21% CP; Table 1).

Ingestion of Terpenes across a Gradient of Energy Concentrations. Lambs consumed terpenes in isonitrogenous (15% CP) diets with increasing concentrations of energy. Four groups of lambs were randomly assigned to four diets across a gradient of energy concentrations (Group 1 = 2.17; Group 2 = 2.55; Group 3 = 3.30; Group 4 = 3.53 Mcal/kg; Table 1). The concentration of protein was that where lambs had the highest intake of terpenes in the previous study.

Statistical Analyses. The statistical design for the ANOVA during exposure was a split-plot with lambs nested within Group (1–4) and day (1–10) as the repeated measure. Intakes were converted to g food ingested/kg metabolic body weight ($kg^{0.75}$). During preference tests, Group (1–4) was the between-animal factor, and lambs were nested within group. Diet (with terpenes, without terpenes, or alfalfa) was the within-animal factor in the analysis. Day (1–2) and period (immediately after exposure, 20-d after exposure) were repeated measures. When F values were significant ($P < 0.05$), means were compared using the LSD test. The ANOVA was performed using the MIXED procedure (Littell et al., 1996).

RESULTS

Ingestion of Terpenes across a Gradient of Protein Concentrations. Intake did not differ for diets with different concentrations of protein without terpenes (main effect; $P = 0.922$; group × day, $P = 0.731$; Figure 1). However, that pattern changed after the addition of terpenes to the diets (group × day, $P = 0.004$). Initially, terpenes caused a marked decline in intake of all diets, but intake then increased differently depending on the protein concentration in the diets (15% > 21% and 9% > 6% CP; Group effect, $P = 0.004$; Figure 1).

Ingestion of Terpenes across a Gradient of Energy Concentrations. When terpenes were absent from the diets (control diets), lambs in Groups 2 (2.55 Mcal/kg) and 3 (3.30 Mcal/kg) ate more food than lambs in Groups 1 (2.17 Mcal/kg) and 4 (3.53 Mcal/kg; Group effect; $P = 0.003$). As with protein, all groups decreased intake of the diets after the addition of terpenes, and intake diverged through time,

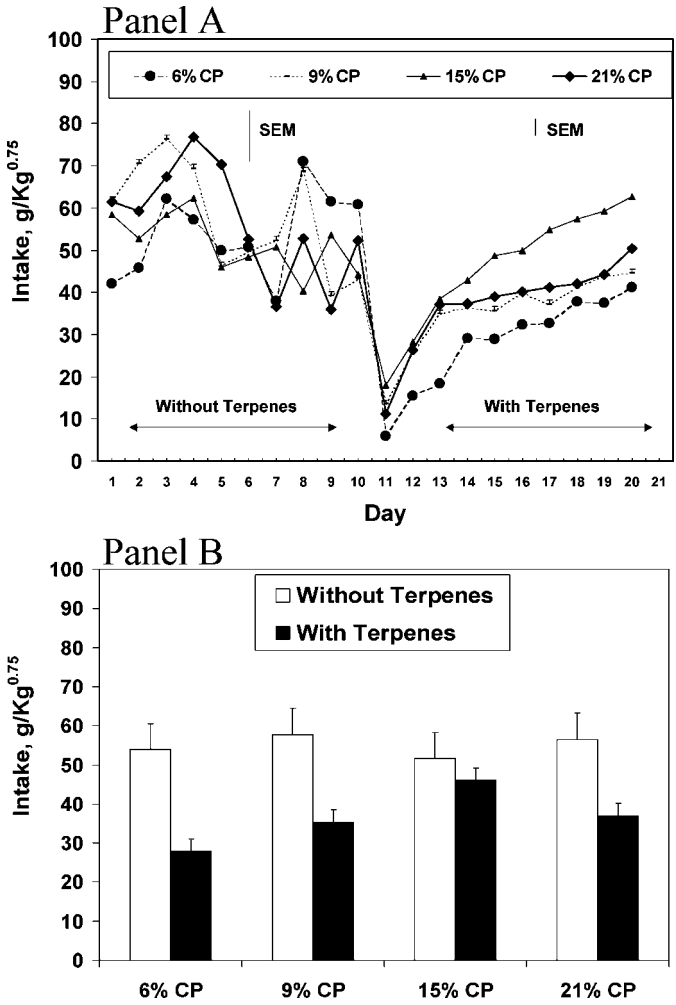

FIG. 1. Intake of diets with or without terpenes across a gradient of protein concentrations. Values are daily means recorded for 8 lambs/diet (Panel A), and averages with standard error bars for each 10-d period, when diets were fed without (without terpenes) or with (with terpenes) terpenes (Panel B).

particularly for lambs in Group 1 vs. Groups 2, 3, and 4 (group $\times$ day, $P < 0.001$; Figure 2); averaged across days, intake of food was Group 4 $>$ Group 3 $=$ Group 2 $>$ Group 1 ($P = 0.012$; Figure 2).

Preference Tests. Lambs avoided the terpene-containing diets, but the pattern of selection was influenced by the nutritional composition of the diets with terpenes

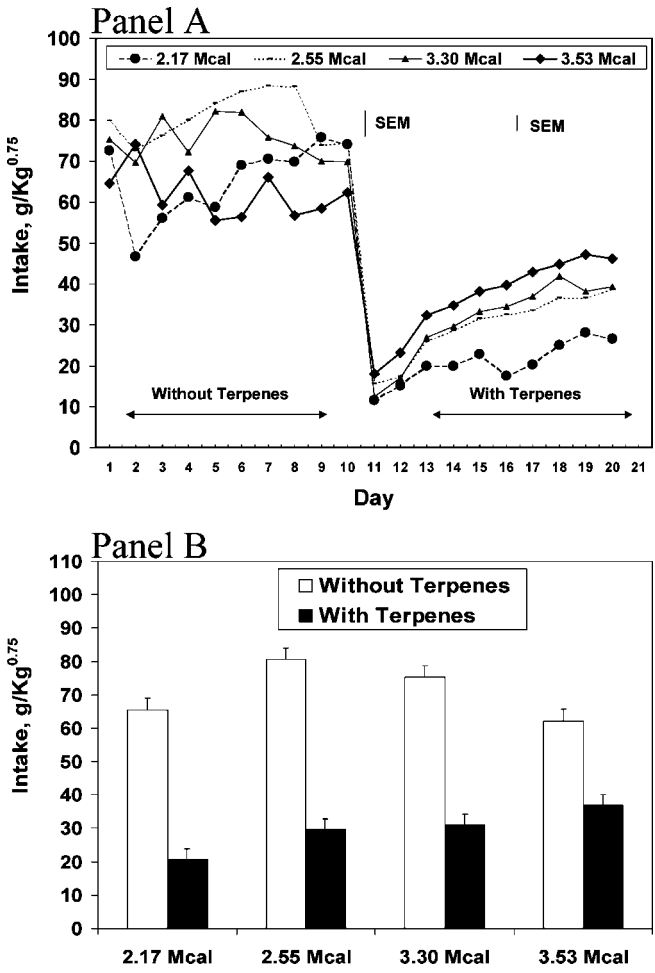

FIG. 2. Intake of diets with or without terpenes across a gradient of energy concentrations. Values are daily means recorded for 8 lambs/diet (Panel A), and averages with standard error bars for each 10-d period, when diets were fed without (without terpenes) or with (with terpenes) terpenes (Panel B).

as well as the nutritional quality of the alternatives (Diet effect; $P < 0.001$; Figure 3).

Preference Tests across a Gradient of Protein Concentrations. When lambs were offered a choice of their respective diets with or without terpenes immediately after exposure, they ate similar amounts of the diet with terpenes ($P > 0.05$). However, 20 d after exposure, only lambs fed the diet with 15% ate more of the

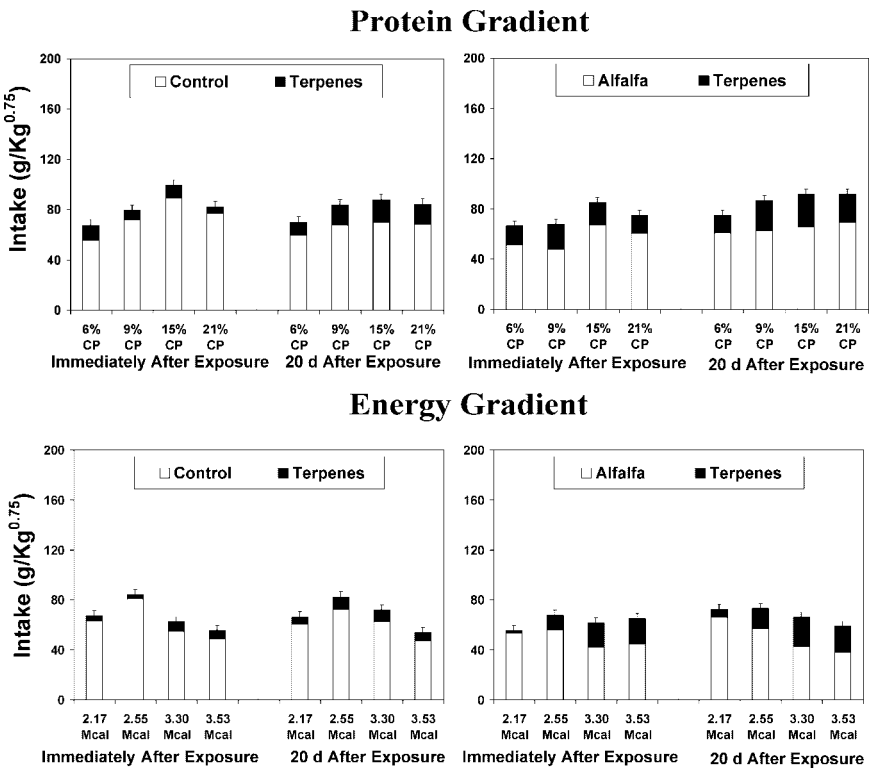

FIG. 3. Intake of diets with (terpenes) and without (control) terpenes, or with terpenes (terpenes) and alfalfa hay (alfalfa) by four groups of lambs during preference tests conducted immediately and 20-d after exposure to diets of different protein (protein gradient) and energy (energy gradient) concentrations. Values are means with standard error bars recorded for 8 lambs/group during 2 consecutive days.

diet with terpenes than lambs fed the diet with 6% CP ($P < 0.05$; Figure 3). Lambs fed 6% CP ate the least of the diet without terpenes in both periods ($P < 0.05$; Figure 3).

When lambs were offered choices between their respective diets with terpenes and alfalfa hay, lambs fed the diet with 9% CP (Group 2) tended to consume more diet with terpenes than lambs fed the diet with 6% CP (Group 1) immediately after exposure ($P = 0.10$). Lambs fed the 6% and 9% CP diets ate less alfalfa than lambs fed the 15% and 21% CP diets. After 20 d, lambs fed 6 % CP ate the least of the diet with terpenes ($P < 0.001$; Figure 3). All lambs ate the same amounts of alfalfa, except that lambs fed 21% CP ate more alfalfa than lambs fed 6% CP ($P < 0.05$; Figure 3).

Preference Tests across a Gradient of Energy Concentrations. When all groups were offered choices between their respective diet with or without terpenes, intake of the terpene diets did not differ among groups either when preference tests were conducted immediately or 20-d after exposure ($P > 0.05$; Figure 3). In both periods, lambs in Group 4 (3.53 Mcal/kg) ate the least amounts of diet without terpenes and lambs in Group 2 (2.55 Mcal/kg) ate the most of the diet without terpenes ($P < 0.05$).

When lambs were offered choices between diets with terpenes and alfalfa hay, lambs fed the diet with 3.53 (Group 4) and 3.30 Mcal/kg (Group 3) ate more diet with terpenes and less alfalfa than lambs fed the diet with 2.17 (Group 1) and 2.55 Mcal/kg (Group 2), both immediately and 20 d after exposure ($P < 0.05$; Figure 3). Lambs in Group 2 ate more terpenes than lambs in Group 1 ($P < 0.05$; Figure 3).

Body Weight Changes. With regard to diets differing in protein, groups did not differ in average daily weight gains ($P = 0.861$), but trends varied along a gradient. Lambs in Groups 1 (6% CP) and 2 (9% CP) lost weight (–22 and –13 g/d, respectively), lambs in Group 3 (15% CP) maintained weight, and lambs in Group 4 (21% CP) gained weight (6 g/d).

With regard to energy, daily weights changed along a gradient (Group effect; $P < 0.001$). Lambs in Group 1 (2.17 Mcal/kg) lost more weight than lambs in Group 2 (2.55 Mcal/kg) (–140 vs. –65 g/d), and lambs in Groups 3 (3.30 Mcal/kg) and 4 (3.53 Mcal/kg) gained weight (5 and 35 g/d).

DISCUSSION

Animals Forced to Consume PSM. We assessed the ability of lambs to consume terpenes across isoenergetic diets that varied in protein, and isonitrogenous diets that varied in energy. Protein and energy availability affected the amount of terpenes lambs ingested. Intakes of diets with terpenes were lowest with diets of 6% CP and 2.17 Mcal/kg, and highest with diets of 15% CP and 3.53 Mcal/kg (Figures 1 and 2). In contrast, when terpenes were absent from the diets, lambs ate similar amounts of diets with different concentrations of protein, and more of diets with intermediate energy (2.55 and 3.30 Mcal/kg) than of either low (2.17 Mcal/kg) or high (3.53 Mcal/kg) concentrations of energy.

Our results are consistent with modeling efforts that suggest the ability to ingest PSM depends on nutrient intake (Illius and Jessop, 1995, 1996). Detoxification processes deplete the body of protein and glucose, and, thus, adequate levels of nutrients are needed to tolerate ingesting PSM (Foley et al., 1995). Supplemental macronutrients increase intake of foods that contain toxins as diverse as lithium chloride (Wang and Provenza, 1996), terpenes (Banner et al., 2000; Villalba et al., 2002b), menthol (Illius and Jessop, 1996), and tannins (Villalba et al., 2002c).

Nonetheless, nutrient availability alone cannot increase tolerance for PSM, as nutritional imbalances can depress ingestion of PSM. Tannic acid affects *Locusta migratoria* performance only when the ratio of protein to carbohydrate in the diet is low (Simpson and Raubenheimer, 2001). Likewise, sheep and goats fed concentrates high in protein, or offered a choice between concentrates high in energy and high in protein, ingest more terpenes (Villalba et al., 2002b) and tannins (Villalba et al., 2002c) than when they receive only concentrates high in energy.

In our study, intake of the terpene-containing diets increased monotonically within the range of energy densities (2.17–3.53 Mcal/kg). In contrast, lambs fed a diet with 21% CP ate less terpene-containing food than lambs fed a diet with 15% CP (Figure 1), which indicates that intake of the terpene-containing diets did not increase monotonically with levels of protein, and that the protein–energy ratio was important. High protein concentrations may overload the animal's deaminating systems (Harper, 1974), and the addition of terpenes might have enhanced the negative effects of excess protein, promoting a decline in food intake relative to diets with lower nitrogen concentrations. Collectively, these results suggest a balanced ratio of protein–energy in the diet can be more important than the absolute concentration of a single nutrient.

Theorists usually regard PSM as fixed constraints on food intake (Belovsky and Schmitz, 1991), but our results argue against that contention (see also Simpson and Raubenheimer, 2001; Behmer et al., 2002). The effects of terpenes depended on the balance of energy and protein. Some also propose that animals cannot consume a food that exceeds a threshold ratio of PSM–nutrient unless they can dilute the ratio by adding other foods or nutrients (Illius and Jessop, 1996). However, by varying protein and energy concentrations we showed that diluting PSM with protein is different from diluting PSM with energy, and that ingesting terpenes as a function of energy and protein density is graded rather than an all-or-none response dependent on a threshold of nutrient supply.

Thus, we predict that when animals are forced to consume an abundant plant high in PSM, the amounts of biomass eaten will depend on the concentrations and balances of energy and protein supplied by the plant. The higher the concentration of nutrients in the plant, along with an adequate balance of protein–energy, the higher the potential amount of plant that can be ingested and the higher the likelihood to sustain maintenance and detoxification costs (Figure 4).

Plants lessen herbivory by reducing nutritive quality (Rhoades, 1979; Haukioja et al., 1991), provided animals are unable to compensate by increasing rates of consumption or time feeding (Augner, 1995). Intake also is restricted by PSM and their interactions with low or imbalanced amounts of nutrients (Figures 1 and 4). Compensatory feeding on a nutritionally imbalanced food, which is needed to satisfy requirements for a limiting nutrient, also can be restricted by PSM (Raubenheimer, 1992). Thus, when alternative plants are scarce, herbivory on abundant, defended plants may decline, even if concentrations of

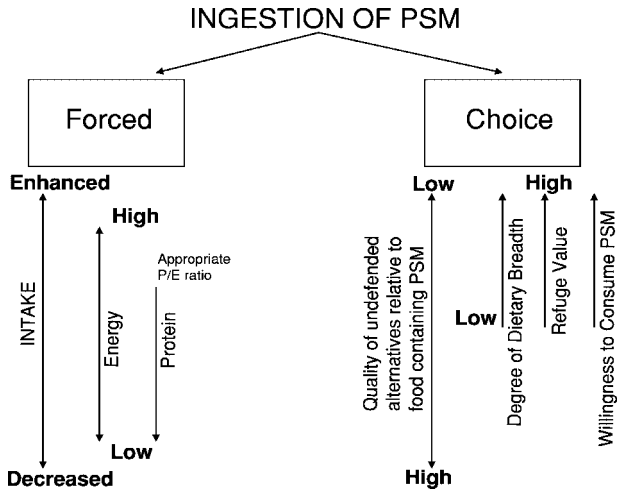

FIG. 4. Conceptual model of ingestion of PSM by herbivores based on results of this study. When animals are forced, due to lack of alternatives, higher concentrations of protein and energy lead to greater intake of PSM, but only when protein–energy ratios are appropriate (forced). When animals have choices of equally abundant defended and undefended plants, their willingness to consume PSM increases only when the quality of undefended alternatives declines relative to the quality of defended plants (choice). This increase in use of defended species increases the degree of dietary breadth due to more uniform use of all foods available, which increases the associational refuge value for undefended species.

PSM do not change, due to dilution or imbalance of nutrients in the plant.

Animals Offered Choices with Foods Containing PSM. All animals ate similar (low) amounts of the terpene-containing foods, regardless of energy or protein contents, when given a choice between diets with or without terpenes. Moreover, the physiological state of the animals—immediately after exposure vs. fed the alfalfa-based diet—did not influence choice, except lambs fed 6% protein ate less diet with terpenes than animals fed 15% protein 20 d after exposure (Figure 3). Thus, while lambs fed diets higher in energy or protein could eat more terpenes when forced (Figures 1 and 2), they did not do so when alternative foods of similar quality without terpenes were available. Thus, contrary to what some propose (Illius and Jessop, 1995, 1996), animals with higher nutrient intake may not always be more willing to eat PSM than animals with lower nutrient intake.

Experience and nutritional context both influence preference (Provenza et al., 2003). Experiences with combinations of PSM-containing foods that are complementary increase preferences for those foods, even when high-quality alternatives

are available *ad libitum* (Baraza et al., 2005; Villalba et al., 2004). Thus, "positive" experiences with PSM due to the presence of appropriate kinds and amounts of nutrients may lead to a higher preference for PSM than "aversive" experiences with PSM created by the absence of appropriate kinds and amounts of nutrients. In the current study, where only food with terpenes was available, lambs preferred less defended foods regardless of plane of nutrition (Figure 3).

Chemical defenses satiate detoxification capabilities of herbivores at critical thresholds of plant abundance. Below these thresholds, local extinction is more likely as species become less abundant because toxin satiation is not achieved and the relative removal of biomass of a species increases with its decline in abundance (Provenza et al., 2003). Results from our study suggest highly defended and rare plants with either high or low nutrient content may escape herbivory, provided that the quality of undefended neighbors is similar to the quality of the defended plant.

When lambs chose between the terpene-containing diets and alfalfa, the protein and energy concentrations of the former affected the amount of terpenes ingested (Figure 3). Energy densities higher than alfalfa, and protein concentrations higher than 6%, increased the likelihood of lambs ingesting the terpene-containing diet. This suggests the amount of defoliation a defended plant will sustain may be influenced by its nutritional quality relative to that of its neighbors. Lambs fed diets with more energy (3.30 and 3.53 Mcal/kg) ingested more terpene than animals fed diets with less energy (2.17 and 2.55 Mcal/kg), and lambs offered the diet with the least energy ingested the least terpenes. Lambs fed 6% CP ingested the least terpenes, but only during the second period of preference tests, after they received alfalfa pellets and barley for 20 d. The energy density of alfalfa was similar to the isoenergetic foods (2.50 Mcal/kg), and its protein concentration was similar to the isonitrogenous foods (16%). Thus, protein but not energy was different when alfalfa was tested against isoenergetic foods [alfalfa had more protein than foods offered to Groups 1 (6%) and 2 (9%), but less protein than the food offered to Group 4 (21%)], whereas energy but not protein was different when alfalfa was tested against isonitrogenous foods [alfalfa had more energy than the food offered to Group 1 (2.17 Mcal/kg), but less energy than foods offered to Groups 3 (3.30 Mcal/kg) and 4 (3.53 Mcal/kg)]. Higher energy densities increased intake of terpenes during the choice with alfalfa (Figure 3), but intake of terpenes did not increase along different protein concentrations tested against alfalfa. Intake of terpenes did not differ immediately after exposure, or among foods with 9%, 15%, or 21% CP, 20 d after exposure (Figure 3). Lambs typically show stronger preferences for energy than for protein (Villalba and Provenza, 1999), probably because ruminants require nearly five times more energy than protein on a daily basis (NRC, 1985), and because they are efficient at recycling nitrogen (Owens, 1988). Thus, lambs evidently did not need the protein provided by alfalfa when they were fed diets of 9% to 21% CP because they had enough protein to detoxify terpenes and

to complement the marginal levels of energy in the test diets (2.55 Mcal/kg) and alfalfa (2.50 Mcal/kg). Higher amounts of protein (21%) did not promote greater intake when animals were forced to eat just one food (Figure 1).

Accounts of partial preferences have received more attention (McNamara and Houston, 1987; Berec and Krivan, 2000) than explanations for their occurrence (but see Dearing et al., 2000 for a contrast between generalists and specialists). Dilution of PSM (Freeland and Janzen, 1974) and nutrient balancing (Westoby, 1978) are the main hypotheses for varied diets in herbivores, but these explanations are not mutually exclusive (Provenza, 1996; Behmer et al., 2002; Singer et al., 2002). The two mechanisms operate simultaneously, with the relative importance of each dictated by the kinds and amounts of PSM and nutrients. Based on our results, a uniform nutritional environment—choice between foods of similar quality with or without terpenes—narrows the diet constituents to the least defended species, regardless of food quality, whereas a diverse nutritional environment may broaden the diet if the quality of defended species is high relative to the quality of undefended species. If the concentration of nutrients in a plant is higher than the concentration of nutrients in an equally abundant and undefended neighbor, an herbivore may ingest higher amounts of the defended plant, and thus higher amounts of PSM. A diverse nutritional environment may also narrow the diet to the less defended plant species if the quality of the defended species is lower than the quality of the undefended neighbor (Figure 4).

Plant neighbors may be an important way plants cope with herbivores. The preference or avoidance of a plant species depends not only on factors internal to the plant, but also on the chemical and physical characteristics of neighbors (Atsatt and O'Dowd, 1976). While little attention has been given to these interactions (Milchunas and Noy-Meir, 2002; but see Hay, 1986; Pfister and Hay, 1988), the kinds and amounts of nutrients and toxins in a plant may influence how herbivores use their neighbors. Plant–plant–herbivore interactions may affect the probability of extinction of a plant chemotype within a plant community, and thus the diversity of plant species (Provenza et al., 2003). External factors for coping with herbivory include associational plant refuges (Atsatt and O'Dowd, 1976), which occur when "a plant that is susceptible to herbivory gains protection from herbivory when it is associated with another plant" (Pfister and Hay, 1988). Within a plant community, defoliation of an individual depends on the surrounding matrix of plant species (Milchunas and Noy-Meir, 2002). Thus, benefits and costs of a defense need to be weighed not only in terms of the individual, but also within the context of the association (Tuomi and Augner, 1993). Refuges may be essential for maintaining the diversity of plant communities and their resilience to changing grazing pressures (Milchunas and Noy-Meir, 2002). Factors that affect refuges include characteristics of the herbivore as well as the plant association. Palatable plants may gain associational protection from unpalatable plants when herbivores are selective between patches and unselective within a patch. On the other hand,

palatable plants are less likely to gain protection if herbivores are selective within a patch (Hjalten et al., 1993).

Nutrient–PSM interactions add another dimension to "palatability" and "unpalatability" within the context of associational plant refuges (Villalba et al., 2002a). Interactions among multiple nutrient and PSM dimensions may explain why certain plant–plant associations are of higher or lower preference to herbivores than predicted from the isolated effects of PSM repellency or plant palatability. Our results suggest undefended plants are more likely to gain protection, even if herbivores are selective within a patch, when the quality (energy density in our study) of the PSM-containing plant is higher than the quality of the undefended plant. In contrast, undefended plants are less protected from herbivory when the qualities of all plants, defended and undefended, in the patch are similar (Figure 4). Thus, the possible increase in herbivory of toxic plants promoted by their higher quality relative to their neighbors may enable palatable and less-competitive species to survive.

In summary, energy and nutrient concentrations influenced ingestion of terpenes in a graded fashion. The amounts of PSM that lambs ingested depended on the nutritional composition of the PSM-containing food relative to the nutritional composition of the alternatives (Figure 4). These interactions between PSM, nutrients, and neighbors can influence preference in ways that cannot be predicted solely by the isolated effects of nutrients or PSM. The effects of nutrients and PSM on herbivores can influence interactions and coexistence of plant species with different kinds and amounts of PSM, nutrients, and different competitive abilities. A formal integration of nutrient–PSM interactions into existing models of foraging behavior and plant community ecology will provide further insights into how protection or susceptibility function within plant communities to increase or decrease species richness.

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GENDER DIMORPHISM AND ALTITUDINAL VARIATION OF SECONDARY COMPOUNDS IN LEAVES OF THE GYNODIOECIOUS SHRUB *Daphne laureola*

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Abstract—In this article, we analyzed the concentration of coumarins in leaves of female and hermaphrodite individuals of the gynodioecious shrub *Daphne laureola*, along an elevational gradient in southern Spain. Combining HPLC and NMR techniques, we identified three different glycosides of 7-methoxycoumarin in leaves of this species. Total coumarin concentration averaged between 60 and 120 mg/g dry weight for mature summer leaves of *D. laureola* growing at six different populations. As predicted by optimal theory, females tended to have a higher concentration of coumarins than hermaphrodites, thus upholding the idea that male reproductive function is costly for hermaphrodites. Furthermore, concentrations in females but not hermaphrodites were positively correlated with increasing population altitude, and the magnitude of gender divergence in coumarin concentration varied among populations, suggesting that the cost of the male function may be context dependent. To our knowledge, this is the first evidence of gender differences in chemical defenses of a gynodioecious species in the field.

Key Words—Coumarins, *Daphne laureola*, elevation, gynodioecy, plant–animal interactions, plant defense.

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INTRODUCTION

In this study, we analyze the relationship between allocation to reproduction and chemical defenses, and its natural variation along an altitudinal gradient, in a long-lived perennial shrub. According to optimal theory, organisms allocate resources to survival, growth, and reproduction, such that their fitness will be maximized (Maynard-Smith, 1978). Plant defenses aim to decrease pathogen and herbivore attacks that may eventually reduce plant survival, growth, and reproduction and, thus, plant allocation to defenses can also be interpreted within optimal theory (Herms and Mattson, 1992). Within this framework, trade-offs (or opportunity costs) between growth, defense, and reproduction should exist whenever resources are limited. However, field measurements of trade-offs and other physiological costs in plant allocation to reproduction and defenses are not straightforward (see Obeso, 2002; Cipollini et al., 2003 for a review of each topic, respectively). Gender dimorphic species split the range of individual variance in reproductive allocation into categories, thus facilitating the assessment of consequences in terms of survival, growth, and defense of a higher reproductive allocation under natural conditions.

In dioecious species, allocation to reproduction in males is commonly higher than in females at flowering, though the opposite is true at fruiting time when the sink of resources to reproduction is maximum (Delph, 1990). Therefore, both the nature and magnitude of differences between genders in resource allocation may change with time (Ågren et al., 1999). Overall, herbivores usually distinguish and prefer males over females of dioecious plant species (Ågren et al., 1999, and references therein) suggesting that differences in reproductive allocation modify other plant features that in turn decrease quality of females as food for herbivores (e.g., Boecklen et al., 1990; Jing and Coley, 1990). Gynodioecy consists of populations having both hermaphrodite and female pollenless individuals. Thus, gender difference in allocation to reproduction is in principle lower and due to the male function only. The costs of male function are apparently more subtle, but still relevant (Eckhart and Seger, 1999), and there is also evidence for male-biased flower and seed predation favoring the maintenance of females in gynodioecious populations (Uno, 1982; Marshall and Ganders, 2001; Collin et al., 2002; reviewed in Ashman, 2002). To our knowledge there are no data available comparing physical or chemical defenses in gynodioecious species (but see Gouyon and Vernet, 1980) and, thus, whether gender differential consumption is mediated through distinct defenses or otherwise, for example through differential attractive properties, is unknown.

We explore the relationship between allocation to reproduction and defense in a gynodioecious species. Hermaphrodite and female *Daphne laureola* plants produce a similar number of flowers and fruits, and fruit size is also similar between genders (Alonso and Herrera, 2001). However, female flowers are smaller

and pollenless, thus leading to a lower allocation to reproduction of female individuals. In southern Spain, *D. laureola* is consumed by several species of Noctuid caterpillars. Defoliation has been related to plant architecture (Alonso and Herrera, 1996) and leaf nutrient composition (Alonso and Herrera, 2003), and caterpillars are able to distinguish among several plant structures (Alonso and Herrera, 2000). The role of allelochemicals in this plant-herbivore interaction remained unstudied. Coumarins are effective chemical defenses against herbivores in other plant species (Berenbaum, 2001 and references therein), and were known to exist in the *Daphne* genus (Hegnauer, 1973; Ulubelen et al., 1986; Zobel and Brown, 1988). Our expectation was that, if the increased reproductive allocation associated with the male function were costly, *D. laureola* females should have a higher concentration of coumarins than hermaphrodite conspecifics. Thus, we evaluated the concentration of the three most abundant coumarin glycosides found in leaves of female and hermaphrodite *D. laureola* individuals.

Furthermore, since the natural concentration of chemical defenses may vary geographically (Johnson and Scriber, 1994), the study was conducted in six different populations selected along an altitudinal gradient, that ranged from 950 to 1800 m asl. Increased exposure to UV radiation and low temperatures at higher altitudes may select for different chemical profiles in plants that in turn can affect herbivores (Johnson and Scriber, 1994; Stratmann, 2003). In particular, contents of UV-B absorbing compounds tend to increase at higher elevation sites in several plant species (Rozema et al., 1997). Coumarins are able to absorb UV radiation (Murray et al., 1982). Thus, we also expected an increase of coumarin concentrations in plants at higher elevation sites.

METHODS AND MATERIALS

Plant Species and Study Area. Daphne laureola L. (Thymelaeaceae) is a long-lived evergreen shrub distributed throughout the Palearctic region and generally found in the understory of coniferous and mixed montane forests in the Mediterranean area. In the Natural Park of Sierras de Cazorla, Segura y Las Villas (Jaén province, south-eastern Spain), where this study was conducted, the species is gynodioecious, and the proportion of female plants varies with site altitude (Alonso and Herrera, 2001).

In June 2002, we collected undamaged leaves from female and hermaphrodite individuals in six different populations comprising the entire altitudinal range of the species at our study area. Study locations were Coto del Valle (950 m elevation), Roblehondo (1235 m), Cañada del Espino (1575 m), Nava de las Correhuelas (1615 m), Cabeza del Tejo (1640 m), and Puerto Llano (1800 m), hereafter referred to as CV, RH, CÑE, NC, CT, and PLL, respectively. Aiming to have three replicates for each combination of gender per population, leaves of each individual ($N = 7$–20) were collected independently and later split into three different sets. Each

replicate had 8–17 g fresh mass. Leaves were collected when plants were bearing mature fruits and all plants were at the same phenological stage despite the fact that the different populations were collected on different dates due to altitudinal variation in plant phenology.

Chemical Analyses. Leaves were washed, dried with filter paper, and stored at −80°C prior to analysis. Frozen samples were weighed, deep-frozen with liquid nitrogen and ground in a coffee mill. As an internal control, 1.5 mg of esculetin (6,7 dihydroxy-coumarin, Aldrich) were added to each sample to evaluate potential process errors. Leaf powder was extracted 2 × 24 hr with methanol (80%), and the combined extract was filtered and concentrated to dryness under reduced pressure. The residue was dissolved in water and cleaned by sequential decantation with chloroform. Coumarins were detected as pale-blue spots on C_{18}-TLC (Alugram® RP-18W/UV_{254}) only in the aqueous phase, that was subsequently concentrated to dryness under reduced pressure to record the weight of the final residue. The residue was suspended in 15 mg of double-distilled water to obtain a 10% concentration of the internal control, esculetin. Three 750 μl aliquots were taken from this solution and centrifuged for 10 min at 12,000 rpm. The supernatant contained the target coumarins. For every aliquot, solid phase extraction was conducted on packed MFE C_{18} 3/500 columns (Análisis Vínicos S.L.) prior to HPLC analysis. On each occasion, a new packed column was gently washed with distilled water. The sample supernatant (250 μl) was loaded and elution started with 750 μl of methanol (85%) that was discarded. A mixture of methanol–water–glacial acetic acid (60:40:1, v/v; 1500 μl) was collected directly into an HPLC vial. Two injections of 10 μl from each vial were analyzed by HPLC, and the average peak area for each compound was used for further statistical analyses (see below).

HPLC was conducted on a Waters 2690 separation module with a Waters 996 PDA detector (Waters Cromatografía S. A., Barcelona, Spain) that allowed coumarin identification by way of their characteristic UV spectrum with two maxima absorption lengths around 260 and 320 nm (Murray et al., 1982). A Waters ODS2-3 μm RP-HPLC column (4.6 mm i.d. × 15 cm length) was used for quantification. Analyses were conducted in isocratic mode at a flow-rate of 1 ml/min, using a mixture of water–methanol–glacial acetic acid (84.8:14.2:1, v/v) as mobile phase (modified from Thompson and Brown, 1984). Double distilled water and HPLC quality solvents were used for the analyses. A calibration regression line was obtained for esculetin by varying the volume injected of two different solutions w/w in methanol. Regression of peak area on amount of esculetin injected explained 99.8% of peak area variation. Analyses of esculetin recovery based on this calibration showed that on average 76% of the esculetin initially added to leaf samples was lost during sample processing. Thus, error for each individual sample was calculated as the ratio between the expected and observed area of esculetin peak.

Each coumarin was purified by liquid chromatography, and the ^{1}H and ^{13}C NMR (DMSO-d_6) obtained on a Bruker AVANCE 500 spectrometer were compared to literature data for molecule identification.

Data Analyses. Statistical analyses were performed using the SAS statistical package (SAS Institute, 1996). Peak areas were transformed into coumarin concentrations assuming that for each sample coumarin quantification had the same recovery error as that observed for esculetin, and using esculetin regression to transform peak areas into quantities. Results obtained for each aliquot were averaged by sample, and concentration was referred to total leaf dry weight of the sample. Differences between genders and populations on concentration of coumarins were analyzed by General Linear Models (Procedure GLM). Gender, population, and their interaction were treated as fixed effects.

RESULTS

The methanolic extract of *D. laureola* leaves contained three major components (Figure 1) that were identified as three different glycosides of 7-methoxycoumarin. The observed molecular structures based on ^{1}H and ^{13}C NMR, and the references where these compounds were previously reported (Konishi et al., 1993;

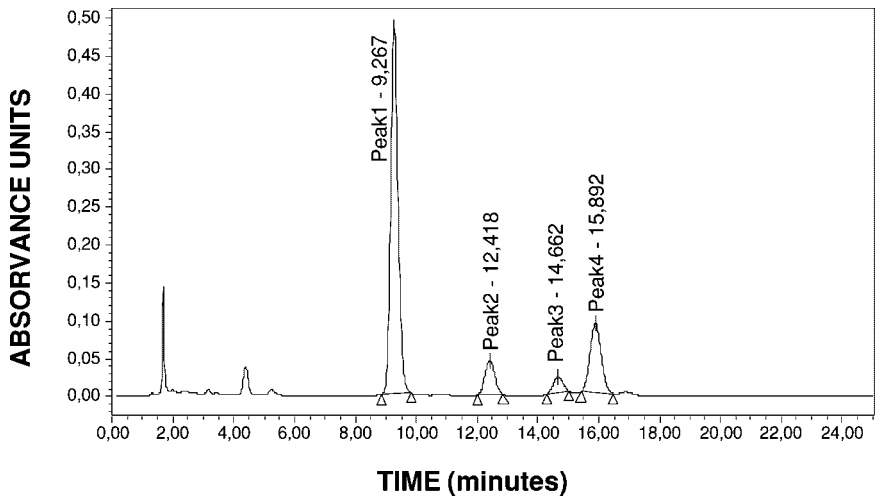


FIG. 1. HPLC chromatogram of the methanolic extract of *D. laureola* leaves with esculetin added, recorded at 320 nm wave length. Key to peak identity: Peak 1: 5-O-β−D-glucosyl-7-methoxy-8-hydroxy coumarin (**1**); Peak 2: 5-O-β−D-glucosyl-(6←1)-β-glucosyl)-7-methoxy-8-hydroxy coumarin (**2**); Peak 3: esculetin; Peak 4: 5-hydroxy-7-methoxy-8-O-β−D-glucosyl coumarin (**3**).

Jung et al., 1994) are shown in Table 1. The major component (**1**) averaged 54,938 (±17,646) ppm leaf dry weight, whereas average concentration of the other two compounds were 10,403 (±4182) ppm and 17,940 (±5787) ppm for (**2**) and (**3**), respectively. Concentrations of all three *D. laureola* coumarins into a sample were positively correlated ($N = 42$; $0.78 < r < 0.91$; $P < 0.001$ in all cases).

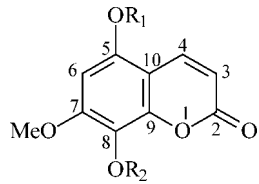
Populations differed in the concentration of the major coumarin glycoside (**1**) ($F_{5,30} = 4.56$, $P = 0.003$; Figure 2). Population × gender interaction was not statistically significant ($F_{5,30} = 1.09$, $P = 0.38$). Females tended to have greater concentrations of compound (**1**) than hermaphrodites, except in the CV population (Figure 2), when samples from this population were excluded from the analysis, differences between genders were statistically significant ($F_{1,24} = 5.76$, $P = 0.025$). Although the patterns were similar, gender differences were less apparent for concentrations of compounds (**2**) and (**3**) (analyses not shown). Populations also differed in the average concentration of compounds (**2**) and (**3**). Plants of the CT population had the highest concentration of both coumarin glycosides (14,471.0 ± 3986.0 ppm and 22,763.8 ± 7203.7 ppm for (**2**) and (**3**), respectively), whereas plants of the RH population showed the lowest concentrations of both (7614.9 ± 1818.4 ppm, and 14,903.7 ± 2355.6 ppm, respectively).

Since the concentration of the three coumarin glycosides was positively correlated, we calculated total concentration of coumarins in *D. laureola* leaves by adding them. The average concentration of total coumarins in female individuals of different populations was positively correlated to site altitude ($N = 6, r = 0.90$, $P = 0.01$; Figure 3). However, such a relationship was not found for hermaphrodite individuals ($N = 6, r = 0.27$, $P = 0.60$; Figure 3).

DISCUSSION

The three most abundant coumarin glycosides found in leaves of *D. laureola* were previously reported from other natural sources. Strangely, compounds (**1**) and (**2**) were isolated from mosses (Jung et al., 1994), compound (**1**) was found in *Polytricum formossum* and *Atrichum undulatum*, and compound (**2**) only in *P. formossum*. Compound (**3**) was previously isolated from leaves of the congeneric *Daphne pseudo-mezereum* (Konishi et al., 1993). No evidence for a similar coumarin daphnetin (7,8-dihydroxy coumarin) was detected in *D. laureola* leaves, despite a former report on its presence in the bark of this species (cf. Murray et al., 1982). This highlights that further analyses are needed to determine the identity and abundance of coumarins in other plant structures. The three compounds found in leaves are 5,7,8 trioxygenated coumarins. Apparently, all coumarins with an oxygen-containing substituent at the 7-position seem to be biosynthetically distinct from those that lack such a function, and derived from *p*-coumaric acid, an intermediate in lignin biosynthesis (Brown, 1970). It is also remarkable that all *D. laureola* coumarins share a 7-methoxy function, differing only in the nature and

TABLE 1. CHEMICAL STRUCTURE AND ^{1}H (J HZ) AND ^{13}C NMR DATA (500 MHZ, DMSO-d_6) OF THE THREE COUMARINS OBTAINED FROM THE METHANOLIC EXTRACT OF *Daphne laureola* LEAVES, AND THE REFERENCES WHERE THESE COMPOUNDS WERE PREVIOUSLY REPORTED



Compound	R_1	R_2	References
1	O-β-1-glc	H	Jung et al. (1994)
2	O-β-1-glc-6←1-β-glc		Jung et al. (1994)
3	H	O-β-1-glc	Konishi et al. (1993)

^{1}H (J Hz) and ^{13}C NMR data

	1		**2**		**3**	
Aglycone	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
2		159.8		161.4		160.3
3	6.21 d (9,7)	110.1	6.24 d (9.8)	111.9	6.11 d (9.6)	109.4
4	8.23 d (9,7)	139.4	8.25 d (9.6)	140.7	8.04 d (9.7)	139.8
5		146.1		147.6		151.9
6	6.91 s	96.5	6.85 s	97.9	6.52 s	95.6
7		150.8		152.4		155.8
8		127.7		–		124.1
9		142.2		143.8		147.9
10		103.5		106.6		102.5
Ome	3.85 s	55.8	3.85 s	57.3	3.84 s	56.2
Glucose						
1’	4.80 m	102.2	4.86 d (7.0)	102.9	4.93 d (7.3)	102.4
2’	3.29 m	73.2	–	74.1	3.29 t (7.9)	74.1
3’	3.30 m	76.2	–	77.1	3.24 t (8.4)	76.5
4’	3.12 m	70.0	–	70.7	3.17 t (8.4)	69.9
5’	3.37 m	77.4	–	76.7	3.08 m	77.2
6’	3.39 m/3.75 m	60.8	–	70.0	3.61 m/3.37 m	60.9
1”			4.17 d (7.6)	105.3		
2”			3.03 t (10.9)	74.3		
3”			3.11 t (8.5)	77.6		
4”			–	70.8		
5”			2.95 t (7.9)	74.3		
6”			–	66.6		

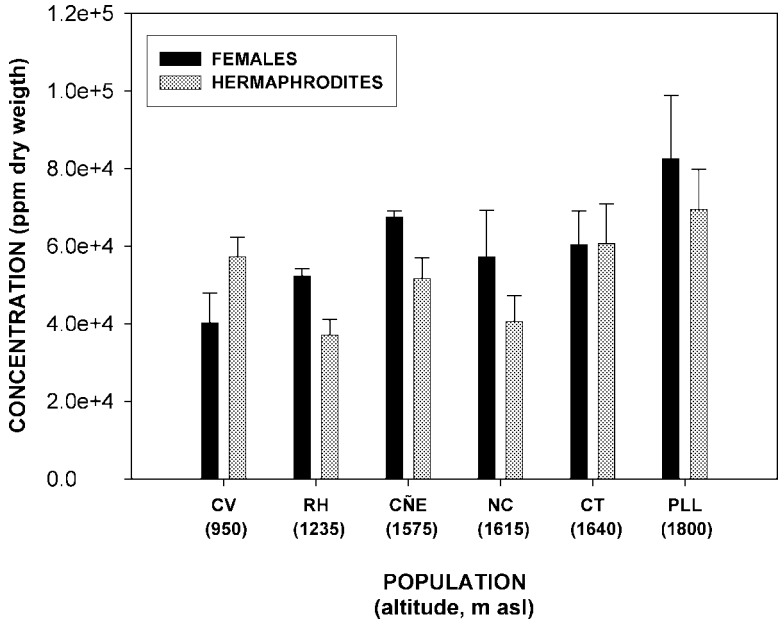


FIG. 2. Average concentration (+SE) of the most abundant coumarin, 5-O-β−D-glucosyl-7-methoxy-8-hydroxy coumarin, in leaves of female and hermaphrodite *D. laureola* individuals from six populations in southern Spain. Note that populations are ordered from lower to higher elevation.

position of the glycoside substituent, suggesting a common biosynthetical pathway. Further studies are needed to elucidate the metabolic relationships between these compounds and other plant physiological processes such as leaf maturation, since it is known that hydroxycoumarin content in *Daphne mezereum* from the Moscow region is maximal during leaf formation and at the end of the growth period (cf. Murray et al., 1982).

Our estimates of total coumarin concentration averaged between 60 and 120 mg/g dry weight for mature summer leaves of *D. laureola*. This figure is ca. 10 times higher than the concentration of dihydroxycoumarins reported for mature summer leaves of *Daphne mezereum* (Zobel and Brown, 1988), although the difference could partially reflect differences in the accuracy of the methods applied. The use of esculetin as internal standard in all samples allowed us to estimate the actual process errors and be confident of our estimates. Moreover, the low rates of standard recovery we observed revealed that further efforts to improve our methods would be important it to detect less abundant compounds.

Female and hermaphrodite individuals of *D. laureola* growing in the same population can have differential concentrations of secondary compounds. As

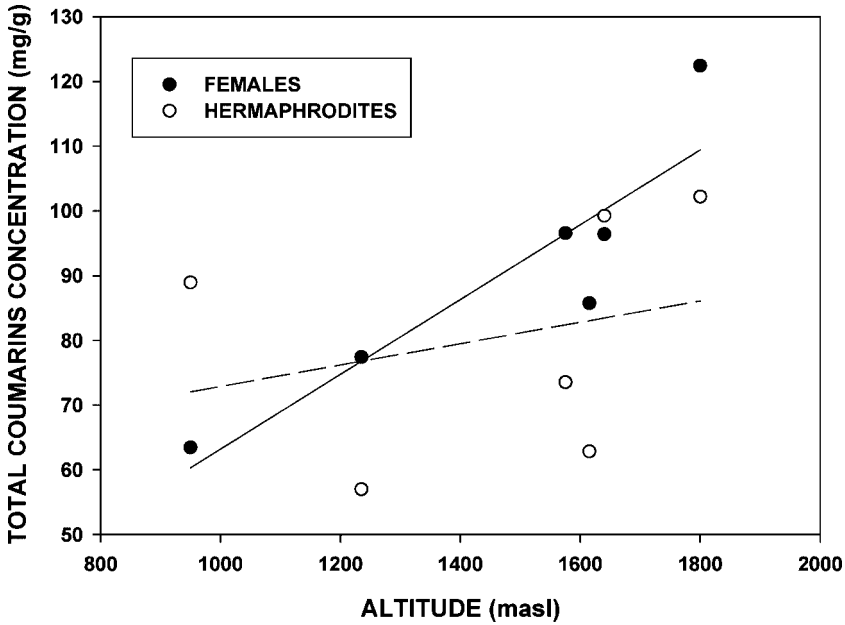


FIG. 3. Average concentration of the sum of all coumarins found in hermaphrodite and female *D. laureola* individuals at six different Spanish populations and the relationship with site altitude (solid line for females and dashed line for hermaphrodites).

expected from optimal theory, due to lower allocation to flowers, female leaves had on average higher concentrations of coumarins, upholding the idea that male function is costly for hermaphrodites (Eckhart and Seger, 1999). However, the magnitude of the difference was not constant, and even in the CV population, the one at the lowest altitude and with the highest proportion of females, the sign of the difference was reversed (Figure 2). Thus, costs of male (and likely also female) reproduction seem to be context dependent.

Finally, we found that average coumarin concentrations differed among *D. laureola* populations within a relatively small region. Heterogeneous spatial distribution of allelochemicals seems to be ubiquitous in both managed and natural systems (Hoy et al., 1998). Occurrence and concentration of plant allelochemicals may vary with latitude, elevation, sun exposure, and other environmental factors (Louda and Rodman, 1983; Dudt and Shure, 1994; Johnson and Scriber, 1994; Salmore and Hunter, 2001; Gómez et al., 2003). A negative relationship between elevation, occurrence, and concentration of some alkaloids (Salmore and Hunter, 2001) and glucosinolates (Louda and Rodman, 1983) has been found in some species, although unrelatedness and nonlinear relationships were found for

different alkaloids and glucosinolates in the same species. Also the content of UV-B absorbing compounds increases with site elevation in several plant species (Rozema et al., 1997). *D. laureola* showed a gender-specific response to site altitude since concentration of coumarins in leaves of female shrubs increased with population altitude, but hermaphrodites did not show a similar altitudinal pattern. On one hand, a higher concentration of coumarins could benefit plants by increasing the UV absorbance in higher elevation sites. Apparently, mostly females would be able to benefit from this advantage, once more supporting the existence of a cost of the male function in this species. Interestingly, some field experiments have shown that plants exposed to ambient solar UV-B radiation are more resistant to herbivorous insects than plants grown under filters that excluded the UV-B component of solar radiation (Stratmann, 2003). In addition to the flavonoids, isoflavonoids, and tannins quoted by Stratmann (2003), coumarins could be also associated with the observed overlapping between plant physiological responses to UV radiation and herbivory. Ongoing studies aimed at specifically evaluating the defensive role of *D. laureola* coumarins against insect defoliation will help to clarify the consequences of gender and altitudinal variation in coumarin concentrations in leaves herein reported.

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BIOCHEMICAL RESPONSES OF CHESTNUT OAK TO A GALLING CYNIPID

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Abstract—We characterized the distribution of nutritional and defensive biochemical traits in galls elicited on chestnut oak (*Quercus prinus* L.) by the gall wasp *Andricus petiolicolus* Basse (Cynipidae) in comparison with gypsy moth-wounded and unwounded leaves. Gall cortex and epidermis exhibited elevated soluble peroxidase (POX) and soluble invertase activities, and greater condensed tannin concentrations than did nutritive tissues or leaves. Nutritive tissue, on which the insect feeds, contained few polyphenols, and lower POX and invertase activities compared with other gall tissues and leaves. Elevated total POX activity arose from a complex pattern of enhanced and suppressed isoform activities in galls. Invertase enzyme activity decreased in all tissues over the course of the 7-d study, although gypsy moth wounding suppressed this decline slightly in ungalled leaves. Our results indicate that the distribution of biochemical defenses in this typical cynipid gall differs significantly from the leaf tissue from which it is formed and support a role for invertases in establishing the gall as a sink. *A. petiolicolus* larvae do not induce, and may suppress, plant defense responses in nutritive tissue, while enzymatic activity and phenolic accumulation are enhanced in gall tissues surrounding feeding sites. These patterns suggest that the gall is manipulated by the insect to enhance its food and protective value.

Key Words—Plant–insect interactions, gall, oak, cynipid, peroxidase, invertase, tannins.

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INTRODUCTION

Galls are specialized plant structures formed when a galling organism alters the development of normal plant tissue (Kinsey, 1920; Felt, 1940). Oak trees support a diverse assemblage of galling insects (Dreger-Jauffret and Shorthouse, 1992), the most prevalent of which are wasps of the family Cynipidae (Kinsey, 1920; Bronner, 1983). Cynipid galls consist of an outer layer of epidermal tissue, a cortex of schlerenchyma, and one or more nutritive tissue chambers that contain and nourish the larvae.

While galls may provide diverse benefits to the insects that elicit them (Price et al., 1987), considerable evidence supports the view that the gall provides a superior food source to the insect (the "Nutrition Hypothesis" [Price et al., 1987; Bronner, 1992]). For example, galling aphids elicit proliferation of phloem elements, on which they feed (Wool et al., 1999). The plant's nutritive value to insect and microbial gallers may be enhanced further *via* formation of strong metabolic sinks in the gall (Bronner, 1977, 1983; Larson and Whitham, 1997). All galls so far examined constitute enhanced sinks for photosynthate (Inbar et al., 1997, Larson and Whitham, 1997). Sink strength is functionally linked to invertase activity in normal plant tissues (Ricardo and Ap Rees, 1970; Leigh et al., 1979; Huber, 1989; Patrick, 1990; Yelle et al., 1991; Scholes et al., 1994; Zhang et al., 1996) and bacterial galls (Weil and Rausch, 1990).

Most galls contain specialized, distinctive "nutritive tissues" on which the insect feeds and which may contain elevated or altered nutrient concentrations (Paclt and Hässler, 1967; Bronner, 1977, 1992; Schonrogge et al., 2000). Putative antiherbivore defenses may be increased or decreased in gall tissues (Hartley, 1998; Nyman and Julkunen-Tiitto, 2000), but recent evidence from sawfly systems indicates that at least phenolic concentrations are reduced in nutritive tissues compared with the rest of the gall or other tissues (Nyman and Julkunen-Tiitto, 2000).

We studied the globular gall elicited on petioles and midribs of chestnut oak (*Quercus prinus* L.) leaves by the cynipid wasp *Andricus petiolicolus* Basse. Like other members of its genus, *A. petiolicolus* larvae chew and progressively destroy the nutritive tissue inside the gall as they develop (Kinsey, 1930; Bronner, 1983). Chewing by free-living insects induces defensive biochemical responses in many tree species (Karban and Baldwin, 1997), including chestnut oak, which responds to gypsy moth (*Lymantria dispar* L.) attack by increasing production of polyphenols in the leaves (Hunter and Schultz, 1995). Plant defense responses may also include increased activity of oxidative enzymes such as peroxidases (POXs) and polyphenol oxidase (PPO), which activate and polymerize polyphenols and are implicated in biochemical responses to insects and pathogens (Vaughn and Duke, 1984; Appel, 1993; Felton et al., 1994; Bi et al., 1997). The Nutrition Hypothesis predicts that such defensive responses should be suppressed in tissues consumed by galling insects.

We examined these aspects of the Nutrition Hypothesis by measuring the concentrations of protein, of putatively defensive phenolics, and activities of oxidative and sink-inducing enzymes in nutritive and non-nutritive tissues of *A. petiolicolus* galls as compared with galled and ungalled leaves, and with leaves wounded by a nongalling chewing insect, the gypsy moth (*Lymantria dispar* L.).

METHODS AND MATERIALS

Tissue Collection. Twenty-six *Q. prinus* L. saplings parasitized by *A. petiolicolus* were selected in late May, 1997 near State College, Centre County, PA, USA. Three to five ungalled and 3–5 galled leaves were collected from each tree on May 24 (Date 1) by plucking the leaf at the base of the petiole. Chilled galls were quickly dissected and separated into cortex, epidermis, and nutritive tissues. Ungalled leaves, galled leaf, and gall tissues were flash frozen in liquid N_2 and stored at -20°C. Nutritive tissue from several trees was pooled to provide enough sample for analysis. Epidermis was separated from cortex for colorimetric analyses of polyphenols. Because of assay sensitivity limitations and the small amounts of nutritive tissues, phenolic contents could not be quantified colorimetrically, so staining was used instead (below).

To assess the impact of wounding on leaf traits, on May 24 (Date 1) we confined 4–7 third-instar gypsy moth larvae within window-screen cages and allowed them to feed on the chestnut oak saplings for 7 d (until May 31, "Date 2"; see Rossiter et al., 1988). Each sapling received 5 cages, and each cage contained both galled and ungalled leaves, producing wounded and unwounded galled and ungalled leaves after 1 wk.

Enzyme and Protein Extraction. Frozen gall and leaf tissue was homogenized in an ice cold mortar and pestle with potassium succinate (KSuc) buffer, pH 5.5, containing 10% (v/v) glycerol and 10% (v/v) polyvinylpolypyrrolidone. Buffer was added to plant tissue at a ratio of 10:1 (v/w), and 10% (v/v) Triton X-100 was added at a rate of 0.8 μl/mg tissue. Nutritive tissue samples were diluted further to obtain enough extract for analysis. All extracts were sonicated for 15 min and centrifuged at 1100 $\times$ g for 15 min at 4°C. The supernatant was removed, stored at -20°C, and later used as the source of protein and enzyme activity. All chemicals were purchased from Sigma Chemical Company, St. Louis, MO, or Bio-Rad Laboratories, Hercules, CA.

Protein Assay. Total protein was measured in each sample extract using the Bradford reagent method (Bio-Rad, Inc.). Bovine serum albumin was used as a standard, and standard curves were prepared with the same concentration of Triton X-100 as in the samples to account for detergent effects on the assay.

Peroxidase: Total Soluble Activity. POX activity was assayed by using a modified version of Bi et al. (1997) spectrophotometric procedure. Twenty μl of extract were added to 980 μl of 88.2 mM H_2O_2 and 4.6 mM guaiacol in 0.1 M KSuc,

pH 5.5, and vortexed for 5 sec. The increase in absorbance due to the formation of tetraguaiacol was monitored for 60 sec at 470 nm.

Peroxidase: Nitrocellulose Blotting. To determine the location of POX in gall tissue layers, sectioned galls were pressed to wet nitrocellulose, and the blot was stained for POX activity according to Spruce et al. (1987). *A. petiolicolus* galls were sliced in half with a scalpel, and the cut face was pressed to the nitrocellulose for 60 sec. After 3–5 water rinses, the nitrocellulose was stained with 20 mM guaiacol, with or without 60 mM H_2O_2, in 0.1 M KSuc, pH 5.5, for 2–5 min. Orange staining indicated the location of POX activity.

Peroxidase: Tissue Staining. POX activity within the gall tissue was examined using diaminobenzidine (DAB) as a substrate (Harris et al., 1994). Galls were sectioned into 1 mm slices and rinsed with water to remove POX that smeared across tissue layers during cutting. Tissue slices were then exposed to the following substrates (in 0.05 M Tris–HCl, pH 7.6): no substrate, 2.3 mM DAB, 2.3 mM DAB + 5.9 mM H_2O_2, 2.3 mM DAB + *Aspergillus niger* catalase. Slices were immersed in the substrate or control solutions for 8 min, rinsed with water, and examined for POX activity indicated by orange–brown staining.

Peroxidase: Isoelectric Focusing. POX isozymes were separated using polyacrylamide gel electrophoresis/isoelectric focusing (IEF) with a Model 111 mini-IEF unit (Bio-Rad, Inc.). Gels were cast using 50 μl 3/10 ampholyte, 200 μl 8/10 ampholyte, and 700 μl 3/5 ampholyte, 5.05 ml H_2O, 2.0 ml 24.25% acrylamide with 0.75% bis-acrylamide, and 2.0 ml 25% glycerol (v/v). Fifty μl of 0.44 M ammonium persulfate (w/v) and 5 μl tetramethylethylenediamine were added to initiate gel polymerization.

One μl of crude extract was added to each well of a 10 well sample template and allowed to diffuse into the gel for 5 min. Isozymes were separated by stepwise increases in voltage of 100, 200, and 450 V for 15, 15, and 45 min, respectively. Following focusing, gels were soaked in 0.1 M KSuc, pH 5.5, for 10 min to equilibrate the pH throughout the gel. POX isozymes were visualized by immersing the gel in substrate solution for 30 min. The substrate solution was made by dissolving 8 mg/ml *o*-dianisidine in methanol, combining 2.5 ml of this solution with 97.5 ml 0.1 M KSuc buffer, pH 5.5, and adding 44.8 μl 30% H_2O_2. Gels were then rinsed 4–6 times in KSuc buffer. Relative isozyme activity was quantified by using a Shimadzu CS-9000U dual-wavelength scanning densitometer.

Polyphenol Oxidase: Total Soluble Activity. We used a spectrophotometric assay for PPO activity developed by Tono and Fujita (1995) that employs caffeic acid as substrate. Ten μl enzyme extract were added directly to a cuvette containing 490 μl of 0.06 mM caffeic acid in 0.1 M KSuc, pH 5.5, shaken 4 times, and the absorbance monitored at 310 nm for 30 sec.

Polyphenol Oxidase: Nitrocellulose Blotting. To determine the location of PPO activity in gall tissue, the same blotting procedure used for POX was repeated

with 14 mM L-DOPA in 0.1 M KSuc, pH 5.5, as a substrate. Orange staining indicated the location of PPO activity.

Polyphenol Oxidase: Tissue Staining. Galls were sectioned as in the POX procedure, and PPO activity was visualized in the gall by immersing tissue slices in 10 mM L-DOPA in 0.1 M KSuc (pH 5.5). Slices were immersed in the substrate solution or a control solution without L-DOPA for 45 min at room temperature.

Soluble Invertase Activity. Soluble invertase activity was determined by using the dinitrosalicylic acid (DNSA) method (Sumner, 1925; Arnold, 1965) with modifications by Miller (1959). Fifty μl of enzyme extract were added to 950 μl 0.1 M sucrose in 0.1 M KSuc, pH 5.5 (Tang et al., 1996), and incubated for 40 min at room temperature. One ml Sumner's reagent (44 mM 3,5-DNSA, 53 mM phenol, 0.25 M NaOH, 4 mM Na_2SO_3) was added to the reaction mixture, and the solution was boiled for 15 min. Before cooling, 330 μl of 1.42 M sodium potassium tartrate were added to the mixture. The absorbance was read at 560 nm on a spectrophotometer and converted to a reducing sugar concentration based on a glucose standard curve. The above procedure was repeated without the 40 min incubation in order to determine the concentration of reducing sugar already present in the enzyme extract; this amount was then subtracted from the final concentration before calculating the enzyme activity. Blanks contained all components except the enzyme extract, for which the sucrose/KSuc solution was substituted.

The results for all enzyme assays were expressed as activity per milligram total protein to reflect the amount of enzyme abundance or activity relative to other proteins, and as activity per gram fresh weight to assess the activity an herbivore or pathogen is likely to encounter in the hydrated plant tissue.

Phenolics: Tissue Concentrations. Leaf, gall cortex, and gall epidermis were assayed for (1) "Folin-reactive polyphenols" with the Folin-Denis assay (Appel et al., 2001), which measures the ability of extracts to reduce a mixture of phosphomolybdic and phosphotungstic acids, (2) *condensed tannins* using the Butanol-HCl assay (Appel et al., 2001), which quantifies hydrolyzed proanthocyanidin residues, and (3) *hydrolyzable tannins* with the potassium iodate method modified for quantitative use (Schultz and Baldwin, 1982), which quantifies galloyl esters. Purified chestnut oak tannins from the same trees were used as standards (Appel et al., 2001), and results are expressed as mg phenolics per mg tissue dry weight.

Phenolics: Tissue Staining. The location of polyphenols in gall tissue was determined by using the nitroso reaction according to Harris et al. (1994). Galls were sliced into 1 mm sections with a scalpel and immersed in equal volumes of 1.2 M $NaNO_3$, 3.3 M urea, and 1.7 M acetic acid for 5 min before adding 2 volumes of 2 M NaOH. Sections were rinsed in water and examined immediately under a Zeiss light microscope for red staining, indicating the presence of polyphenols.

Data Analysis. Chemical variation among chestnut oak tissues (2 gall tissues, ungalled, galled, and gypsy moth-wounded leaves) was subjected to analysis of variance (ANOVA) with the SAS MIXED procedure (SAS Institute, Inc.,

1999). We used an incomplete block design without replication with individual trees as blocks and tissue type, wounding, and date as fixed effects. Because they were pooled from several trees, nutritive tissues were assigned unique block numbers. Differences of adjusted least square means ($\alpha = 0.05$) were used to identify significant differences among the 4 tissue types and 2 dates. One-way ANOVA was used to determine significance of differences among tissues in polyphenol concentrations.

RESULTS

Protein Content. Protein concentrations declined with time in ungalled leaves, but overall variation with date was not statistically significant (Table 1, Figure 1A). Protein concentration in the gall cortex was 25–50% lower than in ungalled leaves; other differences among tissues were not statistically significant (Table 1, Figure 1A). Gypsy moth wounding had no effect on protein concentration of any tissue.

Peroxidase: Total Soluble Activity. Total soluble POX specific activity differed significantly among tissues, but not among dates (Table 1). POX activity in gall cortex + epidermis was double the activity in leaves (galled or ungalled) and 5 times the activity in the nutritive tissue (Figure 1B). POX activity was not altered when leaves were wounded by gypsy moth larvae (Table 1, Figure 1B).

TABLE 1. ANOVA RESULTS FOR CHEMICAL VARIABLES

Variable	Effect	*df* (num., den.)[a]	*F*-value	*P*-value
Protein concentration	Wounding[b]	1, 96	1.70	0.195
	Tissue[c]	3, 96	38.37	<0.001
	Date	1, 96	2.16	0.145
	Wound × Tissue	3, 96	1.37	0.258
	Date × Tissue	3, 96	10.79	<0.001
POX[d] specific activity	Wounding	1, 96	0.25	0.620
	Tissue	3, 96	20.90	<0.001
	Date	1, 96	1.53	0.220
	Wound × Tissue	3, 96	0.54	0.656
	Date × Tissue	3, 96	0.93	0.432
Invertase specific activity	Wounding	1, 95	0.08	0.773
	Tissue	3, 95	54.96	<0.001
	Date	1, 95	30.68	<0.001
	Wound × Tissue	3, 95	1.23	0.302
	Date × Tissue	3, 95	0.96	0.414

[a] Numerator and denominator degrees of freedom for the *F*-test.
[b] Wounding consisted of 1 wk of herbivory by caged gypsy moth larvae.
[c] Tissues were ungalled leaf, galled leaf, gall body, and nutritive tissue.
[d] Peroxidase.

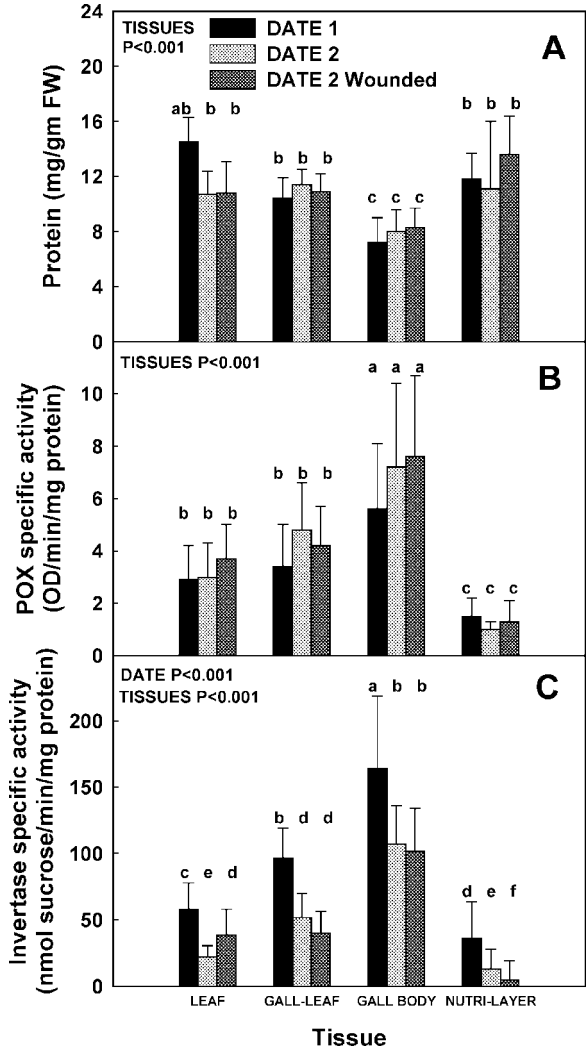

FIG. 1. Means (standard deviation) of total soluble protein concentration (A), peroxidase (POX) specific activity (B), and invertase specific activity (C), by tissue, date, and wounding treatment. Means with the same lowercase letters are not significantly different ($P > 0.05$). Leaf = ungalled leaves; gall-leaf = leaf on which there was one gall; gall body = gall cortex + epidermis; nutri-layer = dissected nutritive tissue from feeding chambers.

Patterns in POX activity and statistical significance were the same when activity was expressed per gram fresh tissue (data not shown).

Peroxidase: Nitrocellulose Blots and Tissue Staining. Nitrocellulose blots of *A. petiolicolus* galls revealed POX activity in the gall cortex immediately surrounding nutritive tissues, but little or none directly within the nutritive tissue (Figure 2A and B). Moderate staining occurred in the gall cortex and intensified in the epidermis of the gall structure. No POX staining was observed when H_2O_2 was excluded from the substrate solution as a control (not shown). Direct POX staining (Figure 2E) was generally consistent with the results of the nitrocellulose blotting. Staining within the nutritive tissue was weak (Figure 2E), while intense staining was observed around the gall epidermis (not shown) and in the cortex just outside the nutritive tissue.

Peroxidase Isozymes. Isozyme activities in leaves and gall cortex + epidermis were examined with IEF (Figure 2F). Activity of several isozymes appeared to be elevated in gall tissues and/or galled leaves compared to leaves on the same tree, while others were depressed. The gel resolution limited our ability to determine statistical significance of most of these differences. However, quantitative scanning densitometry indicated that gall cortex + epidermis contained significantly greater activity of one anionic and one cationic isozyme (Figure 2F, arrows) and lower activities of most other isozymes. This pattern was observed in all 3 sets of chestnut oak leaves and galls measured.

Polyphenol Oxidase: Total Soluble Activity, Nitrocellulose Blots, and Tissue Staining. Although our assays were able to detect PPO in extracts of other plant species under the same conditions (data not shown), our chestnut oak extract exhibited no PPO activity and actually inhibited auto-oxidation of the substrate. In contrast to the POX assay, no PPO staining of the nitrocellulose membrane was observed with L-DOPA as a substrate (not shown). No PPO staining was observed in any gall sections in the absence of H_2O_2 (not shown), indicating that this enzyme is either not present or not detectable by this method in *A. petiolicolus* gall tissue.

Soluble Invertase Activity. Specific activity of soluble invertase differed significantly among tissues and dates (Table 1), with similar patterns observed for invertase activity per unit fresh weight (data not shown). Activity was greatest in gall cortex + epidermis and lowest in nutritive tissue on the first date; galled and ungalled leaves had intermediate activities (Figure 1C). Invertase specific activity declined over the week in all tissues, but there was some indication that gypsy moth wounding may have limited this decline in ungalled leaves (Figure 1C). A *post-hoc* Tukey analysis of wound effects supported this observation (Figure 1C). However, wounding did not have a statistically significant effect on invertase activity across all tissues and dates.

Polyphenol Tissue Concentrations and Staining. Colorimetric assays indicated that gall epidermis had significantly elevated condensed tannin (ANOVA, $F_{2,41} = 20.8$, $P < 0.001$) and nearly-significant elevated Folin-reactive contents

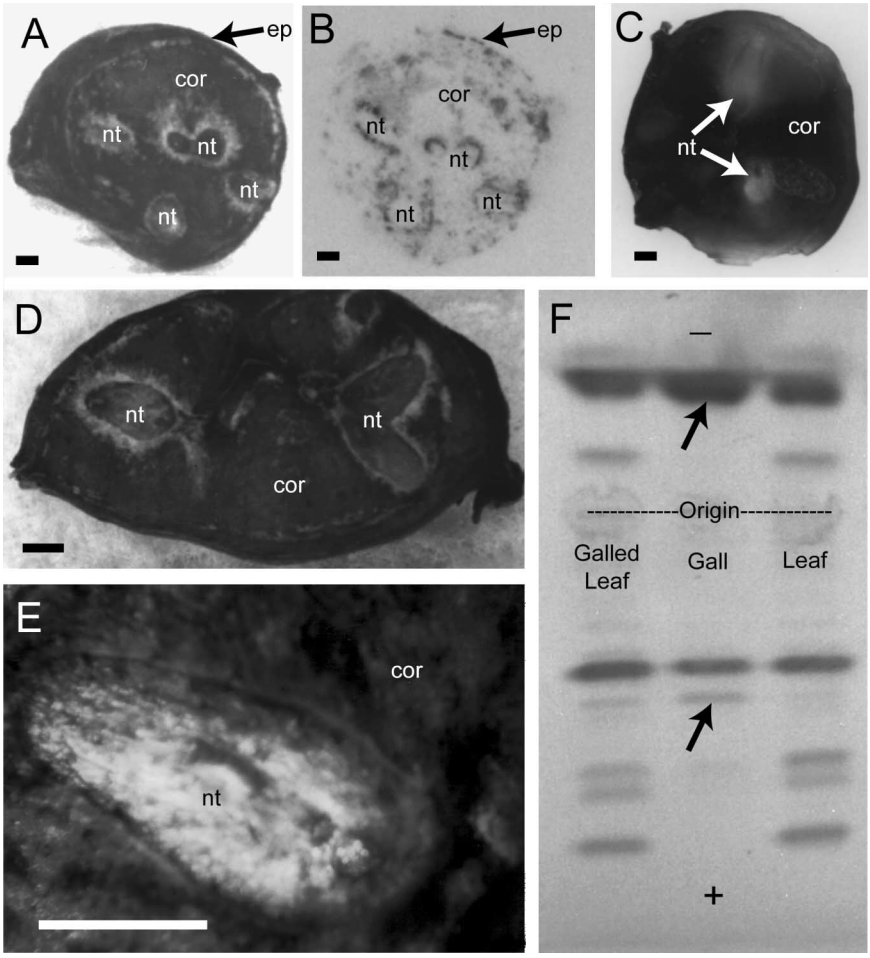

FIG. 2. Unstained *A. petiolicolus* gall section (A) with its nitrocellulose blot (B). In (B), note the absence of peroxidase (POX) staining within, and intense POX activity immediately outside of nutritive tissue (nt). ep = epidermis, cor = cortex. (C) *A. petiolicolus* gall stained for polyphenols. Staining is reduced or absent in the nutritive tissue (arrows). (D) A second unstained *A. petiolicolus* gall section with associated close-up (E) of nutritive tissue stained for POX with DAB. Note lighter color (less POX activity) in nutritive tissue compared to surrounding cortex. All scale bars = 1 mm. (F) IEF gel showing POX isozyme activities in *A. petiolicolus* galls from chestnut oak. "Galled leaf" indicates the leaf on which the gall formed, "gall" indicates gall cortex + epidermis, and "leaf" indicates a control leaf from the same tree with no galls. (–) = direction of migration for cationic POXs, (+) = direction of migration for anionic POXs relative to the origin. Arrows indicate isozymes that were significantly more active in gall tissue.

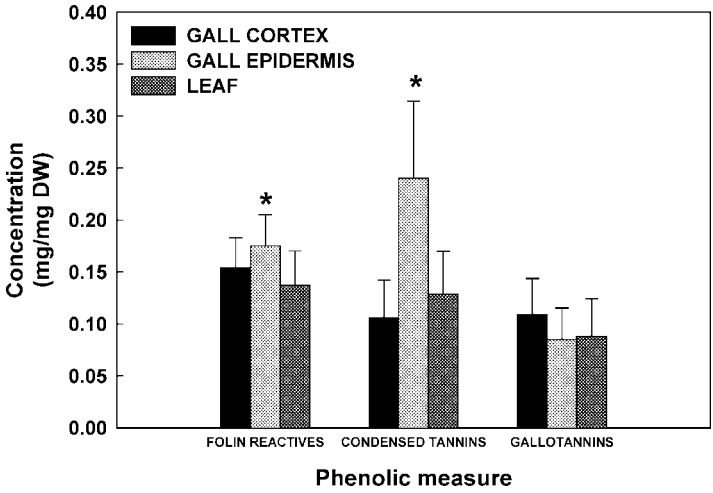

FIG. 3. Mean (standard deviation) polyphenol concentrations of gall tissues and ungalled leaf on last day of study. (*) indicates mean differs at $P = 0.08$ for Folin-reactives, and $P < 0.05$ for condensed tannins.

($F_{2,41} = 2.56,\ P = 0.08$), while cortex phenolic concentrations were indistinguishable from those of the adjacent leaf tissue (Figure 3). Dark red staining for polyphenols with the nitroso reaction revealed lower polyphenol concentrations in the nutritive tissue than in the gall cortex or epidermis (Figure 2C). Globules of polyphenols were present in some cortical cells.

DISCUSSION

Our biochemical characterization of *A. petiolicolus* galls provides support for the Nutrition Hypothesis and reveals a previously undescribed shift in POX isoforms within cynipid gall tissue. The pattern of POX isoform activity in gall cortex + epidermis differed from that of adjacent leaf tissue; some isoforms were more abundant or active in galls, while others were suppressed there. Enhanced activity of particular POX isoforms is frequently associated with responses to wounding, pathogen infection and other stresses (Mäder et al., 1980; Grisebach, 1981; Christensen et al., 1998; Hiraga et al., 2000a,b). Differential activation of various isoforms has been seen in response to pathogens (Lebeda et al., 2001), ethylene and abscisic acid (Kim et al., 2000), cytokinins (Limam et al., 1998), and aphid feeding (Chaman et al., 2001), and diverse stimuli elicit differential accumulation of mRNAs for a wide range of POXs in rice (Hiraga et al., 2000). It is important to note that differences in POX isozyme activity were not detectable in the present study by the spectrophotometric assays of total POX activity.

The distribution of POX in *A. petiolicolus* galls is likely to reduce the food quality of the outer part of the galls for many herbivores and pathogens, but should improve food quality in the tissues the cynipid larva actually eats. Total POX activity (per mg protein) was elevated above that of leaves in gall cortex + epidermis, a finding similar to that of Gopichandran et al. (1992) in thrips galls. In contrast, nutritive tissue had less total POX activity than did leaf and cortex. The results from our nitrocellulose blots (Figure 2B) and direct staining for POX (Figure 2E) confirm that most of the POX activity within galls is concentrated in the cortex and epidermis, rather than the nutritive tissue where it could adversely affect the developing larvae.

Elevated POX within the gall cortex and epidermis could benefit cynipid larvae by deterring other organisms from consuming or entering the gall. POX activity can produce reactive oxygen species (ROS) such as H_2O_2 and O^{2-} (Bolwell, 1996; Bi et al., 1997) and catalyzes the oxidation of *o*-dihydroxyphenolics to *o*-quinones in the presence of H_2O_2 (Kahn, 1985; Bi et al., 1997). Appropriate phenolic substrates for POX are known to be present in many oak galls, particularly those of cynipids (Figure 3; Nierenstein, 1930; Cornell, 1983; Hartley, 1998), and were present at high levels in non-nutritive tissues of *A. petiolicolus* galls (Figure 2C). In the gall cortex, POXs and oxidized phenolics may toughen cell walls through lignin production and cross-linking with cell wall proteins (Gaspar et al., 1982; MacAdam et al., 1992; Lamb and Dixon, 1997).

Because both phenolic compounds and POX activity were found to be localized away from the nutritive tissue, *A. petiolicolus* larvae are likely to consume few POX-associated ROS (Tenhaken et al., 1995; Doke et al., 1996; Lamb and Dixon, 1997; Wojtaszek, 1997) harmful to herbivores (Appel, 1993; Felton et al., 1994; Bi and Felton, 1995). These gross patterns are consistent with the view that gall chemistry may be influenced by the galling insect (Hartley, 1998) to provide protection from herbivores (Janzen, 1977; Schultz, 1992), fungal entomopathogens (Taper et al., 1986; Barrett et al., 1998), or fungal endophytes that can threaten galling insects (Carroll, 1988; Wilson, 1995), while preserving or enhancing the food quality of the nutritive tissues.

Schonrogge et al. (2000, and personal communication) have suggested that cynipid galls resemble plant seeds in tissue structure and/or physiology. Patterns in POX isoform activity (Gijzen et al., 1999) and invertase activity (Wobus and Weber, 1999) seen in developing seeds resemble those we found in galls and suggest that cynipid gall development might incorporate aspects of seed development.

As in crown galls formed by *Agrobacterium* (Weil and Rausch, 1990) and galls of the aphid *Hormaphis hamamelidis* (Rehill and Schultz, 2003), our results implicate elevated invertase activity as a means by which insect galls become sinks. Galls are known to act as sinks for plant assimilates (Bronner, 1977, 1983; Larson and Whitham, 1997), and high levels of soluble invertase are associated with the establishment of sink characteristics (Patrick, 1990; Sturm and Chrispeels, 1990;

Yelle et al., 1991). Within the gall tissue, vacuolar acid invertase may hydrolyze sucrose to provide the hexoses required for elevated metabolic activity (Billet et al., 1977; Zhang et al., 1996), and invertases in the nutritive tissue are known to hydrolyze sucrose to glucose and fructose (Bronner, 1977). While gypsy moth wounding may also elicit invertase activity (Arnold and Schultz, 2002), the increase brought about by gallers was much greater than that caused by 1 wk of gypsy moth feeding in this study.

Although gall formation is associated with significant biochemical changes, not all aspects of plant biochemistry necessarily differ in gall tissues. Previous studies have found increased nitrogen and protein content (Paclt and Hässler, 1967; Schonrogge et al., 2000), extensive protein synthesis (Rohfritsch and Arnold-Rinehart, 1991), and large amounts of soluble amino compounds, ribosomes, and mRNA in the nutritive tissue of other cynipid galls (Bronner, 1977, 1992). However, nutritive tissues of *A. petiolicolus* galls had protein levels similar to those of ungalled leaves, despite having lower protein levels in the cortex. We also found that neither galled nor control chestnut oak tissues contained PPO, although we have measured vigorous PPO activity in tissues from other plant species under the same analytical conditions. Although PPO is present in a number of plants (Richard-Forget and Gauillard, 1997; Shin et al., 1997; Halder et al., 1998) and could reinforce some anti-herbivore functions of POX (Felton et al., 1994; Haruta et al., 2001), it may be absent (Grisebach, 1981) or difficult to extract from some plant tissues (Hsu et al., 1988; Burton and Kirchmann, 1997). We have found previously that PPO activity is low or absent in the leaves of chestnut oak (H. M. Appel and J. C. Schultz, unpublished data), and conclude that this enzyme either is not present in chestnut oak tissue, is present in an inactive form, or is not detectable using the methods employed.

Our data show that *Andricus* galls are biochemically distinct from their host leaves. Altered and redistributed activity of various POX isoforms, concentration of polyphenols away from feeding sites, and the increase in invertase activities in *A. petiolicolus* gall tissue relative to leaves probably combine to improve plant quality for this galling insect. It is also clear that while some responses typically induced by chewing insects are active in *A. petiolicolus* gall cortex or epidermis, they are not induced or are suppressed in the insects' food tissue; this may represent manipulation of host plant quality by the insect (Hartley, 1998; Nyman and Julkunen-Tiitto, 2000).

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BIOFUMIGANT COMPOUNDS RELEASED BY FIELD PENNYCRESS (*Thlaspi arvense*) SEEDMEAL[1]

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Abstract—Defatted field pennycress (*Thlaspi arvense* L.) seedmeal was found to completely inhibit seedling germination/emergence when added to a sandy loam soil containing wheat (*Triticum aestivum* L.) and arugula [*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Mill.) Thell.] seeds at levels of 1.0% w/w or higher. Covering the pots with Petri dishes containing the soil-seedmeal mixture decreased germination of both species at the lowest application rate (0.5% w/w), suggesting that the some of the phytotoxins were volatile. CH_2Cl_2, MeOH, and water extracts of the wetted seedmeal were bioassayed against wheat and sicklepod (*Senna obtusifolia* (L.) H. S. Irwin & Barneby) radicle elongation. Only the CH_2Cl_2 extract was strongly inhibitory to both species. Fractionation of the CH_2Cl_2 extract yielded two major phytotoxins, identified by gas chromatography–mass spectrometry and NMR as 2-propen-1-yl (allyl) isothiocyanate (AITC) and allyl thiocyanate (ATC), which constituted 80.9 and 18.8%, respectively, of the active fraction. When seeds of wheat, arugula and sicklepod were exposed to volatilized AITC and ATC, germination of all three species was completely inhibited by both compounds at concentrations of 5 ppm or less. In field studies, where seedmeal was applied at 0.50, 1.25, and 2.50 kg/m^2 and tarped with black plastic mulch, all of the treatments significantly reduced dry weight of bioassay plants compared to the tarped control, with the highest seedmeal rate decreasing dry matter to less than 10% of the control 30 d after seedmeal application. Field pennycress seedmeal appears to offer excellent

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[1]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

potential as a biofumigant for high-value horticultural crops for both conventional and organic growers.

Key Words—Field pennycress, *Thlaspi arvense*, brassicaceae, seedmeal, glucosinolate, allyl isothiocyanate, allyl thiocyanate, phytotoxicity, soil amendment.

INTRODUCTION

Biofumigation is the exploitation of plant biochemicals with pesticidal properties that may serve as ecologically-friendly alternatives to chemical fumigants such as methyl bromide (Angus et al., 1995). Additionally, organic growers lack most of the soil fumigation/sterilant options available to conventional growers. Several plant families, especially the Brassicaceae or mustard family, have been extensively studied as green manure crops, as mustards have been shown to suppress weeds (Grossman, 1993; Boydston and Hang, 1995; Brown and Morra, 1995; Vaughn and Boydston, 1997; Al-Khatib et al., 1997; Boydston and Vaughn, 2002), soil pathogens (Papavizas, 1966; Lewis and Papavizas, 1971; Papavizas and Lewis, 1971; Ramirez-Villapudua and Munnecke, 1988; Muelchen et al., 1990; Vaughn et al., 1993; Mayton et al., 1996; Brown and Morra, 1997; Williams-Woodward et al., 1997; Olivier et al., 1998; Sarwar et al., 1998), nematodes (Mojtahedi et al., 1991, 1993; Donkin et al., 1995), and insects (Brown et al., 1991) when plant tissues were incorporated into the soil. Mustards are characterized by the presence of glucosinolates, a class of glucose and sulfur-containing organic anions (Figure 1), whose biologically-active degradation products are produced when plant vacuoles are ruptured and the glucosinolates present in vacuoles are hydrolyzed by the enzyme myrosinase (β-thioglucosidase glucohydrolase; EC 3.2.3.1) (VanEtten and Tookey, 1983). These products include substituted isothiocyanates, thiocyanates, nitriles, and oxazolidinethiones that vary depending on the side-chain substitution, pH, and iron concentration (Cole, 1976; Fenwick et al., 1983; Uda et al., 1986; Chew, 1988). Many of these hydrolysis products have pesticidal properties (Vaughn, 1999). Seedmeals from glucosinolate-containing plants have also been identified as potential soil amendments for weed and insect control (Brown and Morra, 1995; Vaughn et al., 1996; Walker, 1996; Elberson et al., 1997; Vaughn and Berhow, 1999). The wide range of glucosinolates found in the Brassicaceae provides the opportunity to select those species that have the highest biofumigation potential (Kirkegaard and Sarwar, 1999; Warton et al., 2001).

Field pennycress (*Thlaspi arvense* L.) is a weedy annual/winter annual species of the Brassicaceae that is a native of Europe but has a wide distribution throughout temperate North America (Rollins, 1993). Field pennycress is considered a major agricultural weed that competes with crops causing significant yield losses (Holm et al., 1997). The plant produces an unpleasant, garlic-like odor that has caused it to be widely known as stinkweed (Mitich, 1996). Each plant may produce

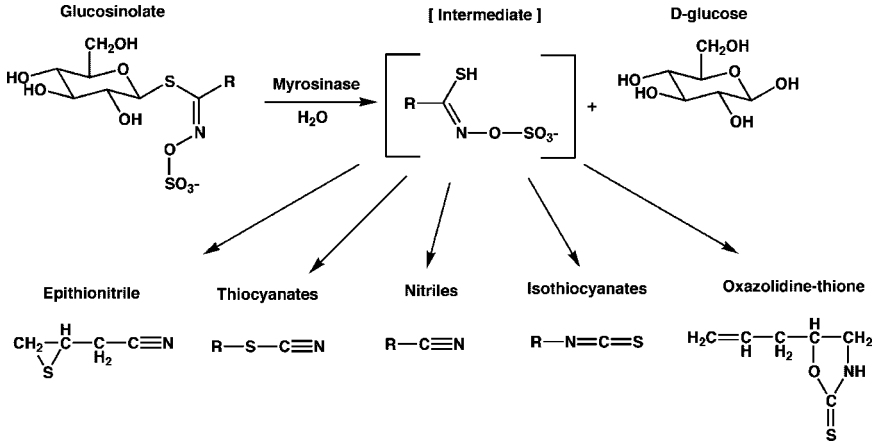

FIG. 1. The general structure of glucosinolates and their enzymatic degradation products. Adapted from Rask et al. (2000).

up to 15,000 seeds, and fields heavily infested with field pennycress yield up to 1345 kg seed/ha (Best and McIntyre, 1975). Field pennycress seed contains the glucosinolate sinigrin (Daxenbichler et al., 1991). Sinigrin can be hydrolyzed by endogenous myrosinase to form principally allyl isothiocyanate (AITC), a potent biocide that may be involved in allelopathy (Vaughn and Berhow, 1999), with allyl nitrile, allyl thiocyanate (ATC), and epithiopropylnitrile also being formed at other pH values or in the presence of reducing compounds (Cole, 1976; Springett and Adams, 1989). However, Lüthy, and Benn (1977) found that field pennycress seed flour extracts converted radiolabeled sinigrin into ATC without any AITC being formed. While AITC has been shown to be an effective biocide against a wide range of organisms, relatively little data have been published concerning the biocidal activity of ATC (Shofran et al., 1998; Tsao et al., 2002). Our interest in using glucosinolate-containing seedmeals as biofumigants prompted us to further investigate the use of field pennycress seedmeal as a soil amendment for weed suppression in high-value, low-acreage crops, and if active, to identify those compounds responsible for phytotoxicity.

METHODS AND MATERIALS

Seedmeal Bioassays. Field pennycress seeds were harvested on June 30, 2003 from a native population present in a limited-tillage soybean field near Hanna City, IL, USA Seeds were dried at room temperature for at least 30 d before use in bioassays. Seeds were ground in coffee grinder and defatted with hexane for 24 hr in a Soxhlet apparatus. The dry seedmeal was stable for an extended period

(>30 d at room temperature), but was used within 2 d of being defatted in all experiments. Seedmeal was thoroughly mixed with soil (Onarga sandy loam; Typic Argiudoll) at rates of 0.5, 1.0, and 5.0% (w/w). The seedmeal-soil mixture was added to 200-ml cups, and 10 seeds of wheat (*Triticum aestivum* L.) and arugula [*Eruca vesicaria* (L.) Cav. subsp. *sativa* (Mill.) Thell.] seeds (both of which exhibit nearly 100% germination) were added and covered with approximately 3 cm of the soil-seedmeal mixture. Nonamended soil was used as the control. Each cup received 10 ml of a solution containing 1g/l thiabendazole (excessive saprophytic fungal growth occurred at the higher seedmeal rates without this fungicide that we thought might influence the results), and then additional water was added to bring the soil to field capacity. Half of the treatments were covered with plastic Petri dishes for the initial 72 hr to prevent loss of potential volatile compounds, similar to what would be done with tarping in the field. All cups were placed in a growth chamber set at a 16 hr, 25°C d/8 hr, 20°C night regime. Emerged seedlings were counted after 14 d, and the above ground tissue of the emerged seedlings were counted. Each treatment was replicated five times in a completely randomized design, and the experiment was repeated. Data were subjected to analysis of variance (SAS, Cary, North Carolina).

Extract Preparation. One hundred grams of defatted seedmeal were wetted with 100 ml ddH_2O and allowed to sit in a beaker covered with aluminum foil for 4 hr. The wet seedmeal was extracted with three successive 250 ml aliquots of CH_2Cl_2. The CH_2Cl_2-extracted seedmeal was dried in a fume hood at room temperature to remove residual solvent, and then extracted $\times 3$ with 250 ml aliquots of MeOH. Extracts from both solvents were pooled and concentrated by rotoevaporation. A water extract was obtained by soaking the solvent-extracted seedmeal in 250 ml of distilled water overnight in a refrigerator at 2°C, after which the marc was washed with two additional 250 ml aliquots, and the extracts lyophilized. The crude CH_2Cl_2 extract subsequently found to be active in the bioassays was separated on a lipophilic Sephadex LH-20 (Supelco, Inc., Bellefonte, PA) column into three separate fractions using 100% $CHCl_3$; 50% $CHCl_3$/50% MeOH; and 100% MeOH as solvents.

Seedling Radicle Elongation Bioassay. Wheat and sicklepod (*Senna obtusifolia* (L.) H. S. Irwin & Barneby) seeds were surface sterilized with 0.5% commercial chlorine bleach for 15 min, after which they were rinsed with and then soaked in sterile distilled water for 2 hr. Seeds were wrapped in sterile paper towels saturated with water and incubated overnight in darkness at 25°C. All crude and column extracts were assayed by adding extracts to which solvents had been completely evaporated to autoclaved water agar (1.0% w/v containing 500 mg/l chloramphenicol) at the rate of 1 mg extract/ml agar (this concentration is generally effective at identifying active fractions), after the agar had cooled to ~40°C. Fifteen ml of the agar-extract mixtures were placed in 9.0-cm plastic Petri dishes, and five germinated seedlings were placed on the agar in the Petri dishes. The dishes

were incubated in darkness at 25°C on 45° slants for 24–48 hr, then evaluated for inhibition of radicle growth.

Chromatography and Spectroscopy. Gas chromatography–mass spectrometry (GC–MS) was performed using an Hewlett-Packard (HP) 6890 GC system attached to an HP 5972A Mass Selective Detector. Quantitation of compounds was performed on an HP 5890 gas chrmoatograph equipped with a flame ionization detector. Columns used in both systems were fused silica HP-5MS capillaries (0.25-μm-film thickness, 30 m $\times$ 0.25 mm ID). The GC operating parameters were as follows: splitless injection mode; temperature programmed from 40° to 315°C at 5°C/min with a 20-min initial temperature hold; He carrier gas flow rate at 1.1 ml/min, with the injector temperature set at 100°C, as ATC has been reported to decompose to AITC at higher injection temperatures (Lüthy and Benn, 1977). Spectra were compared with known standards or by computer with the Wiley/NBS Mass Spectral Registry (McLafferty and Stauffer, 1989). Proton and carbon-13 spectra were obtained on a Bruker Avance 500 spectrometer (Billerica, MA, USA) equipped with a 5-mm inverse broadband Z-gradient probe (^{1}H NMR, 500 MHz, ^{13}C, 125 MHz). The software used to obtain the NMR data was ICON 3.5. The solvent was deuterated chloroform.

Effect of Volatilized AITC and ATC on Seed Germination. AITC and ATC, the active compounds identified in the extract, were tested for inhibition of seed germination of wheat, annual ryegrass (*Lolium multiflorum* Lam.), sicklepod, arugula, and velvetleaf (*Abutilon theophrasti* Medicus). Due to the difficulties involved in isolating and purifying large amounts of the compounds from seedmeal extracts, synthetic AITC and ATC were used in these tests. ATC was synthesized according to the method of Slater (1992) while AITC was purchased from Sigma. Ten seeds of each species were placed into 9-cm Petri dishes on Whatman No. 1 filter paper disks saturated with sterile distilled water. The dishes were then placed into 2.4 l glass desiccators containing a filter paper disk onto which AITC or ATC were added (neat) on a volume compound-headspace volume basis at rates of 0.0, 0.1, 0.5, 1.0, 5.0, 10.0, and 20.0 ppm. Flasks were placed in darkness in a growth chamber at 25°C for 4 d, after which germination was scored. Each treatment consisted of three replicates per species and the experiment was repeated.

Isolation and Quantification of Glucosinolates from Seedmeal. For quantitation of seedmeal glucosinolates, a modification of a high-performance liquid chromatography (HPLC) method developed by Betz and Fox (1994) was used. Replicates of defatted seedmeal (5.0 g) were added to 200 ml boiling 70% (v/v) MeOH with stirring for 15 min, cooled, and then filtered through Whatman No. 2 filter paper. The marc was washed twice with 50 ml aliquots of 70% MeOH. The resulting extract was concentrated to 5–10 ml by rotoevaporation, and diluted to 25 ml to form a working solution. The extract was run on a Finnegan Thermoquest P4000 HPLC system (San Jose, CA) using a C_{18} column (250 $\times$ 4.6 mm^2;

RP-18, 5; Licrosorb, Alltech, Deerfield, IL). Peaks were detected with a Finnegan Thermoquest P6000 photodiode array detector set at 237 nm. The initial mobile phase conditions were 12% methanol/88% aqueous 0.005M THS at a flow rate of 1 ml/min. The binary gradient was developed to 70% methanol/30% aqueous 0.005 M THS for 20 min, and held at these conditions for an additional 15 min. Quantitation was determined from a standard curve prepared from pure sinigrin (Sigma, St. Louis, MO). Each treatment was run in triplicate and the experiment was repeated.

Field Studies. Seedmeal was tested for its ability to suppress plant germination and growth when applied to tarped field plots. Experiments were conducted in a randomized complete block design with four replications (each replicate measured 0.75 m by 1.5 m) on a field site soil which was an Orthents complex containing a silt loam surface of 30–40 cm underlaid with a silt clay loam. The site was conventionally tilled 1 wk prior to experiments. In addition to native weed seeds present in the soil, each plot was spiked prior to seedmeal incorporation with 75 g of a bioassay seed mixture containing 1:1:1 amounts of annual ryegrass, white mustard (*Sinapis alba* L.), and sicklepod seeds. Defatted seedmeal prepared as previously described was applied to test plots at rates of 0.5, 1.0, and 2.5 kg/m^2 by sprinkling the seedmeal evenly over the test plots and incorporating the seedmeal into the soil with pitchforks to a depth of approximately 10 cm. The plots were then watered (approximately 5 l per plot) thoroughly, and covered with polyethylene plastic sheets (0.1-mm thickness, 0.91 m by 2 m; Sunbelt Plastics, Minneapolis, MN) that were covered with soil at the edges to prevent loss of volatiles. Controls consisted of untarped plots and tarped plots without seedmeal. Tarps were removed after 7 d, and all plots were watered as needed. After 30 d, the plant biomass of the plots was determined by digging up and washing all of the plants after which they were placed in a 50°C drying oven for 48 hr before being weighed. Data were subjected to analysis of variance (SAS, Cary, NC). Dry weight mean separation was performed using the Student–Newman–Keuls multiple range test ($P < 0.1$).

RESULTS AND DISCUSSION

Bioassays. After 14 d, field pennycress seedmeal added to soil completely inhibited germination of wheat and arugula at rates of 1.0% or higher (Table 1). Covering the cups increased inhibition at the 0.5% rate over uncovered meal at the same rate, suggesting that some or all of the phytotoxins were volatile. Indeed, when the covers were removed after 72 hr, all of the cups possessed a distinctive garlic-like odor regardless of application rate, which could also be detected initially from the seedmeal after watering. At the 5% rate, this odor was still detectable after 3 d from uncovered cups as well as covered ones.

Identification of the Phytotoxins. The crude CH_2Cl_2 extract strongly inhibited sicklepod and wheat radicle elongation, while the MeOH and water extracts had no

TABLE 1. INHIBITION OF WHEAT AND ARUGULA GERMINATION BY FIELD PENNYCRESS SEEDMEAL

	Germination (% of control)[a]					
Species	0.5% covered	0.5% uncovered	1.0% covered	1.0% uncovered	5.0% covered	5.0% uncovered
Wheat	15 ± 2.9b	40 ± 4.1c	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a
Arugula	32 ± 3.2b	73 ± 4.0c	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a

[a] Mean ± SE values within a row followed by the same letter were not significantly different by the Student–Newman–Keuls multiple range test ($P < 0.1$).

effect. Fractionation of the crude CH_2Cl_2 extract on a Sephadex LH-20 column yielded one fraction (100% $CHCl_3$) that was inhibitory to radicle elongation in bioassays when tested at 1 mg extract/ml agar. This fraction contained two major peaks as determined by GC–FID. The compounds were identified by GC–MS spectra and by NMR analysis as AITC and ATC. Prominent diagnostic mass spectral ions and their relative intensities for ATC are as follows: EI–MS [*m/z* (%)]: 99 (M^+, 91), 86 (6), 84 (10), 72 (35), 58(12), 41 (100). Prominent diagnostic mass spectral ions and their relative intensities for AITC are as follows: EI–MS [*m/z* (%)]: 99 (M^+, 100), 98 (9), 72 (28), 71 (8), 41 (47). The AITC and ATC peaks accounted for 80.9 and 19.1%, respectively, of the active fraction as quantitated by GC–FID. ^{1}H NMR and ^{13}C spectra for ATC are as follows: ^{1}H NMR δ ($CDCl_3$): 5.86 (1H, m) CH=; 5.32 (1H, d, $J = 16.8$ Hz) CH =; 5.28 (1H, d, $J = 10$ Hz); 3.49 (2H, d, $J = 7.3$ Hz) CH_2. ^{13}C NMR δ ($CDCl_3$): 130.5 (C=); 121.4 (CH_2=); 111.7 (C); 36.7 (CH_2). ^{1}H NMR and ^{13}C spectra for AITC are as follows: ^{1}H NMR δ 5.78 (1H, m) CH=; 5.33 (1H, d, $J = 16.9$ Hz) CH =; 5.22 (1H, d, $J = 10.3$ Hz) CH =; 4.08 (2H, m) CH_2. ^{13}C NMR δ ($CDCl_3$): 132 (C); 130.3 (CH=); 117.7 (CH_2=); 47.1 (CH_2). In the proton NMR spectrum of ATC, the aliphatic methylene protons are observed at δ 3.49, in the AITC spectrum the methylene protons resonate at δ 4.08. Differences in the ^{13}C spectra for the two compounds are striking. The resonance for the nonprotonated carbon in ATC is observed at δ 111.7. In AITC, the nonprotonated carbon is observed as a small, broad signal at δ 132.0. The changes in aliphatic methylene resonances also demonstrate the difference between the two compounds.

Intact Glucosinolate Analysis. Analysis of intact glucosinolates from defatted seedmeal by HPLC found only one major glucosinolate peak, tentatively identified by retention time as sinigrin. LC–MS analysis of the peak confirmed the mass of the unknown to be 358, affirming that the compound was indeed sinigrin. Total amounts of sinigrin contained in the seedmeal were found to be 39.7 ± 3.8 mg sinigrin/g defatted seedmeal.

Inhibition of Seed Germination by AITC and ATC. Both AITC and ATC were inhibitory to seed germination, with 5 ppm levels of both compounds completely

TABLE 2. MINIMUM INHIBITORY CONCENTRATIONS OF AITC AND ATC

	Concentration (ppm)				
Volatile	Wheat	Ryegrass	Arugula	Sicklepod	Velvetleaf
AITC	1	0.5	5	5	1
ATC	1	1	5	1	0.5

inhibiting the germination of the five species tested (Table 2). The lowest level tested (0.1 ppm) did not inhibit the germination of any of the species, although annual ryegrass seed germination was completely inhibited by both compounds at 0.5 ppm levels. Interestingly, arugula, the only bioassay species to possess the glucosinolate-myrosinase system, was the most tolerant of the five species to both compounds. Although thiocyanates have been shown to have activity against plants, they are generally thought to be less active than their respective isothiocyanates (Vaughn, 1999). However, Shofren et al. (1998) found that ATC had similar inhibitory activity to AITC against several bacteria, while Tsao et al. (2002) found that the LC_{50} (concentration required to kill 50% of the population) values of ATC as a fumigant against house fly (*Musca domestica* L.) and lesser grain borer (*Rhyzopertha dominica* Fabricius) adults was lower than AITC.

Field Studies. At all three rates tested, field pennycress seedmeal reduced plant biomass as compared to both the tarped and untarped controls (Figure 2). At the highest rate of incorporated seedmeal, biomass was reduced to less than 9% of the tarped control and less than 8% of the untarped control. All of the treatments exhibited visual reductions in seed germination compared to controls, and in the two highest treatment levels the ground remained completely bare in the center of each plot at the time of harvest. This seems to indicate that volatile compounds were primarily responsible for the inhibition, as leakage of volatiles near the edges of the tarps would decrease their concentrations compared to the centers. At the 0.5 kg/m^2 rate, there was some fungal growth found upon tarp removal near the plot edges that was lacking at the higher rates, although visible evidence of this fungi dissapeared within several days after removal of the tarps. Tarping alone appeared to promote rapid seed germination, possibly due to increased soil temperatures under the tarps, but many of the seedlings that had germinated either died after tarp removal or took longer to recover.

Using glucosinolate-containing plant material and processed seedmeals offer excellent potential in controlling weeds and soilborne pests, whether for organic or conventional growers. Because the bioactive degradation products dissapate rapidly, they pose less environmental risk than compounds that persist for greater periods of time. Field pennycress seedmeal offers potential as a biofumigant if the

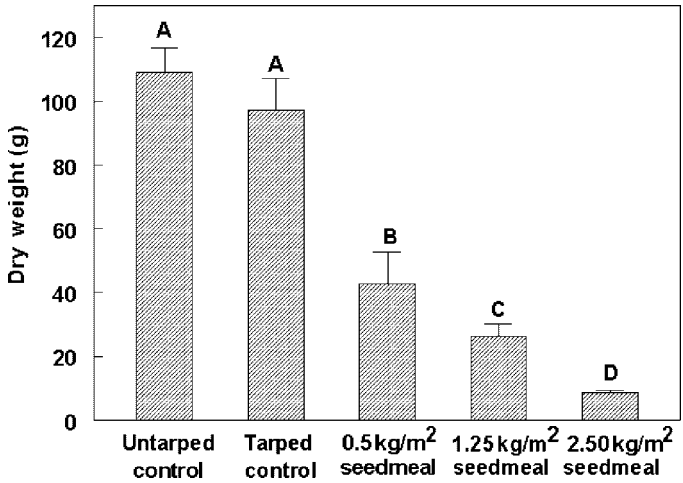

FIG. 2. Weed biomass in field plots 30 d after seedmeal application. Bars represent 1 S. E. Treatments with the same letter are not significantly different by the Student–Newman–Keuls multiple range test ($P < 0.1$).

agronomic obstacles associated with growing the plant as a crop are solved. Additionally, unlike synthetic fumigants, field pennycress seedmeal also is an excellent organic fertilizer, containing approximately 4% nitrogen.

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CHANGES OVER TIME IN THE ALLELOCHEMICAL CONTENT OF TEN CULTIVARS OF RYE (*Secale cereale* L.)

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Abstract—Published studies focused on characterizing the allelopathy-based weed suppression by rye cover crop mulch have provided varying and inconsistent estimates of weed suppression. Studies were initiated to examine several factors that could influence the weed suppressiveness of rye: kill date, cultivar, and soil fertility. Ten cultivars of rye were planted with four rates of nitrogen fertilization, and tissue from each of these treatment combinations was harvested three times during the growing season. Concentrations of a known rye allelochemical DIBOA (2,4-dihydroxy-1,4-(2H)benzoxazine-3-one) were quantified from the harvested rye tissue using high performance liquid chromatography (HPLC). Phytotoxicity observed from aqueous extracts of the harvested rye tissue correlated with the levels of DIBOA recovered in harvested tissue. The amount of DIBOA in rye tissue varied depending on harvest date and rye cultivar, but was generally lower with all cultivars when rye was harvested later in the season. However, the late maturing variety 'Wheeler' retained greater concentrations of DIBOA in comparison to other rye cultivars when harvested later in the season. The decline in DIBOA concentrations as rye matures, and the fact that many rye cultivars mature at different rates may help explain why estimates of weed suppression from allelopathic agents in rye have varied so widely in the literature.

Key Words—Allelopathy, cover crop, residue, redroot pigweed (*Amaranthus retroflexus* L.), goosegrass (*Eleusine indica* L. Gaertn.), 2,4-dihydroxy-1,4-(2H)benzoxazine-3-one, DIBOA, maturity, phenology, rye (*Secale cereale* L.).

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INTRODUCTION

Rye cover crop residues have been well studied for their weed suppressive capabilities (Barnes and Putnam, 1983; Masiunas et al., 1995; Creamer et al., 1996; Nagabhushana et al., 2001). Reviews of this literature have noted that estimates of weed control attributable to rye mulch allelopathy have varied considerably between studies (Weston, 1996; Foley, 1999). Foley (1999) suggested that these differences could be due to the use of different cultivars of rye. Support for this theory was found in a recent study of an important allelochemical, DIBOA. Among eight cultivars of rye, there was over a 10-fold difference in the concentration of DIBOA (Burgos et al., 1999). While the relative contribution of DIBOA to rye allelopathy is still under debate (Blum, 1995), its high phytotoxicity to many weeds and relative abundance in the plant have often singled it out for study (Barnes et al., 1987; Niemeyer, 1988). The large differences among cultivars in the production of this compound may explain the discrepancies in weed suppression among studies that utilized different cultivars.

Another factor that may be causing discrepancies among studies is differences in the date at which the rye is killed. The typical date of kill varies considerably among regions depending on temperatures and soil moisture, and also which crop is being planted after the rye. As a result, the rye residues from various studies differ dramatically in the age of the tissue and the developmental stage at which it was killed. Age and developmental stage have proven important factors in greenhouse studies of DIBOA production in rye (Burgos et al., 1999). These factors may also interact with the cultivar effect to determine allelochemical concentrations. Some cultivars are slower to develop phenologically, with as much as a month's difference in flowering time having been observed in fall-sown rye (Reberg-Horton, 2002). The biggest difference in the development of cultivars is seen between winter and facultative types of rye. Winter types will only flower and complete their life cycle if sown in the fall. Facultative types can be planted in spring or fall and will develop normally. The facultative types also appear to develop more rapidly in the southern US (Reberg-Horton et al., 2003). The impact of differing developmental rates on allelochemical concentrations has not been explored.

Fertility is another factor that may be impacting allelochemical production. In the only study examining the effect of fertility on rye allelopathy, Mwaja et al. (1995) found that low to moderate rates of fertilization led to higher DIBOA production than higher rates. This effect was also evidenced in their bioassays of the rye tissues.

How to measure the impact of any of these factors on allelopathy has been much debated in the literature (Blum et al., 1999; Inderjit and Weston, 2000). Many studies have utilized weed seedling bioassays due to their simplicity and ability to screen for general phytotoxicity, without invoking a bias about which compounds are the most important. Critics, however, argue that most bioassays

do a poor job simulating natural systems and often overestimate the toxicity of plant extracts. Another approach is to quantify individual compounds that have proven to be phytotoxic. For this approach to be reliable, extensive prior research is required to identify the likely toxic compounds. Fortunately, rye allelopathy has been well researched with the identification of multiple allelopathic compounds including the extensively studied benzoxazinone, DIBOA (Shilling et al., 1986; Barnes et al., 1987). Not only is this allelochemical highly toxic to most weed seedlings (Barnes et al., 1987; Burgos and Talbert, 2000), it is also produced in abundance by rye (Burgos et al., 1999). By using DIBOA quantification in parallel with bioassays, this study was able to examine allelochemical production both at the specific compound and general toxicity level. Both methods were used to examine how date of kill, cultivar, and fertility affect rye allelopathy.

METHODS AND MATERIALS

The experimental design was a split plot with the main plots laid out in randomized complete blocks. The main plots were fertilized with four rates of nitrogen: 0, 22, 45, or 90 kg N ha^{-1} applied as ammonium nitrate (34-0-0). The nitrogen was spread with a Gandy drop spreader and incorporated prior to planting. Ten rye cultivars comprised the subplot factor: 'Aroostook,' 'Bates,' 'Elbon,' 'Maton,' 'WinterGrazer 70,' 'Wrens 96,' 'Wrens Abruzzi,' 'Wheeler,' 'Bonel,' and NC unnamed. NC unnamed is not an actual cultivar, but comes from a large seed distribution chain with the variety unstated.

Plots were located at the Center for Environmental Farming Systems in Goldsboro, North Carolina on a well-drained, Wickham loamy sand (fine-loamy, mixed, semiactive, thermic Typic Hapludult). The rye was planted on raised 1.2 m beds by using an Almaco grain drill with an 18 cm row spacing at 124.3 kg ha^{-1} with 6 rows fitting on each bed. They were seeded on November 16th, 2000 in plots that were 3 beds wide and 9.1 m long. Aboveground biomass was measured by clipping shoot tissue at ground level in 0.5 m^2 quadrants, placed in the center 4 rows of a bed. Samples were taken on March 1, April 5, and April 23, 2001. Tissue was placed in cotton bags and dried at 60°C for 2 d in a forced-air oven and weighed. A portion of this tissue was reserved for bioassays and quantifying DIBOA, making sure that whole plants were kept so that all shoot tissues were fairly represented. Tissue was ground using a Wiley mill with a 1 mm mesh size and stored at room temperature in the dark.

Bioassay Methods

Bioassays were conducted on all cultivar and sample date combinations and on 2 of the 4 nitrogen rates: the 22 and 90 kg N ha^{-1} treatments. To make the rye

extracts, 1 g of ground tissue was added to 40 ml de-ionized water, placed on a shaker for one hr, vacuum filtered through Whatman no. 1 filter paper, and kept for 12 hr at 4°C. Five ml aliquots of the extract were added to 90 mm diam by 15 mm high culture dishes containing three Whatman no. 1 filter papers. Ten seeds of redroot pigweed or 25 seeds of goosegrass were placed on the filter papers, and the dishes were sealed with parafilm and incubated in a germination chamber at 28°C with constant light. Root elongation of each seedling was measured to the nearest mm after 72 hr.

A 1:40 ratio (wt/v) for the extracts was chosen based on past research with the technique that showed optimal separation of the cultivars at this concentration (Reberg-Horton, unpublished). However, tissue from the March 1 sampling date was so inhibitory that many petri dishes had no seeds germinating. These samples were subsequently bioassayed at a dilution of 1:160 (wt/v).

Data were square root transformed before being analyzed over time using a Repeated Measures ANOVA model (SAS Institute, 2002). Preplanned orthogonal contrasts were used to test for difference among cultivars. Choices of comparisons to make were based on previous work with these cultivars (Reberg-Horton et al., 2001). The Wilk's Lamda statistic was used to test the effect of time and all interactions with time. Root measurements were converted to percentage of untreated plants after the statistical analysis.

Quantification of Diboa

The interactions of all combinations of cultivars, nitrogen rates, and time on DIBOA levels were not quantified. Three of the 4 nitrogen rates were used: 0, 45, and 90 kg N ha^{-1}. Four cultivars were chosen for DIBOA quantification based on the bioassay results and past research. Two of these cultivars, 'Wheeler' and NC unnamed, are winter types of rye, and the shoot tissue had demonstrated relatively high levels of allelochemical activity in preliminary work done the year prior. The other two cultivars, 'Bonel' and 'Wrens Abruzzi,' are facultative types of rye, meaning they can be planted in the fall or spring. 'Bonel' was chosen due to published reports of high DIBOA production (Burgos et al., 1999) and 'Wrens Abruzzi' due to its popularity in the South. The decision to choose 2 facultative and 2 winter types was based on observations of the growth and development of these different classes over time. Facultative types produce more biomass early in the season and flower earlier than winter types.

DIBOA content in the tissue was determined by using a protocol modified from previously published methods (Yenish et al., 1995; Melansom et al., 1997). Ground tissue (0.5 g, see above) was first extracted in water, and then incubated for 1 hr (to hydrolyze the DIBOA-glycoside into DIBOA). Methanol was then added (to 60%, v/v) and the mixture vigorously agitated. The extract was then filtered through 2 layers of Miracloth, and centrifuged at 20,000 $\times$ g for 20 min. The

supernatant was retained and extracted twice with a 2-fold (v/v) excess of ethyl-acetate, and the combined nonpolar phases were dried under vacuum (Speed-Vac). The dried sample was re-solubilized with 0.5 ml mobile phase (see below) and filtered through a 0.2 μ filter.

Chromatographic separation of the extracts was conducted by using an isocratic mobile phase consisting of 40% methanol and 60% water (0.02% phosphoric acid). The chromatography was facilitated by using a Waters system high performance liquid chromatography (HPLC), and a Beckman Ultrasphere ODS, 5 μ, C18 column (4.6 $\times$ 250 mm^2). The detector used was a Perkin Elmer variable wavelength, UV–Vis detector, monitored at 254 nm. DIBOA was identified and quantified utilizing a chemically synthesized analytical standard (provided by Dr. Scott Chilton, NCSU). BOA was identified and quantified using a standard purchased from Sigma Chem. Co.

The HPLC method was found to effectively resolve BOA, DIBOA, and the DIBOA-glycoside. DIBOA was the principal benzoxazinone found in the extracts, and only trace levels of DIBOA-glycoside and BOA were observed. The identity of the DIBOA glycoside was deduced by incubating subsamples of a rye extract in the presence of almond glucosidase (Sigma Chem. Co.) for different times. The successive decrease in the area of the peak eluting at 6 min, and the corresponding increase in the DIBOA peak at 8 min indicated that the 6 min peak was the DIBOA glycoside. (Subsequent, efforts with GC-MS have established that the 6 min peak is the DIBOA glycoside, details to be published).

The DIBOA content per gram of dry tissue derived from the HPLC analysis was multiplied by the biomass production for each plot to generate estimates of the rate of DIBOA being produced on a per unit area basis. This variable is likely a useful predictor of weed suppression in the field. DIBOA rates and concentrations were analyzed over time by using a Repeated Measures Analysis ANOVA (SAS, 2002). Preplanned orthogonal contrasts were used to compare winter and facultative types and within type differences. The Wilk's Lamda statistic was used to test the effect of time and all interactions with time.

Harvesting by Developmental Stage

A separate study was conducted simultaneously using the same 10 rye cultivars. The study was planted at the Cunningham Research and Extension Center in Kinston, North Carolina. A three replicate completely randomized design was utilized. The rye was planted on October 20, 2000 in single row plots 1.2-m long, spaced 0.3-m apart. Twenty-two kg N ha^{-1} was applied as a top dressing in early February 2002. A key difference between the two studies is when the tissue was harvested. In this study, tissue was not harvested at specific dates, but whenever each cultivar reached the early boot stage (Feeke's growth stage 9). Using this

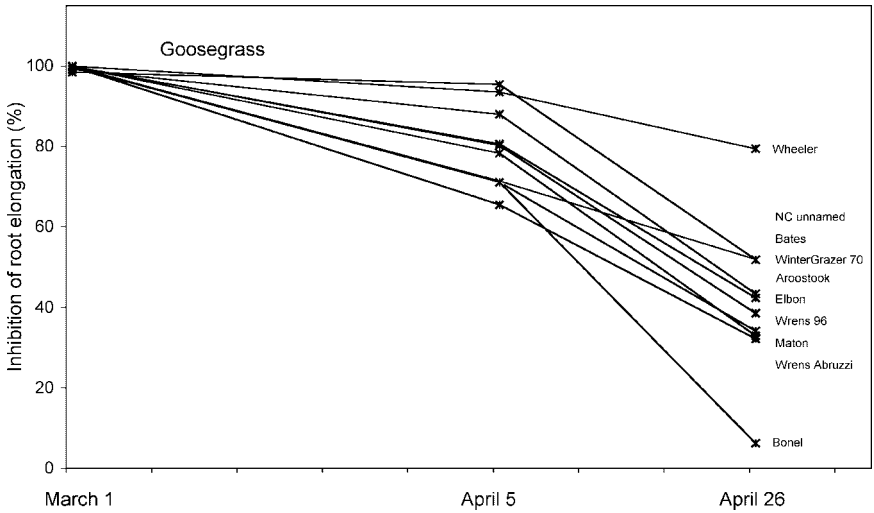

FIG. 1. Inhibition of goosegrass root elongation in shoot tissue extracts form different rye varieties.

criterion, the harvest date varied from March 12 for 'Wrens Abruzzi' to April 11 for 'Wheeler.'

RESULTS AND DISCUSSION

Bioassays

Root elongation of goosegrass (Figure 1) and pigweed (Figure 2) was inhibited by the aqueous extracts from all rye cultivars. Tissue extracts from all varieties on the March 1st harvest inhibited root elongation of both test species by greater than 95%. The relative phytotoxicity of extracts for all cultivars progressively decreased over time. This decrease was rapid and was significant at the 0.001 level for both weed species. By the April 5th harvest, inhibition of pigweed and goosegrass root elongation ranged between 60 and 95%. At the April 26th harvest, the relative phytotoxicity had decreased, with 20–60% inhibition for pigweed, and 5–75% for goosegrass. The rate of decreasing phytotoxicity was similar for extracts from all cultivars when pigweed was the indicator species (Figure 2). Goosegrass, however, revealed differences between the cultivars in the rate at which phytotoxicity decreased (Figure 1). 'Wheeler' had a slower rate of decrease in phytotoxicity in comparison to the other 9 cultivars when goosegrass was the indicator species ($P = 0.004$). The declining phytotoxicity of the extracts as the growing season progressed suggested that a time dependent factor at the time of kill,

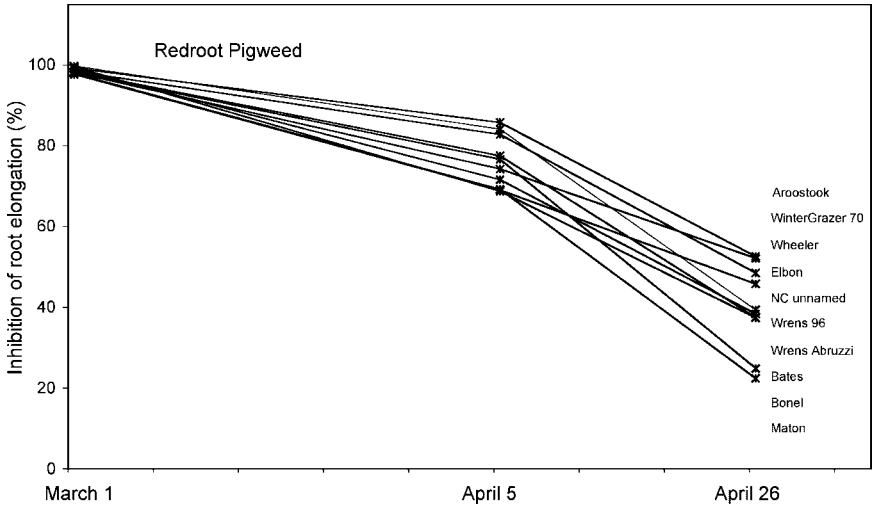

FIG. 2. Inhibition of pigweed root elongation in shoot tissue extracts form different rye varieties.

e.g., developmental stage of growth, is important in determining the allelopathic potential of rye tissue.

DIBOA

DIBOA concentrations likewise varied over time and among the cultivars (Figure 3). DIBOA concentrations in rye tissue decreased over time ($P < 0.001$) in a pattern similar to changes in the phytotoxicity of the extracts at different harvests (Figures 1 and 2). At the March 1st harvest, 'Bonel' contained the highest amount of DIBOA (1.8 mg g^{-1} dry wt), and 'Wrens Abruzzi' contained the least (1.0 mg g^{-1} dry wt). At the April 5th harvest, 'Wheeler' and NC Unspecified contained between 0.7 and 0.9 mg g^{-1} dry wt, and 'Wrens Abruzzi' and 'Bonel' contained 0.2–0.3 mg g^{-1} dry wt. By the April 26th harvest, all varieties contained less than 0.2 mg g^{-1} dry wt. Though the DIBOA concentrations in all varieties declined over time, the rate of decline varied with the cultivar. The changes in the winter types ('Wheeler' and NC Unspecified) were different from the facultative types over time ($P < 0.001$). The winter types declined more slowly between March 1 and April 5, but all cultivars had similar levels by April 26th.

Another factor that distinguishes the winter and facultative types of rye is evident in the graph reporting growth stage changes over time (Figure 4). 'Wheeler' and NC Unspecified are the slowest to develop phenologically. At each sampling date, these two winter types were at an earlier stage of development than the other

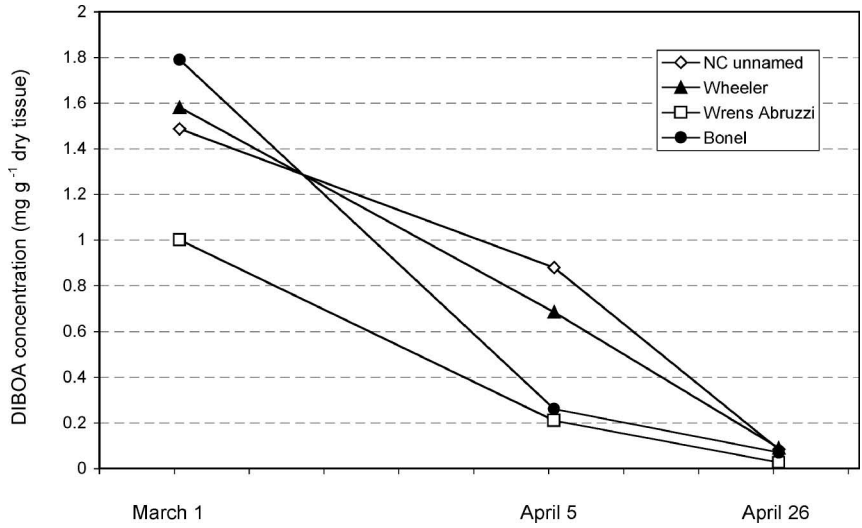

FIG. 3. Changes in DIBOA concentration in shoot tissue from different rye varieties over time.

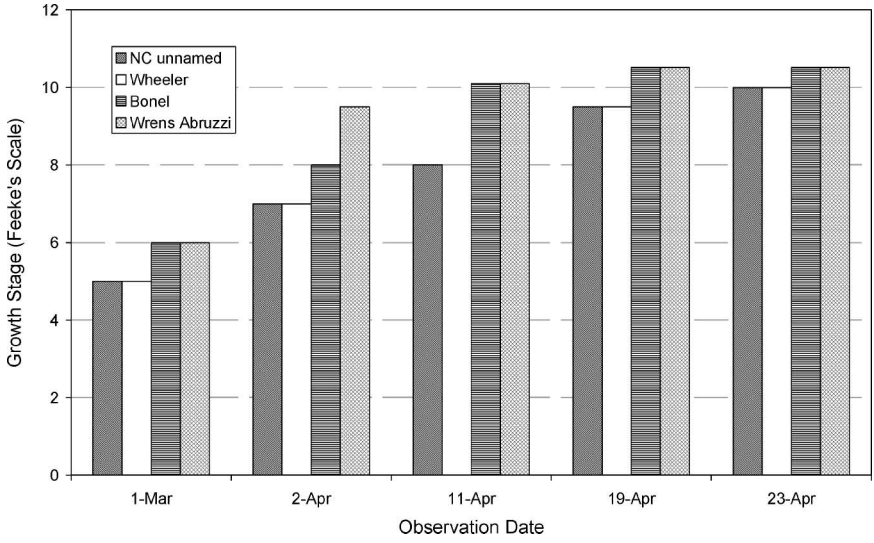

FIG. 4. Relative changes in the phenology of 10 rye cultivars over time. The average growth stage (Feekes scale) was determined visually in each experimental plot.

TABLE 1. ROOT GROWTH INHIBITION BIOASSAYS OF EXTRACTS FROM DIFFERENT RYE CULTIVARS HARVESTED AT THE SAME GROWTH STAGE (FEEKE'S STAGE 9)

Variety	Harvest date	Inhibition of root elongation (%)	
		Pigweed	Goosegrass
NC unnamed	(4/2–4/11)	8 a[a]	77[b]
Aroostook	(3/25–4/2)	46 abc	80
Wheeler	(4/11)	50 abcd	91
Bonel	(3/25)	58 bcd	72
Wrens 96	(3/19)	61 bcd	80
WinterGrazer 70	(3/19)	63 bcd	85
Elbon	(3/19–3/25)	63 bcd	89
Maton	(3/25)	69 bcd	85
Bates	(3/19–3/25)	78 cd	87
Wrens Abruzzi	(3/12)	92 d	94

[a]Means followed by the same letter are not significantly different according to Fisher's Protected LSD ($P < 0.05$) test.
[b]Goosegrass bioassays found no significant differences.

cultivars. Because the allelochemical content in rye tissue was greater earlier in the season and decreased over time, the levels may be related to the developmental stage of growth. The winter types of rye had slower phenological development, and had higher levels of DIBOA later in the season when compared to the facultative types (Figure 3). This suggests that the relative rates of maturation of winter and facultative varieties play a role in the change of DIBOA content.

At the Kinston study site, cultivars were harvested at the same developmental stage (Feeke's Stage 9, or 'boot stage'), but on different harvest dates (Table 1). The results from this experiment further emphasized that the varieties had distinct developmental profiles over time because 'Wheeler' and NC Unspecified reached boot stage by April 11, nearly 1 mo later than 'Wrens Abruzzi,' which reached boot stage on March 12. The other varieties reached the boot stage during March.

When the varieties were harvested at the same growth stage, even though there was a 1-mo period between the harvest of the first and last varieties, reaching Feeke's 9, inhibition of goosegrass by extracts from the different varieties was not different (Table 2). However, there was variation in the inhibition of pigweed root elongation by extracts of the different varieties. If the pigweed inhibition data for each variety from Table 2 is graphed according to each harvest date, there is a negative correlation ($R^2 = 0.67$) between phytotoxicity and date of harvest, with the earlier harvests more toxic. If the developmental stage of growth were the only determining factor in allelochemical production, then harvesting at the

TABLE 2. ROOT GROWTH INHIBITION BIOASSAYS OF EXTRACTS (1:160, W/V DILUTION) FROM DIFFERENT RYE CULTIVARS HARVESTED MARCH 1

Cultivar	Inhibition of root elongation (%)	
	Pigweed	Goosegrass
Maton	9 a[a]	29[b]
Elbon	16 ab	29
Bates	20 abc	32
Wrens Abruzzi	21 abc	19
NC unnamed	22 abc	28
WinterGrazer 70	23 abc	29
Wheeler	24 bc	26
Bonel	27 bc	33
Wrens 96	30 bc	30
Aroostook	31 c	35

[a] Means followed by the same letter are not significantly different according to Fisher's Protected LSD ($P < 0.05$) test.
[b] Pigweed bioassays found no significant differences.

same growth stage should have eliminated the differences between varieties, as observed with the goosegrass data. Remaining differences between varieties could be due to the environmental conditions previous to harvest. During the month over which varieties reached the boot stage, temperature, rainfall, and soil moisture were changing rapidly. The varieties may also differ due to genetic factors that are not related to developmental rate. The pigweed data suggest that developmental stage is not the only factor affecting a cultivars allelopathic potential.

While the general phytotoxicity of the rye tissue (as measured by the bioassay) and DIBOA content decreased over time, biomass increased (Table 3). The field rate (kg ha^{-1}) of allelochemical(s) that would be (potentially) available for weed suppression is a function of both biomass and allelochemical content of the tissue. The field rate of DIBOA increased between March 1 and April 5 for the winter types but decreased during the same time for facultative types ($P < 0.001$, Figure 5). After reaching a maximum level, the DIBOA rates in the winter types decreased. DIBOA rates for all cultivars were similar by the last sampling on April 23.

The rye extracts from the March 1 harvest were so toxic to the seedlings at 1:40 dilution, that all the varieties inhibited growth >95% (Figures 1 and 2). The same tissue was subsequently bioassayed at a dilution of 1:160 (Table 2). The more dilute extracts were still toxic, with inhibition of either goosegrass or redroot pigweed ranging from 9 to 35% for all the rye varieties. The more dilute bioassay from the March 1 harvest also allowed for treatment mean separation with goose

TABLE 3. SHOOT BIOMASS (mg ha^{-1}) OVER TIME

Cultivar	March 1st	April 5th	April 26th
Wheeler	1.5 c[a]	5.9 de	10.9 a
Bonel	1.7 bc	7.0 abc	10.8 ab
Wrens 96	1.8 ab	7.7 a	10.4 abc
Wrens Abruzzi	2.0 a	7.2 ab	10.0 abc
Maton	1.8 ab	6.9 bc	10.0 abc
WinterGrazer 70	0.6 e	5.2 e	9.7 bc
Aroostook	1.1 d	6.4 cd	9.5 c
Elbon	1.8 ab	7.2 ab	9.2 cd
Bates	1.1 d	7.0 cd	9.2 cd
NC unspecified	0.8 e	4.4 f	8.3 d

[a] Means followed by the same letter are not significantly different according to Fisher's Protected LSD ($P < 0.05$) test.

grass, but not pigweed. No clear division between winter and facultative types can be seen at this date. All cultivars were between Feeke's Stage 5 and 7. Given the high toxicity of the young tissue relative to that of the later harvests, using the early-harvest 1:160 data as a predictor of field performance of the different cultivars may be unreliable.

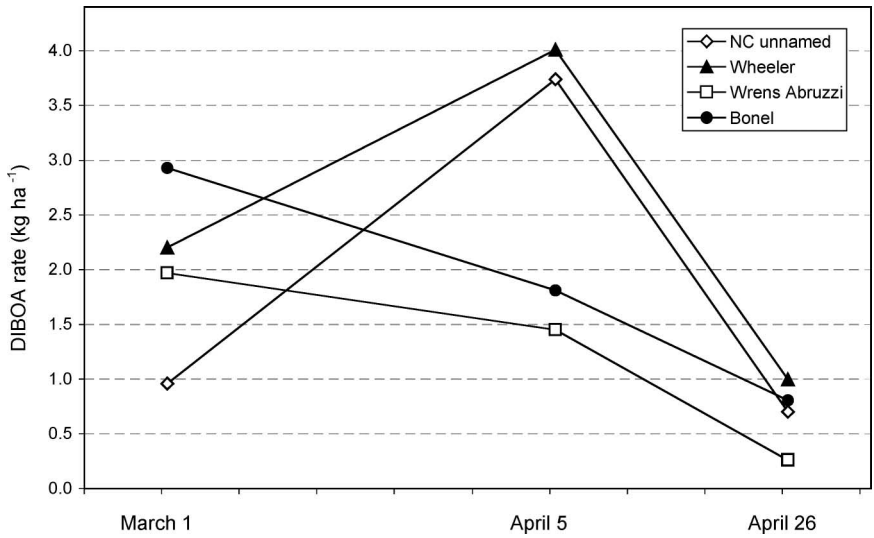

FIG. 5. DIBOA rate (concentration × biomass) for different rye varieties over time.

Fertility Effects

The rate of N fertilization had no effect on biomass accumulation for any of the varieties (data not shown). Soil N was tested on February 9, 2001, which demonstrated a high level of total soil N in the 0 N treatments (40–70 kg N ha^{-1} at 0–30 cm depth; and 65–75 kg N ha^{-1} at the 30–60 cm depth: see Reberg-Horton, 2002 for details). Others have reported fertility levels in greenhouse grown rye influenced allelochemical production (Mwaja et al., 1995). Given the high N levels and biomass production at the 0 N rate, we conclude that the residual N in the field was too high to either observe a growth response or induce low N stress. In addition, the bioassays failed to detect any N effects. However, a significant effect of N was observed in the DIBOA levels ($P = 0.008$), but only at one point in time. 'Wheeler' had higher DIBOA concentrations (LSD, $\alpha = 0.05$) in the 0 N rate at the March 1 sampling (data not shown). No differences in DIBOA levels due to N treatments were present at the other sample dates. Because neither an N growth response nor evidence of N stress was observed in this study, a fair test of N effects on allelopathy was not achieved. The observation that DIBOA levels were higher at the 0 N treatments was not expected, but the results parallel the observations of Mwaja et al. (1995). We can only speculate as to the mechanism for a greater DIBOA level at low N, but because no N stress was apparent, a stress-induced increase in DIBOA is an unlikely hypothesis.

Time and Cultivar Effects

These data demonstrated that the allelochemical content in field-grown rye changed over the growing season. For any given variety, comparing the kill dates selected, the younger tissue produced greater phytotoxic effects on selected bioassay species and contained a greater amount of DIBOA than older tissue. These results confirm other research that describe a temporal profile of benzoxazinones (BX) levels in rye and other species (Nakagawa et al., 1995; Collantes et al., 1998; Burgos et al., 1999; Copaja et al., 1999; Cambier et al., 2000; Gianoli et al., 2000). BX biosynthesis begins after germination, and the content (g g^{-1} dry wt) increases over time to some maximum level, and then decreases. In the greenhouse, Burgos et al. (1999) found the maximum to be about 60 d after germination. In corn, the maximum BX content occurs between 7 and 10 d. The date or growth stage at which the maximum DIBOA/allelochemical content in field grown rye occurs is not known. The factors that regulate the allelochemical content in cover crops needs to be defined to obtain optimum weed suppression from a cover crop mulch.

While date of kill was a critical factor, all cultivars were not affected equally. Winter types of rye, such as 'Wheeler,' had higher concentrations of allelochemicals later into the season. 'Wheeler's' ability to retain these high concentrations may be due to its later maturation, although more research is needed to accurately compare the differences between winter and facultative types of rye.

It is likely that the allelopathic components in the cover crop mulch that function in weed growth suppression represent a mixture, rather than a single chemical. Several potential allelochemical compounds have been identified in rye (Zuniga et al., 1983; Shilling et al., 1986; Barnes et al., 1987). Though information of the complex of allelochemical compounds is limited, there is a growing body of information about DIBOA and related chemicals (Friebe, 2001; Gierl and Frey, 2001; Sicker et. al., 2001). The temporal changes in the content of the individual allelochemicals in rye may be different, but our data demonstrate that the total phytotoxic potential of rye extracts declines over time, as does the DIBOA content. The observation that the DIBOA and general allelochemical content of rye decreased over the growing season was verified by additional studies the following year (data not shown). Given the phytotoxicity of DIBOA to multiple weed species (Barnes et al., 1987), the similarities between the quantity of DIBOA found in leaf tissue and the phytotoxicity demonstrated in the bioassay suggest an important role for DIBOA.

Allelochemical content is not the only factor in determining how much weed control will be provided by rye mulch. The quantity of mulch determines how much weed control will be obtained through physical suppression (Teasdale and Mohler, 2000). NC Unspecified is a low biomass producer (Table 3), and this may negate the impact of high DIBOA rates. However, 'Wheeler' maintained high DIBOA levels and produced high biomass, and, thus, may be a better choice for weed management. Crops that can be planted early to mid April will be able to capitalize on the high DIBOA rates provided by winter types of rye.

The weed suppression provided by cover crop mulch is most likely due to both the allelochemical content in the tissue and the total biomass present. Thus, the dramatic changes in the rate of DIBOA in the field (Figure 5) over time and among varieties suggests that the specific variety used, and time at which the cover crop is killed play a critical factor in weed suppression, and a knowledge of the tissue levels of allelochemicals is necessary for optimum weed management.

Further work is needed to elucidate factors that regulate allelochemical content in rye. Opportunities for delaying maturation of rye also need to be explored. Possibilities include later plantings, repeated mowing, plant growth regulators, and breeding for later maturation. The implications of these temporal changes in allelochemical content, and their interaction with different cultivars, have not been fully integrated into cover crop utilization.

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CONTENTS OF SOLUBLE, CELL-WALL-BOUND AND EXUDED PHLOROTANNINS IN THE BROWN ALGA *Fucus vesiculosus*, WITH IMPLICATIONS ON THEIR ECOLOGICAL FUNCTIONS

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Abstract—Phlorotannins are ubiquitous secondary metabolites in brown algae that are phenotypically plastic and suggested to have multiple ecological roles. Traditionally, phlorotannins have been quantified as total soluble phlorotannins. Here, we modify a quantification procedure to measure, for the first time, the amount of cell-wall-bound phlorotannins. We also optimize the quantification of soluble phlorotannins. We use these methods to study the responses of soluble and cell-wall-bound phlorotannin to nutrient enrichment in growing and nongrowing parts of the brown alga *Fucus vesiculosus*. We also examine the effects of nutrient shortage and herbivory on the rate of phlorotannin exudation. Concentrations of cell-wall-bound phlorotannins were much lower than concentrations of soluble phlorotannins; we also found that nutrient treatment over a period of 41 days affected only soluble phlorotannins. Concentrations of each phlorotannin type correlated positively between growing and nongrowing parts of individual seaweeds. However, within nongrowing thalli, soluble and cell-wall-bound phlorotannins were negatively correlated, whereas within growing thalli there was no correlation. Phlorotannins were exuded from the thallus in all treatments. Herbivory increased exudation, while a lack of nutrients had no effect on exudation. Because the amount of cell-wall-bound phlorotannins is much smaller than the amount of soluble phlorotannins, the major function of phlorotannins appears to be a secondary one.

Key Words—Phenolics, phlorotannins, plant defense, *Fucus vesiculosus*, brown alga, cell-wall-bound, exudation.

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INTRODUCTION

Tannins are a subgroup of phenolic compounds that are produced as secondary metabolites by a host of diverse plant species. These water-soluble compounds differ from other phenolics in their ability to bind proteins (Stern et al., 1996; Strack, 1997; Harborne et al., 1999). Tannins can be divided into soluble tannins, occurring in cytoplasm or within cell organelles, and insoluble tannins, bound to the cell wall (Strack et al., 1988; Peng et al., 1991). Phlorotannins, a subgroup of tannins, are produced entirely by polymerization of phloroglucinol (1,3,5-trihydroxybenzene) (Ragan and Glombitza, 1986; Waterman and Mole, 1994; Arnold and Targett, 1998), which is the product of the acetate–malonate pathway, also called the polyketide pathway (Waterman and Mole, 1994).

Phlorotannins are known only from brown algae (Phaeophyceae), where soluble phlorotannins can constitute up to 25% dry weight (e.g., Targett et al., 1992; Van Alstyne et al., 1999). They are suggested to have multiple ecological roles: some phlorotannins act as chemical defenses against herbivory (e.g., Steinberg, 1988; Targett and Arnold, 1998; Arnold and Targett, 2000; Pavia and Toth, 2000a) and as antifouling substances (Sieburth and Conover, 1965; Wikström and Pavia, 2004), although the evidence for this function is equivocal (Jennings and Steinberg, 1997). Concentrations of phlorotannins show phenotypic plasticity in response to environmental parameters, such as salinity, nutrient and light availability, ultraviolet irradiation, and intensity of herbivory (Yates and Peckol, 1993; Peckol et al., 1996; Pavia et al., 1997; Pavia and Toth, 2000b; Honkanen et al., 2002; Swanson and Druehl, 2002). This implies that the pools of soluble phlorotannins are not stable but rather in a state of flux, and their concentration may be determined by the balance between rates of synthesis and turnover (Arnold and Targett, 2000). Existing data on phlorotannins are based solely on soluble phlorotannins, which limits our knowledge of factors controlling their metabolism and degradation.

Soluble phlorotannins are stored in cell organelles, physodes, which are round to elliptical, highly mobile, vesicle-like, strongly refractive bodies observed in the cytoplasm of brown algae (Ragan and Glombitza, 1986; Schoenwaelder, 2002). Phlorotannins are suggested to transform into components of the cell walls of brown algae when physodes fuse with the cell membrane and the phlorotannins are secreted into the cell wall, complexing finally with alginic acid (Arnold and Targett, 2003). The cell walls of brown algae are mainly composed of polysaccharides: alginic acid, alginates (carboxylated polysaccharides, salts of alginic acid), and fucans (sulfated polysaccharides), which comprise up to 40% of the thallus dry weight (Mabeu and Kloareg, 1987; van den Hoeck et al., 1995). Schoenwalder and Clayton (1998) have suggested that phlorotannins are likely to be integral structural components of brown-algal cell walls. There are few quantitative data on cell-wall-bound phenolics or their functional roles (but see Strack et al., 1989; Peng et al., 1991; Viriot et al., 1993). One possible generalization is that phenolic compounds are bound into the cell wall during maturation (Peng et al., 1991).

Resource availability is expected to have an impact on algal growth and secondary metabolism. Resource-based hypotheses assume that under conditions of good nutrient availability, the allocation of carbon will shift from the production of secondary metabolites to growth (e.g., Bryant et al., 1983; Herms and Mattson, 1992). Often, negative correlations have been found between the contents of available nutrients and the production of soluble phenolic compounds, both in terrestrial plants (reviewed by Herms and Mattson, 1992) and brown algae, although much spatial and temporal variation in this relationship exists (e.g., Yates and Peckol, 1993; Steinberg, 1995; Peckol et al., 1996; Pavia and Toth, 2000b). Variation in the contents of soluble phlorotannins has also been found among different parts of algae. In actively growing meristems, soluble phlorotannins have often been found in lower concentrations than in older vegetative parts (Jennings and Steinberg, 1997; Van Alstyne et al., 1999).

In addition to soluble and cell-wall-bound phenolics, brown seaweeds release phlorotannins into the surrounding water directly via exudation (Carlson and Carlson, 1984; Ragan and Glombitza, 1986; Jennings and Steinberg, 1994; Toth and Pavia, 2000; Swanson and Druehl, 2002). Phlorotannins may also be released indirectly as a consequence of grazing or tissue erosion. The occurrence of phlorotannin excretion in healthy, relatively unstressed algal tissues *in situ* has been confirmed in several studies (Sieburth and Jensen, 1969; Ragan and Jensen, 1979; Swanson and Druehl, 2002). However, few data are available on quantities of exuded phlorotannins (but see Toth and Pavia, 2000) or on their ecological function.

In this study, we modified a quantification procedure for cell-wall-bound phlorotannins, optimized the quantification of soluble phlorotannins, and used these procedures to examine the contents of soluble, cell-wall-bound, and exuded phlorotannins in the brown alga *Fucus vesiculosus* (L.). In two manipulative experiments, we explored the environmental variation of these different types of phlorotannins. In the first, we compared the contents of phlorotannins between growing and nongrowing parts of the thallus in order to clarify the consequences of active growth for the dynamics of soluble vs. insoluble phlorotannins. We also tested the effect of nutrient enrichment. In the second experiment, we quantified the amount of exuded phlorotannins, and studied the effects of nutrient shortage and grazing action by the isopod *Idotea baltica* (Pallas) on the amount of exudation.

METHODS AND MATERIALS

Study Organisms

F. vesiculosus is a perennial, brown alga dominating the littoral rocky bottom of our study area in the Archipelago Sea, SW Finland. The thallus grows by dichotomous branching; it consists of both vegetative apices and, during the

reproductive period, generative apices. *F. vesiculosus* has an apical meristem, so growth occurs only at the blade tips. Individuals live for many years, so most of the thallus is nongrowing tissue. In our study area, the alga has only one important herbivore, the isopod *I. baltica*; this is a generalist feeder, but prefers *F. vesiculosus* over other potential host species (Jormalainen et al., 2001).

Experimental Growing Conditions of Algae

Effect of Ambient Nutrients on Soluble and Cell-Wall-Bound Phlorotannins in Growing and Nongrowing Thalli. We conducted the study at the Archipelago Research Institute of the University of Turku (60° 14′ N, 21° 40′ E). On June 10, 1999, we collected 20 *F. vesiculosus* individuals from one population in the Archipelago Sea of Finland and randomly assigned them to 10 microcosms of 60 l, two individuals in each. Microcosms were located outdoors, with natural light and diurnal rhythm, and were protected from rainfall by a thin plastic cover, permeable to UV light; they had continuous seawater through-flow of 200 l/d. Five microcosms were randomly assigned to a nutrient-enrichment treatment, while the others served as controls. The fertilizer [nutrient content of dry mass: N 20% ($NO_2 + NO_3$ 3.7%, NH_4 11.3%), P 2.0%, K 8.0%, Mg 1.0%, S 9.0%, B 0.03%, Fe 0.1% (Kemira, Agro OY)] was applied by automatic feeders, which added 0.03 g of the fertilizer into the pools three times a day. The mean ($\pm SE$, $N = 2$) content of total nitrogen, $NO_2 + NO_3$, NH_4, and PO_4 (μg/l) in the control microcosms was 255 ($\pm$120.1), 6 ($\pm$3.4), 1 ($\pm$2.6), 8 ($\pm$11.8), and mean ($\pm SE$, $N = 5$) in the fertilized ones were 420 ($\pm$90.6), 12 ($\pm$1.8), 7 ($\pm$1.7), 25 ($\pm$8.7), respectively. Samples of water for chemical analysis were collected immediately after collecting the algae for the chemical analyses. We did not conduct statistical contrasts on nutrient concentrations due to low replication of control-tank measurements and, consequently, low statistical power. However, the existence of the treatment effect was confirmed because the algae responded to our manipulation. At the end of the experiment (20 July), we took samples for analyses of soluble and cell-wall-bound phlorotannins from each individual (now numbering 17: 9 individuals from the nutrient-enrichment treatment and 8 from the control), from both the apical parts that had grown during the experiment, and the older parts (nongrowing) of the thallus. Samples were freeze-dried, homogenized, and stored at –20°C until analysis. Contents of soluble and cell-wall-bound phlorotannins were measured as described in subsequent paragraphs.

Effects of Nutrient Shortage and Herbivory on Exudation of Phlorotannins. For this experiment, we collected four *F. vesiculosus* individuals, split each individual into three equal-sized pieces (approximately 40 g fresh weight), and placed them individually into plastic, transparent, 2.5-l aquaria. The aquaria were aerated and placed under greenhouse light (400 W sodium vapor lamps, Sylvania, MA, USA). Pieces of algae were randomized among three different treatments in such

a way that one piece from each individual was assigned to each treatment. The first treatment was a control, to which nutrients were added every second day [$c(NH_3)$ = 120 μg/l, V(added) = 1.5 ml; $c(NO_3)$ = 40 μg/l, V(added) = 1.0 ml; $c(PO_4)$ = 30 μg/l, V(added) = 0.75 ml] to maintain availability of nutrients during the experiment. In the second treatment, no nutrients were added. Because algae in closed aquaria, when light is not limiting, quickly deplete the ambient nutrients, the algae in this treatment faced a nutrient shortage. In the third treatment, five *I. baltica* individuals were placed in each aquarium to graze. As in the control, nutrients were made available by nutrient additions. The experiment was maintained for 14 d, after which the water from each aquarium was filtered (pore 0.45 μm diam.) and stored in dark bottles at 4°C for a few days before a half-liter aliquot was taken from each sample, evaporated to an exact volume of 100 ml, and the amount of exuded phlorotannins measured as described subsequently.

Extraction and Quantification Methods

Optimizing Extraction Efficiency. Freeze dried and ground *F. vesiculosus* powder was extracted with eight different extractants (Figure 1) to examine extraction efficiency of soluble phlorotannins. Three replicates for each extractant were extracted ×4 described as follows. Freeze dried and ground algal aliquots (200 mg dry weight) were weighed and placed into screw-top test tubes (16 mm diam. and length 100 mm). Samples were treated with 10 ml of extractant for 1 hr with continuous shaking, after which they were centrifuged (10 min at 3200g). Supernatants were collected and a fresh extractant aliquot was added. After the fourth extraction, supernatants from each replicate for a specific extractant-type were collected into one container, thus producing only one independent assessment for each extractant type. However, the sample thus obtained was composed of three independently weighed and extracted subsamples prior to pooling of the samples. After volatile solvents were removed in the fume hood, both aqueous extracts and insoluble residues in the extraction tubes were freeze dried. Freeze-dried extracts were dissolved in a known amount of water, their phlorotannin content was analyzed, and the residuals were stored in a freezer; the insoluble residues were also weighed and stored in the freezer. In a separate test, we tested the efficiency of the extraction of phlorotannins by performing consecutive extractions with 70% aqueous acetone and analyzing each extract separately.

Soluble Phlorotannins. Dried and ground aliquots (200 mg dry weight) from the first experiment were extracted ×4 (1 hr) with 10 ml of 70% aqueous acetone. Samples were centrifuged (10 min at 3200g), and the supernatants were combined. Acetone was removed in open air, and the aqueous extracts and insoluble residues in the extraction tubes were freeze dried. Extracts of soluble phlorotannins were dissolved in water (2 × 2.5 ml) and stored in a freezer.

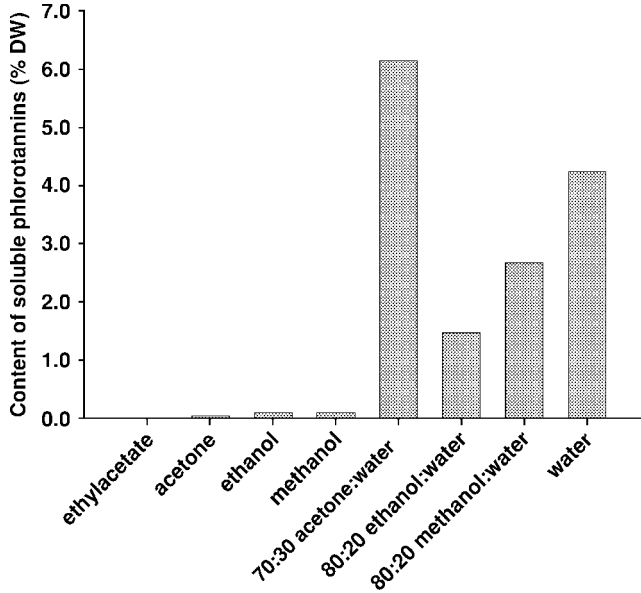

FIG. 1. Acquisition (*as a mean of three replicates*) of soluble phlorotannins with the different extractants. Extractants are ranked by increasing order of polarity according to Hildebrand solubility parameters (Barton, 1983): ethyl acetate (polarity δ (SI) 18.6 $MPa^{1/2}$), ethanol (26.0), methanol (29.6), acetone (20.2), 70% aqueous acetone (28.5), 80% aqueous ethanol (41.1), 80% aqueous methanol (42.4), water (47.9).

In order to remove the remaining soluble phlorotannins from the freeze-dried insoluble residues, they were treated (according to Strack, 1989) with the following solvents (10 ml) for 15 min with stirring followed by centrifugation (5 min at 2400*g*): (1) MeOH; (2–4) H_2O; (5–9) MeOH; (10,11) Me_2CO; (12, 13) Et_2O. After this washing, the remaining insoluble residues (cell-wall fraction) were oven dried at 60°C for 1 hr.

Insoluble Phlorotannins. Phenolic compounds bind with four major types of bonds—hydrophobic, hydrogen, ionic, and covalent—in order of increasing strength (Appel, 1993). Possible linkages between the cell wall (alginic acid) and phlorotannins are the ester bond and the hemiacetal bond, both of which are covalent, irreversible bonds, thus requiring strong conditions to degrade.

Cell-wall-bound phlorotannins were quantified with a modified alkaline-degradation method described by Strack et al. (1989), which was originally developed to degrade phenolics other than phlorotannins. In addition to alkaline degradation, an acidic-degradation method (Peng et al., 1991) was tested: samples were treated with methanol hydrochloric acid (9:1, v/v) at 120°C for 160 min. Preliminary tests showed alkaline degradation to be slightly more effective than

acidic degradation (data not shown); we, therefore, chose to continue with the alkaline-degradation method. To modify the method to suit our purposes, we tested the reaction time, the strength of the sodium hydroxide (NaOH) solution, the number of repeats, and the volume of acid addition.

The total insoluble residue of the alga remaining after the extraction and washing of soluble phlorotannins was suspended in 8 ml of 1 M aqueous NaOH solution (80°C) and stirred for 2.5 hr at room temperature. Samples were centrifuged (5 min at 2400*g*), and 1 ml aliquots were neutralized with 45 μl of H_3PO_4; the remaining NaOH was discarded. The alkaline treatment was repeated $\times 4$, with the aliquots of each treatment analyzed separately.

Spectrophotometric Determination of Phlorotannins. The contents of total phlorotannins in the extracts of soluble and cell-wall-bound phlorotannins were determined according to a modification of the Folin–Ciocalteu method (Waterman and Mole, 1994), using phloroglucinol (ICN Biomedicals Inc.) as a standard agent. Samples were diluted taking into account the measurable range of the spectrophotometer [e.g., a 0.05 ml aliquot of extract of soluble phenolics (samples from the first experiment) was mixed with 4.95 ml water]. A 1.0 ml aliquot of the diluted sample was mixed in a test tube with 1.0 ml of 1 N Folin-Ciocalteu reagent (Merck). The mixture was allowed to stand for 3 min, after which 2.0 ml 20% Na_2CO_3 were added. Samples were incubated in the dark at room temperature for 45 min and centrifuged for 8 min (1600*g*), after which the absorbance of the supernatant was measured at 730 nm on a Unicam Helios ε UV–VIS spectrometer. Total phenolic content is expressed as percent dry weight (soluble and cell-wall-bound phlorotannins) or as percent dry weight per day (exuded phlorotannins).

RESULTS

Method Development

Soluble Phlorotannins. The most efficient extractant was 70% aqueous acetone (Figure 1). Quantitatively, most of the soluble phlorotannins (78% of the total amount in 13 consecutive extracts) were acquired during the first extraction (Figure 2a). After the extraction tubes were allowed to stand in the refrigerator overnight, the amount of phlorotannins extracted (fifth and ninth repeat) was slightly higher than in the preceding extraction. On the basis of this result, we decided to use only four extractions, which could be completed in a single day. These four extractions yield 93.5–95% of the amount of soluble phlorotannins acquired with the 13 consecutive extracts. When analyzing soluble phlorotannins from ecological experiments, we used four extractions.

The amount of soluble phlorotannins from the first study (34 samples, pooled over all data) was 7.99% ($\pm$0.23). After extraction of soluble phlorotannins, before analyzing the insoluble ones, the residue was washed with several extractants in

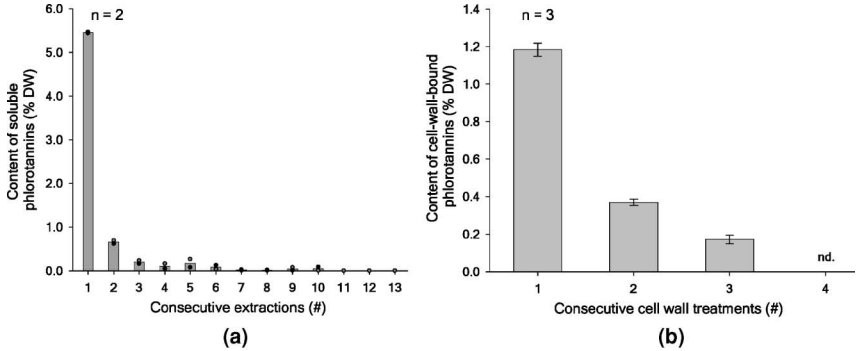

FIG. 2. Acquisition of soluble (values and their mean) (a) and cell-wall-bound (mean ± *SD*) (b) phlorotannins in consecutive extractions. nd. = not detectable.

order to remove all remaining soluble phlorotannins. Extractants were combined and the mean content (±*SE*) of remaining soluble phlorotannins (34 samples, pooled over all data) was 0.36% (±0.03) of dry weight.

Insoluble Phlorotannins. The alkaline degradation of cell-wall-bound phenolics originally included acid addition, i.e., the reduction of pH in the insoluble phenolics liquor aliquot from 13 to 2. The effect of pH on the amount of phlorotannins measured with the Folin–Ciocalteu method was tested with solutions rich in phlorotannins. The effect of this pH range on phlorotannin content is almost linear; the variation between the high and low ends of the range is about 2% in phloroglucinol units (Figure 3). Because soluble phlorotannin extracts were in

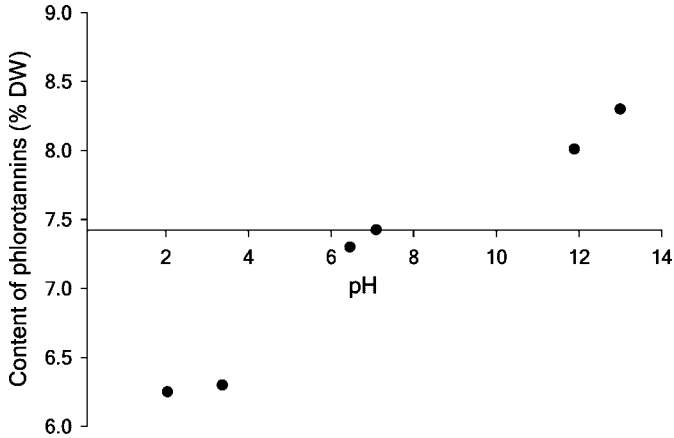

FIG. 3. Effect of pH in the crude extract on quantification of phlorotannins with Folin–Ciocalteu method.

distilled water, i.e., near neutral pH, we added acid to neutralize the solution of phlorotannins that were degraded from the cell wall in alkaline conditions. This allowed a comparison of the quantifications of these two phlorotannin fractions.

The final results were obtained by combining the results of the first three treatments, since in the fourth repeat, they were no longer at a detectable level. Similar to soluble phlorotannins, most of the cell-wall-bound phlorotannins (68%) were released by the first alkaline treatment (Figure 2b).

Experimental Growing Conditions of Algae

Effect of Ambient Nutrients on Soluble and Insoluble Phlorotannins in Growing and Nongrowing Thalli. The amount of phlorotannins in the cytoplasm was significantly higher than in the cell wall (Figure 4, Table 1). The average content of soluble phlorotannins was 8.0 ± 0.2% (mean ± *SE*), while that of insoluble

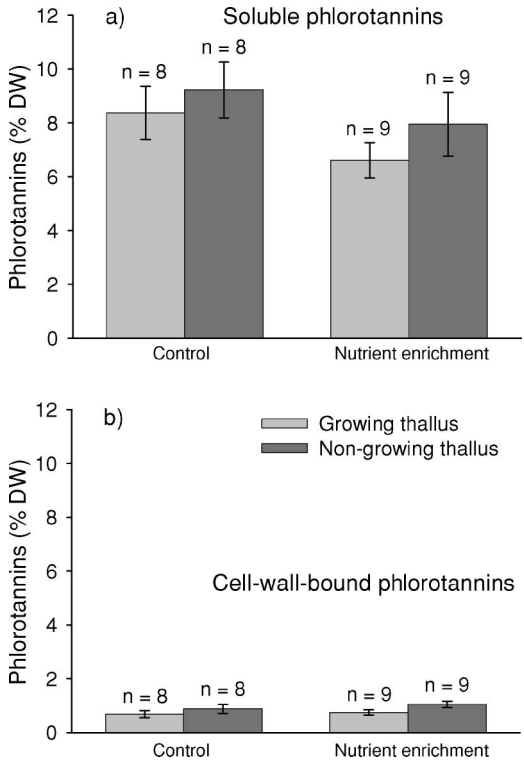

FIG. 4. Mean (±*SE*) content of soluble and cell-wall-bound phlorotannins from growing and nongrowing parts of algae grown in control or nutrient enrichment treatment.

TABLE 1. ANOVA OF REPEATED MEASURES STATISTICS ON THE EFFECTS OF NUTRIENT ENRICHMENT AND THALLUS TYPE (GROWING AND NONGROWING THALLI) ON THE CONCENTRATION OF SOLUBLE AND CELL-WALL-BOUND PHLOROTANNINS IN *F. vesiculosus*

	Soluble phlorotannins			Cell-wall-bound phlorotannins		
	df	*F*	*P*	df	*F*	*P*
Between-*subject effects*						
Nutrient enrichment[a]	1	6.72	<0.05	1	1.87	0.20
Aquaria (nutrient enrichment)	9	2.42	0.15	9	0.97	0.54
Error	6			6		
Within-*subject effects*						
Thallus type	1	17.85	<0.01	1	46.30	<0.001
Thallus type × nutrient enrichment[b]	1	0.70	0.42	1	1.74	0.06
Thallus type × aquaria (nutrient enrichment)	9	1.70	0.27	9	0.37	0.91
Error (thallus type)	6			6		

[a] Aquaria (nutrient enrichment) as an error term.
[b] Thallus type × aquaria (nutrient enrichment) as an error term.

phlorotannins was only 0.84 ± 0.03%. The concentrations of both were lower in growing tissue than in nongrowing tissue (Figure 4, Table 1). Nutrient enrichment reduced the content of soluble phlorotannins in both growing and nongrowing thalli (Figure 4, Table 1). However, nutrient enrichment did not have a significant effect on the content of cell-wall-bound phlorotannins in either type of thallus (Figure 4, Table 1). Algal growth rates, measured in terms of numbers of vegetative apices, did not differ among treatments (final number of vegetative apices in control treatment: 212.0 ± 16.1, $N = 9$; in nutrient enhancement treatment: 200.5 ± 15.3, $N = 10$; ANCOVA, initial number of apices as a covariate, nutrient effect: $F_{1,9} = 0.07$, *ns*).

In nongrowing thalli, there was a negative correlation between soluble and cell-wall-bound phlorotannins (Figure 5a), whereas in growing thalli we found no such correlation (Figure 5b). There was a positive correlation between the contents of soluble and cell-wall-bound phlorotannins in both nongrowing and growing thallus (Figures 5c and d).

Effects of Nutrient Shortage and Herbivory on Exudation of Phlorotannins. Algae growing under control conditions with abundant nutrients exuded phlorotannins into seawater at a rate of 0.49×10^{-3} % dry weight × d^{-1}. The amount of exudation varied in three treatments (Figure 6; one-way ANOVA $F_{2,9} = 10.47$, $P < 0.01$). When algae were grazed by *Idotea*, the amount of exuded phlorotannins was over twice that of the other treatments (Tukey *a posteriori* comparisons with other groups; $P < 0.05$). Phlorotannin exudation was not affected by nutrient shortage (Tukey: NS). Soluble phlorotannins did not differ among the treatments in either growing or nongrowing thalli (mean ±*SE* in control, nutrient shortage

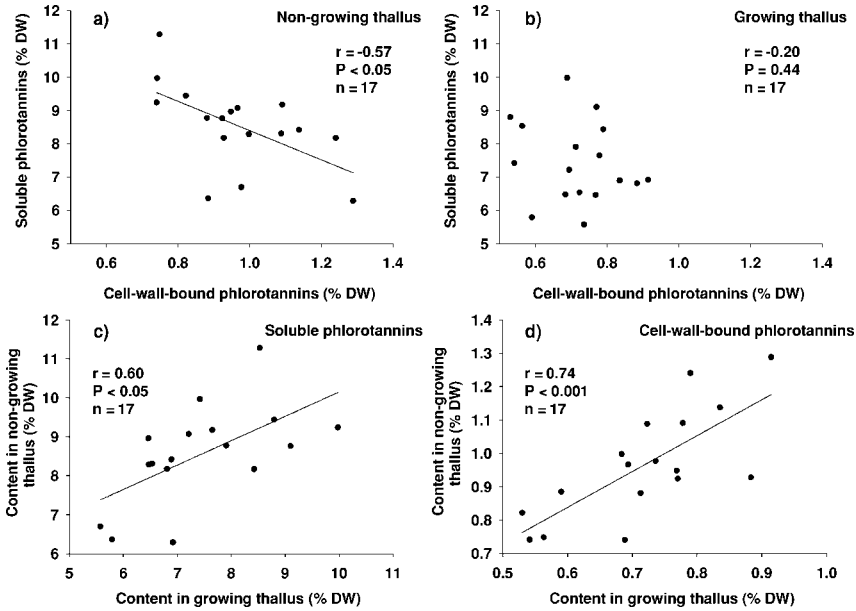

FIG. 5. Correlations between soluble and cell-wall-bound phlorotannins in (a) nongrowing and (b) growing parts of *F. vesiculosus*, and correlations of (c) soluble and (d) cell-wall-bound phlorotannins between nongrowing and growing parts of the thallus.

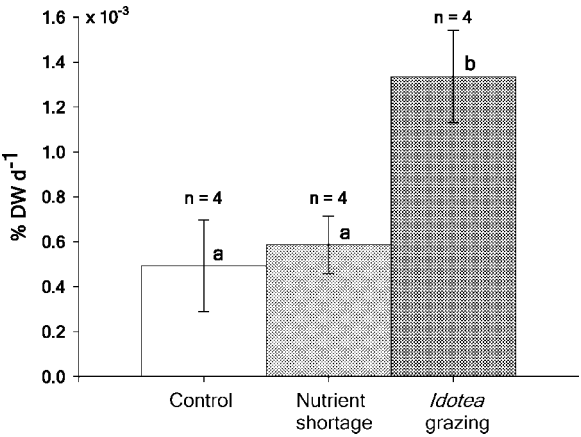

FIG. 6. Amount (mean ± *SE*) of exuded phlorotannins from *F. vesiculosus* in three different treatments.

and grazing treatments, respectively: 8.0 ± 0.53, 7.8 ± 0.61, 7.9 ± 0.29, ANOVA: $F_{2,8} = 0.06$, $P = 0.94$). The difference between nongrowing and growing tissue, on the other hand, was significant (8.5 ± 0.34 and 7.3 ± 0.32, respectively; ANOVA: $F_{1,8} = 8.97$, $P < 0.05$).

DISCUSSION

Alkaline degradation was effective in releasing cell-wall-bound phlorotannins so that they could be quantified; after three consecutive treatments, all detectable phlorotannins were released. Alkaline degradation is also repeatable as judged by the small variance among the three replicates. We cannot, however, exclude the possibility that there may be compounds classifiable as phlorotannins still left in the cell wall due to tight bonding, which were not released by the method. The method used here, however, was more effective than the one of Peng et al. (1991), implying that it is capable of efficiently releasing cell-wall-bound phlorotannins. We also suggest that the washing treatments prior to alkaline degradation minimize the amount of soluble phlorotannins in the fraction of insoluble phlorotannins (see Salminen, 2003).

Both the polarity of the extractant and the solubility of the compounds of interest are crucial for the yield of polyphenols, and extraction of different phenolics from different plant material requires different polarities (Waterman and Mole, 1994; van Beek, 2002). Phenolic compounds are usually most soluble in extractants less polar than water. The general recommendation for extractant selection is a mixture of water and methanol, ethanol, or acetone (Waterman and Mole, 1994).

The extraction efficiency of a solvent generally increased with increasing polarity. An exception to this trend is 70% aqueous acetone—which was the most efficient extractant, better than expected on the basis of its polarity. It was followed in efficiency by water, the most polar extractant among those tested. It may be that acetone increases the total yield by inhibiting interactions between tannin and proteins during extraction (Hagerman, 1988) or even by breaking hydrogen bonds (Porter, 1989). In the analyses of proanthocyanidins, monomers and low-molecular weight compounds can be generally extracted with anhydrous organic solvents, while the addition of water to the extractant increases the solubility of higher molecular weight compounds (Rohr, 2002). We also found a marked difference between extractions with anhydrous organic solvents and solvents containing water. The solvents most commonly used to extract phlorotannins from *F. vesiculosus* have been aqueous mixtures of ethanol and acetone (e.g., Ragan and Glombitza, 1986), and in several studies the efficiencies of different solvents in extracting tannins have been similar to ours (Hagerman, 1988; Keinänen, 1993; Salminen, 2003). The solubility of most phenolic compounds in polar extractant enables for instance the use of LC-ESI-MS, which requires at least semipolar conditions, to analyze polyphenols (Wang and Cole, 1997).

Ecological experiments revealed that in both growing and nongrowing tissue of *F. vesiculosus*, the amount of phlorotannins in soluble form within the cells was greater by approximately an order-of-magnitude than those bound to the cell wall. This is because they are either bound only in very small amounts, or in the process of binding may have been transformed chemically into other compounds that cannot be quantified as phlorotannins. In addition, a part of the phlorotannins passes through the cell wall and is exuded. Although phlorotannins, like other tannins, are terminal products of their synthetic pathway, they can further degrade, for instance, via demethylation, hydration, or oxidation (Strack, 1997). They may bind temporarily to the cell wall, and then undergo turnover or degradation, e.g., into cell-wall building compounds, complexing with alginic acid. Based on the small amount of phlorotannins found in the cell wall, they seem to play a minor role in cell-wall construction as such; if, however, degradation regularly occurs in the cell wall, they may function as important transitional compounds in cell-wall construction, as suggested by Arnold and Targett (2003).

In higher plants, it is suggested that phenolic compounds are esterified or etherified into cell-wall components (Lozovaya et al., 1999), while phlorotannins, due to their structure, may become involved in the cell wall via esterification or a hemiacetal reaction. One suggested function may be in cell-wall hardening (Schoenwaelder and Wiencke, 2000). Thallus toughness may function as a defense against herbivory (reviewed in Lucas et al., 2000), but it may also be part of the necessary adaptation to life in the intertidal littoral zone, where on the one hand wave exposure, and on the other periodical desiccation set demands on the structural strength of the thallus.

The lack of correlation between soluble and cell-wall-bound phlorotannins in growing parts of algae may be an indication of active phlorotannin production. In growing parts, phlorotannins are produced intensely, probably in the cytoplasm or chloroplasts like other phenolic compounds, and subsequently stored in physodes (Schoenwalder, 2002). Although phlorotannins are both exuded and bound to the cell wall, the high rate of production may explain the excess of soluble phlorotannins.

We assume that phlorotannin production does not stop as the thallus gets older. Rather, production decreases because of the thickening of the thallus and the consequent decrease in photosynthesis. A trade-off develops when phlorotannins are bound to the cell wall and soluble phlorotannins are no longer effectively replenished by synthesis. However, it is worth noting that the levels of both soluble and cell-wall-bound phlorotannins remain higher than those in actively growing parts. On the other hand, phlorotannins can differ qualitatively at different stages of age: in young thallus, they may be rather short oligomeric forms. As the thallus ages, they may polymerize into longer and more complex forms, which may be more difficult to degrade or exude through the cell wall. Disappearance of soluble phlorotannins from cytoplasm happens rather fast, which is primarily explained

with excretion and incorporation into the cell wall (Arnold and Targett, 2000). Detailed information, however, awaits the development of a suitable chromatographic method for separating individual phlorotannin oligomers and polymers.

Nutrient enrichment had an effect only on soluble phlorotannins, indicating that phlorotannins bound to the cell wall may be relatively stable with respect to environmental variation. A constant and minor amount of phlorotannins appear to be bound to the cell wall, regardless of the amount produced, which varies with environmental conditions. Nutrient enrichment decreased the amount of soluble phlorotannins. While such a nutrient effect is often explained by an increase in growth at the expense of allocation to the production of soluble phlorotannins, this was not the case in our experiment, since growth rate was not affected. In through-flow mesocosms, nutrient enrichment commonly leads to increased growth of epibiota (Honkanen and Jormalainen, unpublished data; Jormalainen et al., 2003); this, in turn, causes shading and may thereby explain the decrease in phlorotannin production. As such, the dynamic behavior of soluble phlorotannins together with the relative stability of cell-wall-bound phlorotannins supports an approach that uses soluble phlorotannins as the basis for studying phlorotannin plasticity.

There was considerable variability among individuals in the contents of both soluble and cell-wall-bound phlorotannins; some individuals have a concentration only half that in others. Variation in the contents of soluble phlorotannins is to a large extent genetic (Jormalainen et al., 2003), but variation in cell-wall-bound phlorotannins has never been measured. Since soluble and cell-wall-bound phlorotannins were not positively correlated, higher production does not necessarily mean a thicker cell wall. The variation in the contents of cell-wall-bound phlorotannins seems to be due to different causes and it may be related to genetic or phenotypic variation in cell-wall characteristics. When there was a large amount of phlorotannins—soluble or cell-wall-bound—in growing parts, there was also a large amount in nongrowing parts.

In all of our treatments, we found exudation of phlorotannins into the water. The amount of exudation was not affected by nutrient shortage. Since a shortage of nutrients may slow the growth rate and increase the amount of secondary metabolites (Herms and Mattson, 1992) our result suggests that a diminished need of phlorotannins for cell-wall construction would not lead to an increase in their exudation. The amount of exudation may be relatively independent of the growth rate, for instance if exudation also takes place in the old, nongrowing thallus that comprises a major part of the algal biomass. However, exudation did increase compared to the other treatments when the alga was grazed by *Idotea*. There are two possible explanations for the increase. The feeding process may induce an indirect defense in algae, i.e., increased excretion of phlorotannins into the water. By indirect defenses, plants reduce losses to herbivory by facilitating the enemies of herbivores (Karban and Baldwin, 1997). There is evidence from terrestrial plant–herbivore systems for indirect defenses based on volatile plant metabolites

(reviewed in Sabelis et al., 1999; Dicke and van Loon, 2000; Dicke et al., 2003). Alternatively, phlorotannins end up in the water without any induction in the alga, as a consequence of the breaking of the surface, chewing action, or digestion of *Idotea*. In both cases, the consequence of herbivory is an increase in exudation, and it is possible that predators or other plants use exuded phlorotannins as a signal of the presence of herbivores. If the defense were induced, however, we would expect an increase in phlorotannin production, and also in the level of soluble phlorotannins found in the algae. The content of soluble phlorotannins found in both growing and nongrowing parts of the alga, however, showed no change, thus supporting the latter explanation.

In summary, the quantification of phlorotannins from *F. vesiculosus* revealed only a small amount bound to the cell wall, while the bulk was found in soluble form in the cytoplasm. This suggests that the chemical role of phlorotannins is mainly secondary; in other words, there is no evidence for a role as primary metabolites, but they may have adaptive functions in interactions with other organisms or the abiotic environment. Phlorotannins, after degradation, may become part of the cell wall, and thereby have a function as primary metabolites, but at least quantitatively this is minimal. If, however, degradation to other, nonphenolic compounds occurs commonly in binding to the cell wall, this conclusion needs reassessment. Clarification of the biosynthetic pathway of phlorotannins, the enzyme systems involved, and the turnover and degradation processes is needed before our understanding of the functional role of these compounds is complete.

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ROLE OF HOST PLANT VOLATILES IN MATE LOCATION FOR THREE SPECIES OF LONGHORNED BEETLES

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Abstract—Stressed woody plants represent an ephemeral and unpredictable resource for larvae of some species of longhorned beetles (Coleoptera: Cerambycidae) because prime subcortical tissues are rapidly degraded by a guild of xylophagous competitors. Selection favors efficient mechanisms of host and mate location to expedite colonization of hosts by larvae. Based on previous research, we hypothesize that mate location in some species of the subfamily Cerambycinae involves three sequential behavioral stages: (1) both sexes are attracted to larval hosts by plant volatiles; (2) males attract females over shorter distances with pheromones; and (3) males recognize females by contact pheromones in their epicuticular wax layer. We already have evidence of second-stage and third-stage behaviors in three species in this subfamily whose xylophagous larvae feed in hardwood trees: *Xylotrechus colonus*, *Megacyllene caryae*, and *Neoclytus mucronatus mucronatus*. In this report, we evaluate the first behavioral stage of mate location behavior (i.e., independent response of both sexes to host plant volatiles) for the same three species. Supporting our hypothesis, both males and females responded to volatiles emanating from hickory logs in Y-tube olfactometer bioassays.

Key Words—Host location, mating behavior, pheromone, Cerambycidae, *Xylotrechus, Megacyllene*, *Neoclytus*.

INTRODUCTION

A better understanding of host and mate location is critical for developing management strategies of longhorned beetles (Coleoptera: Cerambycidae), among the most important pests of woody plants in natural and managed systems world wide

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(Hanks, 1999). Disparate studies of many species in this family have suggested that the sexes are brought together by a mutual attraction to the larval host rather than by long-range pheromones (reviewed by Hanks, 1999). Recent research on several species in the subfamily Cerambycinae, however, has revealed that males produce pheromones that operate over short or moderate distances (reviewed by Lacey et al., 2004). The pheromones are comprised of single or multiple compounds based on a common structural motif: straight chains of six, eight, or ten carbons with hydroxyl or carbonyl groups at C_2 and C_3.

We have documented the first aggregation pheromone in a longhorned beetle, that of the cerambycine species *Neoclytus acuminatus acuminatus* (F.) (Lacey et al., 2004), and the structure of the single active component conforms to this structural motif. Nevertheless, both sexes of *N. a. acuminatus* also are attracted in the field by volatiles emanating from larval hosts, dying hardwoods (Lacey et al., 2004). Adults of other cerambycine species also respond to plant volatiles (e.g., Hanks et al., 1996; Fettköther et al., 2000), and males use their antennae to recognize females by contact chemoreception of cuticular hydrocarbons (Ginzel and Hanks, 2003; Ginzel et al., 2003). We, therefore, hypothesize that mate location and recognition in cerambycine species involves three sequential behavioral stages: (1) both sexes are independently attracted to larval hosts by plant volatiles; (2) males attract females over shorter distances with pheromones; and (3) males recognize females by contact pheromones.

In this paper, we evaluate the first stage of mate location in three species of cerambycines by testing the hypothesis that adult males and females are independently attracted to volatiles produced by larval hosts. We have evidence of second-stage behaviors in two species: male *Xylotrechus colonus* F. and *Megacyllene caryae* (Gahan) produce compounds consistent with the pheromone structural motif, and we are evaluating their activity (E. S. Lacey, J. G. Millar, L. M. Hanks, unpublished data). We predict that males of the third species, *Neoclytus mucronatus mucronatus* (F.), also produce pheromones because it is a congener of *N. a. acuminatus* and the males display a "perching" behavior that is associated with release of pheromones in that species (Lacey et al., 2004). We have documented the third-stage behaviors for all three species: males use their antennae to recognize females by contact chemoreception (Ginzel and Hanks, 2003; Ginzel et al., 2003).

All three study species are native to eastern North America and their larvae commonly feed in stressed or moribund hickories (Linsley, 1964). Both sexes of all three species congregate on freshly cut logs of hickory in the area of the study (Ginzel et al., 2003; unpub. data). This behavior could be cued by host plant volatiles or entirely by long-range pheromones. Larvae of *X. colonus* develop in many species of hardwoods, and the crepuscular adults are active from May to September (Linsley, 1964; Ginzel et al., 2003). Larval hosts of *M. caryae* include several species of hardwoods, but especially hickory, and adults are diurnal, aposematically-colored wasp mimics that are active from April to June

(Linsley, 1964). Larvae of *N. m. mucronatus* develop in hickories, and the adults are crepuscular and nocturnal and active in June and July (Linsley, 1964).

METHODS AND MATERIALS

We collected adult beetles of all three species from felled shagbark hickories, *Carya glabra* (Mill) Sweet, or reared them from the logs. Adult *X. colonus* were collected at dusk from trees felled in May and June 1998 at Allerton Park, Piatt County, IL. Adult *M. caryae* emerged in March–April 2001 from logs of a tree felled in Athens County, OH, in Spring 2000. Adult *N. m. mucronatus* emerged in May 2003 from logs of a tree felled at Allerton Park in June 2002.

We housed beetles individually in the laboratory in cylindrical cages of aluminum window screen (9 cm diam, 12 cm tall) with clean 9-cm glass Petri dishes at top and bottom. Every 2–3 days we provided fresh 10% sucrose solution in a glass vial into which was inserted a cotton dental roll (Patterson Dental, South Edina, MN). Reared beetles could have mated with a few individuals before they were caged, but the mating history of field-captured beetles was unknown. Beetles used in bioassays had been isolated in cages for at least 24 hr and were active.

We measured the response of walking adult beetles to volatiles from logs of shagbark hickory with a glass Y-tube olfactometer (6 cm diam, main tube 26 cm long, arm length 22 cm, angle between arms 70) positioned on a table with the arms directed toward north-facing windows. Bioassays were conducted under laboratory conditions and natural light during the normal activity periods of the species: late afternoon and evening (*X. colonus* and *N. mucronatus*) or morning (*M. caryae*). To bioassay *X. colonus*, we placed a freshly cut hickory log (~15 cm diam, 30 cm long) in a Plexiglas® box (30 × 30 × 120 cm tall) with the open bottom sealed by standing it in ~10 cm of water. Air entered the box through a hose connector at the top, and air was drawn from the box through another hose connector on the opposite side of the top that was connected with 1 cm i.d. Teflon® tubing to one arm of the Y-tube. An empty box of identical design was connected to the other arm of the Y-tube as a control. To bioassay *M. caryae* and *N. mucronatus*, we placed a hickory log (~8 cm diam, 15 cm long) in a plastic cylinder (10 cm diam, 20 cm long) sealed at one end with aluminum foil with a ~3 cm diam hole to allow air to enter and the other end connected with Teflon® tubing to one arm of the Y-tube. An identical cylinder containing moistened cotton dental rolls served as a control. Air was drawn through these systems (~1.7 l/min) with a 1 hp vacuum cleaner (Shop-vac®, Williamsport, PA) on a variable power supply, and entering air was purified with ~450 g of activated charcoal.

Odor sources were randomized between arms of the olfactometer for each bioassay to control for location effects, and the olfactometer was rinsed with acetone and air dried between trials. A beetle was released in the olfactometer at the downwind end of the main tube and responded by walking at least

TABLE 1. PERCENTAGES OF FEMALE AND MALE LONGHORN BEETLES OF THREE SPECIES THAT RESPONDED IN AN OLFACTOMETER TO VOLATILES EMANATING FROM HICKORY LOGS VERSUS A BLANK CONTROL

Species	Sex	N	# Responding	% Responding		χ^2statistic (P)
				Hickory	Control	
Xylotrechus colonus	Female	36	30	77	23	8.53 ($P < 0.01$)
	Male	34	30	80	20	10.8 ($P < 0.01$)
Megacyllene caryae	Female	36	30	80	20	10.8 ($P < 0.01$)
	Male	40	30	73	27	6.53 ($P < 0.01$)
Neoclytus m. mucronatus	Female	25	25	80	20	9.0 ($P < 0.01$)
	Male	30	30	83	27	13.3 ($P < 0.001$)

16 cm down one of the arms. Beetles that did not enter either arm of the olfactometer within 15 min were recorded as "no response." We tested 25–40 males and females of each species and compared percentages of beetles responding to hickory volatiles with percentages responding to the control with a χ^2 goodness-of-fit test.

RESULTS AND DISCUSSION

Adult beetles of all three species showed a significant response to plant volatiles, with an average of ~80% of each sex responding to volatiles produced by hickory logs (Table 1). These findings support the hypothesis that the sexes are brought together by a mutual attraction to volatiles of the larval host. These data also lend support to our proposed three-stage behavioral sequence of mate location.

Stressed and dying host trees become available to wood borers sporadically and unpredictably when they are damaged or weakened by such environmental factors as wind, lightning strike, fire, and water deficit (reviewed by Hanks, 1999). Their quality as hosts declines rapidly, however, as subcortical tissues are degraded by xylophagous competitors, including buprestid and scolytid beetles, as well as by other cerambycid species. Thus, the quality of larval nutrition depends on the timing of colonization by larvae, and selection favors behaviors in adults that expedite that process, including mutual attraction of males and females to the larval host, and brief copulation followed immediately by oviposition. Adults of other species of the Cerambycinae that require stressed hardwood hosts show similar reproductive behaviors, presumably due to convergent selective pressures (Hanks, 1999).

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BIOMIMETIC MEASUREMENT OF ALLELOCHEMICAL DYNAMICS IN THE RHIZOSPHERE

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Abstract—Polydimethylsiloxane (PDMS) materials were used to quantify levels of the photosynthesis inhibitor sorgoleone in the undisturbed rhizosphere of sorghum plants. The materials used included stir bars coated with PDMS (stir bar sorptive extraction), technical grade optical fiber coated with a thin film of PDMS (matrix-solid phase microextraction), and PDMS tubing. PDMS tubing retained the most sorgoleone. As analyzed by high performance liquid chromatography, amounts of sorgoleone retained on the PDMS materials increased with time. Other materials tested (polyurethane foam plugs, C18 and Tenax disks, and resin capsules) proved less suitable, as they were subject to sometimes extensive penetration by fine root hairs. These results demonstrate the potential for PDMS-based materials to monitor the release of allelochemicals in the undisturbed rhizosphere of allelopathic plants. Unlike extraction procedures that recover all available compounds present in the soil, PDMS functions in a manner more analogous to plant roots in sorbing compounds from soil solution or root exudates. Information on chemical dynamics in the rhizosphere is crucial for evaluating specific hypotheses of allelopathic effects, understanding allelopathic mechanisms, and assessing the importance of allelopathic processes in plant communities.

Key Words—Methods, *Sorghum bicolor x Sorghum sudanense*, sorgoleone, rhizosphere chemistry, polydimethylsiloxane, stir bar sorptive extraction, matrix-SPME, biomimetic extraction, allelopathy.

INTRODUCTION

The knowledge of the chemical basis of allelopathy has advanced greatly since Putnam and Tang's (1986) observation that "chemistry has been the Achilles' heel of allelopathy." Several allelochemicals with toxicity rivaling that of synthetic herbicides are known, including α-terthienyl (Campbell et al., 1982), artemisinin

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(Duke et al., 1987), and sorgoleone (Einhellig and Souza, 1992; Nimbal et al., 1996). α-Terthienyl has been found in the rhizosphere beneath the source plants (Campbell et al., 1982; Tang et al., 1987; Martin and Weidenhamer, 1995; Thijs, 1999), and sorgoleone exudation by sorghum roots has been the subject of numerous investigations (Einhellig and Souza, 1992; Czarnota et al., 2001, 2003).

Unfortunately, analysis of rhizosphere chemistry has lagged behind advances in natural product characterization. The general lack of understanding of the qualitative and quantitative dynamics of allelochemicals in the rhizosphere is perhaps the present Achilles' heel for research in this field (Weidenhamer, 1996). The need for such information has been emphasized repeatedly. Fuerst and Putnam (1983) considered knowledge of the amount of toxin released to the environment and taken up by the target plant crucial to proving a hypothesis of allelopathy. Radosevich and Holt (1984) concurred, arguing "research in this area must be designed specifically to prove that a toxic substance is produced and that it accumulates or persists long enough at concentrations in the environment sufficient to inhibit development of other plants." Williamson and Weidenhamer (1990) went a step further, arguing that knowledge of concentrations alone is not enough because soil concentrations reflect the current balance of input vs. output rates for a compound. Plant roots compete with both microorganisms and other processes to remove allelochemicals from soil solution, and the rate at which allelochemical concentrations are replenished will determine how much is available for plant uptake. Allelochemical flux rates are, thus, likely to be a key component of toxicity.

Blum et al. (1999) assert that the research focus in studies of allelopathy needs to shift to the soil, and "specifically the barrier of the rhizosphere through which allelochemicals must pass." In a recent review of rhizosphere chemistry, Bertin et al. (2003) note that root exudates vary greatly in both chemical composition and biological effects, and argue for the development of techniques that will deepen our understanding of the chemical and biological dynamics of the rhizosphere. The results of Tang et al. (1987) and Weidenhamer and Romeo (2004) underscore these points. Tang et al. (1987) found much higher concentrations of 5-(4-hydroxy-1-butynyl)-2,2-bithienyl (BBT-OH) relative to other thiophenes in the rhizosphere than in roots of *Tagetes patula*, and concluded that analysis of the rhizosphere was more important than analyses of plant tissues for studies of allelopathy. Weidenhamer and Romeo (2004) showed that soil microorganisms rapidly convert the hydroquinone glycoside arbutin to hydroquinone and then to benzoquinone. The latter two compounds are suspected to be the allelopathic agents of the Florida scrub perennial *Polygonella myriophylla*, but they do not occur in free form in the plant itself. The question, then, is what methods can be used to gain insight to the chemical dynamics of the rhizosphere.

Tang and Young (1982) developed a method to analyze compounds produced by plant root systems. Plants are grown in open-bottomed pots in sand, and water and/or nutrient solutions circulate through a trap containing XAD-4

resin. Hydrophobic compounds are eluted from the XAD-4 resin with methanol and identified by standard methods. Tang (1986) pointed out that with typical soil extractions, it is impossible to be certain that all fine roots have been removed from soil beneath potentially allelopathic plants prior to analyses, and, thus, one cannot be sure that the compounds extracted are actually from the soil rather than from root fragments. The Tang and Young method has the virtue of monitoring allelochemicals in root exudates, and has been successfully employed in the investigation of root exudates of several species (Brown et al., 1983; Stevens and Tang, 1986; Tang and Zhang, 1986). Müller et al. (1993) adapted the method to preparative-scale by using a 250 g bed of XAD-4 resin. However, for the quantitative measurement of allelochemical release rates over time, the Tang and Young technique has serious disadvantages. First, the ecological realism of the technique is limited by the requirement to use sand as the growing medium and the abnormally high flow of water through the root zone. Second, and perhaps more importantly, it is not applicable to field situations.

Environmental researchers have devised a number of approaches to attack a similar problem—the qualitative and quantitative measurement of bioavailable concentrations of hydrophobic organic pollutants in surface and ground water and sediment. Several of these methods offer two key advantages over the trapping technique of Tang and Young (1982): simplicity, and potential adaptability to field monitoring.

Materials used in the study of anthropogenic pollutants include adsorbents such as Tenax-TA (Yeom et al., 1996), octadecyl-modified (C18) silica disks (Verhaar et al., 1995; Tang et al., 1999; Krauss and Wilcke, 2001), polyurethane foam (PUF, Farag and El-Shahawi, 1991), and various encapsulated carbonaceous resins (Johns and Skogley, 1994; Morse et al., 2000). The polymer polydimethylsiloxane (PDMS) has also found many uses in environmental analysis. In contrast to *adsorptive* materials in which analytes adsorb to the surface, PDMS is classified as a *sorptive* material in which analytes actually dissolve into the PDMS phase (Baltussen et al., 2002). PDMS is used in a number of forms. In solid phase microextraction (SPME), a fiber coated with a sorptive or adsorptive phase, often PDMS, is used to extract volatile organics from water or headspace. These are then analyzed by gas chromatography (GC) after thermal desorption, or by high performance liquid chromatography (HPLC) after liquid desorption (Arthur and Pawliszyn, 1990). A wide variety of fiber coatings and applications have been developed (Pawliszyn, 1999). However, the fragility of the fiber limits its utility in soil analysis to the sampling of volatiles in pore spaces. A recently developed alternative to SPME with potential applications to rhizosphere analysis is matrix-SPME (Mayer et al., 2000). In this technique, a technical grade optical fiber coated with a thin film of PDMS was used to extract PCBs and other persistent and bioaccumulative pollutants from sediment. The low cost of optical fiber allows for disposable usage, which is not practical with regular SPME because of the

high cost of the individual fibers. Stir bar sorptive extraction (SBSE) is a recently developed method in which a stir bar is coated with a layer of PDMS (Baltussen et al., 1999). After concentration of the analytes on the stir bar, it is desorbed with solvent or thermally. The larger volume of the PDMS phase compared to SPME allows for higher recovery and as much as a 500-fold increase in sensitivity with SBSE (Baltussen et al., 1999). Reported applications include the extraction of water (Baltussen et al., 1999; Popp et al., 2003), direct analysis of fruit by insertion into strawberries (Kreck et al., 2001), and analysis of volatiles emitted by living plants (Vercammen et al., 2000).

The objective of this study was to evaluate the ability of several materials to analyze the undisturbed rhizosphere of living plants. The materials compared were: (a) SBSE (PDMS coated stir bar); (b) matrix-SPME using PDMS coated optical fibers; (c) PDMS tubing; (d) Tenax TA disks; (e) C18 disks; (f) PUF plugs; and (g) capsules containing XAD-7 or Ambersorb 563 resin. Sorghum was chosen as the plant species for this study. The highly phytotoxic sorgoleone is known to be the principal component of the root exudate. Varieties of sorghum that produce greater or lesser quantities of sorgoleone are known (Nimbal et al., 1996). Furthermore, root exudates can be readily analyzed for sorgoleone content by HPLC (Czarnota et al., 2003).

METHODS AND MATERIALS

Chemicals. All chemicals used were reagent grade. Chromatography solvents were HPLC grade.

Sorgoleone Isolation and Purification. The isolation and purification procedure followed that of Rimando et al. (1998). Seeds of *Sorghum bicolor* (L.) Moench cv. Bundle King, a hybrid forage sorghum (Browning Seed Co., Plainview, TX), were sown in a flat containing a sandy loam soil. Seeds were laid on a fiberglass screen and covered with approximately 1 cm soil. After 20 d, the roots beneath the fiberglass screen were cut, washed with water to remove adhering soil, and blotted dry. Roots were extracted twice by covering with methylene chloride (0.25% acetic acid, v/v) for 2 min. The resulting extract was dried with granular sodium sulfate, filtered, and concentrated by rotary evaporation. A crude extract of 41 mg was obtained from 103 g (fresh mass) roots. Sorgoleone was purified by preparative thin layer chromatography on silica gel using hexane/isopropanol (90:10) as the developing solvent. The UV spectrum, TLC, and HPLC profiles of the purified material matched those reported in the literature (Czarnota et al., 2003). This purification procedure does not eliminate several minor analogs of sorgoleone that have been reported (Kagan et al., 2003) but these have low concentrations. Sorghum root exudates contain 85–90% pure sorgoleone by weight (Nimbal et al., 1996).

Sorghum Growth Conditions. Approximately 20 seeds of *Sorghum bicolor x sudanense* cv. High Sugar, an experimental sorghum–sudangrass hybrid (Browning Seed Co., Plainview, TX), were planted in Spinks soil (a sandy, mixed mesic Lamellic Hapludalf) in 3.8 l polyethylene nursery pots. This variety was chosen on the basis of a preliminary screening study of sorghum and sorghum–sudangrass hybrid cultivars in which it produced the greatest quantities of sorgoleone. The pot bottom was covered with fiberglass screen and a 2.5-cm layer of gravel to hold in the soil.

Plants were grown in a greenhouse during January–April 2004. In general, daytime temperatures ranged from 21°C to 30°C and nighttime temperatures from 14°C to 18°C. Temperature extremes were 13°C and 43°C. Pots were laid out in three replicates in a randomized block design. Pots were thinned to a density of four seedlings 11 days after planting (DAP). Plants were watered as needed. Natural sunlight was supplemented by 3 hr of mixed metal halide and sodium vapor lighting beginning 28 DAP. Plants received 300 ml of a general purpose fertilizer solution (Expert gardener®all purpose plant food, 15-30-15 plus B, Cu, Fe, Mn, Mo, and Zn) at 25, 33, and 47 DAP to supplement soil nutrients. Plants were harvested and extractant materials removed and analyzed (see below) on 29, 55, and 88 DAP. Fresh shoot and root biomass of all pots were measured at harvest.

Rhizosphere Adsorbents and Sorbents. Suppliers, conditioning procedures, placement in soil, and extraction methods are summarized in Table 1. A different extractant was buried in each pot, and extractants were removed only at harvest. There were three replicates of each treatment per harvest, and extractants buried in unplanted pots served as background controls. Once prepared, all extracts were transferred to autosampler vials. For extracts of 350 μl volume or less, microvial inserts were used. All samples were analyzed in duplicate.

HPLC Analysis. HPLC procedures followed those of Czarnota et al. (2003). The HPLC system included a Spectra Physics Model 8800 ternary gradient pump, manual Rheodyne injection valve, Econosphere (Alltech Associates) octadecylsilyl (C18) column (250 $\times$ 4 mm^2, 5 μm particle size), and a Dionex variable wavelength detector set at 280 nm. A Varian 4270 integrator was used for peak integration and retention time measurements. Compounds were eluted at 1.75 ml/min with a gradient of (A) water containing 2.5% (v/v) acetic acid and (B) acetonitrile. Gradient conditions were: 0–15 min, 45% A/55% B isocratic; 15–22 min 55–100% B linear gradient, 22–25 min 100% B, 25–26 min 100–55% B, 26–30 min 45% A/55% B equilibration. The injection volume was 25 μl.

Samples from the final harvest were analyzed with the same column on an Agilent diode array HPLC that included a model 1050 quaternary pump, model 1050 autosampler, and model 1100 diode array detector. Samples were analyzed isocratically (45% A/55% B) at a flow rate of 1 ml/min. The monitoring wavelength was 280 nm and injection volume was 50 μl.

TABLE 1. SUMMARY OF CONDITIONING PROCEDURES, PLACEMENT IN SOIL AND EXTRACTION METHODS FOR ADSORBENT AND SORBENT MATERIALS SELECTED FOR THIS STUDY

Material	Supplier	Conditioning	Soil placement	Extraction
Tenax-TA membranes (47 mm)	Alltech associates (Deerfield, IL)	Soaked in excess methanol 5 min (4×), followed by water rinse (4×)	5 cm depth over fiberglass screen	Extracted in 5 ml acetonitrile overnight. Evaporated to dryness and taken up in 500 μl acetonitrile
Empore C18 disks (47 mm)	3 M filtration products (St. Paul, MN)	Rinsed with 2 × 10 ml methanol, 6 × 10 ml water	5 cm depth over fiberglass screen	Same as Tenax-TA
Polyurethane foam plugs, (4.5 cm hr × 3.5 cm d)	Fisher scientific (Fairlawn, NJ)	Successive 24 hr, Soxhlet extraction in acetone and methanol. Stored dry in sealed jar	Centered in pot at 5 cm depth	Same as Tenax-TA, but with 25 ml acetonitrile
Envi-2 capsules, 2 cm diam, containing Ambersorb 563 non-ionic carbonaceous resin, 1100 m^2 surface area	Unibest (Bozeman, MT)	Washed with shaking in methanol, acetonitrile, followed by five successive rinses with water	Centered in pot at 5 cm depth	Same as Tenax-TA, but with 7 ml acetonitrile
Envi-2 capsules, 2 cm diam, containing XAD-7 resin, approx. 1100 m^2 surface area	Unibest (Bozeman, MT)	Washed with shaking in methanol, acetonitrile, followed by five successive rinses with water	Centered in pot at 5 cm depth	Same as Tenax-TA, but with 7 ml acetonitrile
PDMS-coated stir bar (SBSE, Gerstel "Twister," 10 mm)	Gerstel, Inc. (Baltimore, MD)	None for first use	Placed at 5 cm depth over fiberglass screen with Teflon-coated magnetic stir bar	250 μl acetonitrile, 30 min
PDMS tubing (Silastic, 0.30 mm ID × 0.64 mm OD)	Dow corning, Inc. (Midland, MI)	Soaked in acetonitrile 30 min, washed with water	Four 10 cm strands placed at 5 cm depth over fiberglass screen	500 μl acetonitrile, 30 min
PDMS-coated optical fiber (Matrix-SPME, core diamter 200 μm, PDMS coating 15 μm)	Fiberguide industries (Stirling, NJ)	Nylon coating removed by dipping in hot (180°C) propylene glycol. Cut in 10 cm lengths and soaked in acetonitrile 30 min, washed with water	Four fibers inserted into center of pots to depth of 9 cm, leaving 1 cm exposed for later removal	350 μl acetonitrile, 30 min

HPLC-MS Analysis. To confirm the presence of sorgoleone in the rhizosphere extracts, one sample and standard were analyzed by HPLC-Mass Spectrometry (MS). Experiments were performed on a Micromass LC-TOF(tm) II (Micromass, Wythenshawe, UK) mass spectrometer equipped with an orthogonal electrospray source (Z-spray) operated in positive ion mode. Sodium iodide was used for mass calibration for a calibration range of m/z 100–2000. Optimal ESI conditions were: capillary voltage 3000 V, source temperature 110°C, and a cone voltage of 55 V. The ESI gas was nitrogen. All ions transmitted into the pusher region of the TOF analyzer were scanned over m/z (100–500) with a 1 sec integration time. Data were acquired in continuum mode during the LC run.

The HPLC system consisted of a Waters Alliance 2690 Separations Module (Waters, Milford, MA). A Supelco (St. Louis, MO) Discovery C18 HPLC Column ($250 \times 4.6\,\text{mm}^2$, 5 μm particle size) was used for the separation. A 100 ml injection was made of the samples. The mobile phase [45% water containing 2.5% (v/v) acetic acid and 55% acetonitrile] flow rate was maintained at 1 ml/min and was split postcolumn by using a microsplitter valve (Upchurch Scientific, Oak Harbor, WA) to ~20 μl/min for introduction to the ESI source.

RESULTS

Sorgoleone quantitation was linear (r^2 values 0.97–0.99) over a range of 0.1–50 mg/l^{-1} for both peak area vs. concentration and log area vs. log concentration. The approximate detection limit was 0.25 ng per injection (corresponding to a concentration of 0.01 mg/l^{-1} on the HPLC system used for the first two harvests). Chromatograms of both a standard and SBSE sample are presented in Figure 1.

The non-PDMS materials proved to be problematic to work with. The capsules, foam plugs, and disks were all penetrated by sorghum roots to greater or lesser extent. Analyses of these materials are, therefore, suspect, given that any sorgoleone recovered might be from root fragments that could not be removed rather than from sorgoleone adsorbed from the rhizosphere by these extractants. Also, several blanks showed contaminant peaks that overlapped sorgoleone. For these reasons, results for the analysis of the non-PDMS materials are not presented. However, PUF plugs as well as the C18 and Tenax disks all showed reddish-purple stains suggestive of sorgoleone.

All three PDMS-based materials trapped measurable amounts of sorgoleone that increased with time (Table 2, Figure 2). Sorgoleone in the samples was identified on the basis of its retention time compared to the known standard. Where UV spectra of peaks could be obtained (diode array HPLC, final harvest), these were consistent with sorgoleone. LC–MS analysis of a sorgoleone standard and one of the SBSE samples from the first harvest confirmed the presence of sorgoleone (m/z 359) in the rhizosphere extract. The PDMS tubing gave the highest recovery of the three PDMS materials used, and the amounts recovered

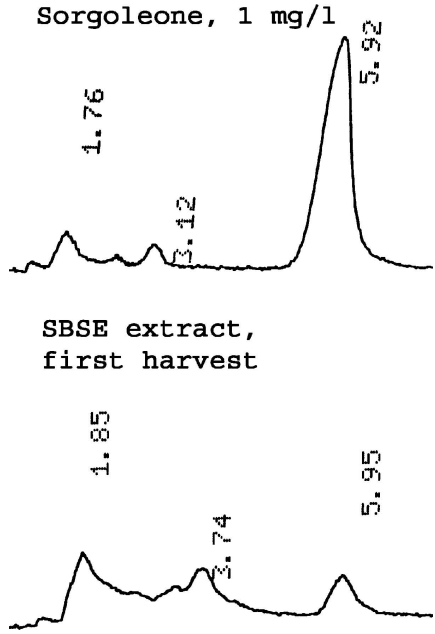

FIG. 1. HPLC chromatograms of sorgoleone standard (1 mg/l^{-1}, injection corresponds to 25 ng sorgoleone) and SBSE rhizosphere extract from the first harvest (determined to have concentration of 0.17 mg/l^{-1}). The peak in this sample was confirmed as sorgoleone by LC–MS.

correlate well with sorghum plant growth over the 88 days of the experiment (Figure 3).

DISCUSSION

These results demonstrate PDMS can be used to sorb and measure root-exuded allelochemicals in the undisturbed rhizosphere of living plants. While this experiment was conducted in pots, all of the PDMS materials used could readily be taken into the field. Both PDMS tubing and optical fiber are fairly inexpensive and, therefore, applicable to large-scale studies.

Of the three PDMS-based materials tested, PDMS tubing recovered higher amounts of sorgoleone and was also more consistent in recovering some sorgoleone in all samples (Table 2). However, because of the different placement of the optical fiber (vertically, rather than being buried) and the different volumes and surface areas of the PDMS materials used in this study, conclusions cannot be drawn about which material was most efficient in extracting sorgoleone. This

TABLE 2. RECOVERY OF SORGOLEONE (ng) from PDMS Materials for all Replicates

Harvest (DAP)	Replicates (ng sorgoleone)			Mean[a]
Optical fiber				
29	68.3	nd[b]	nd	22.7
55	nd	5.4	9.2	7.3
88	261.8	58.7	lost	160.2
Stir bar sorptive extraction				
29	15.5	42.4	nd	19.3
55	35.5	63.4	nd	33.0
88	180.5	16.8	83.8	93.7
PDMS tubing				
29	24.5	17.1	19.5	19.6
55	50.8	26.5	31.0	36.1
88	272.1	198.4	315.5	262.0

[a] Means and standard errors were calculated using values of zero for samples below detection limits.
[b] nd = not detected.

experiment was not designed to compare extraction efficiencies of the different PDMS materials, but rather to demonstrate the feasibility of using PDMS to monitor allelochemical dynamics in the rhizosphere. PDMS tubing had both a larger PDMS phase volume and surface area than the optical fiber and PDMS-coated stir bars. The four 10 cm strands of tubing had a surface area of 812 mm^2, compared to 243 mm^2 for the optical fiber and 69 mm^2 for the stir bar. Approximate PDMS volumes were 241, 55, and 7 μl for the tubing, stir bar, and optical fiber, respectively.

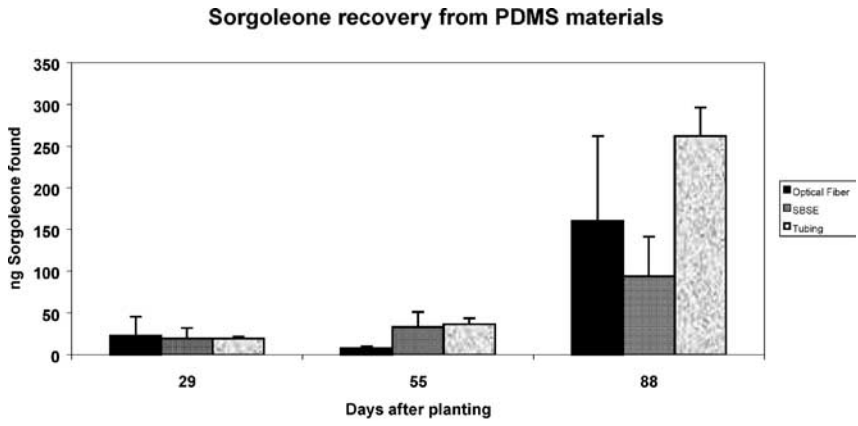

FIG. 2. Mean recovery of sorgoleone with PDMS materials. Bars indicate standard errors.

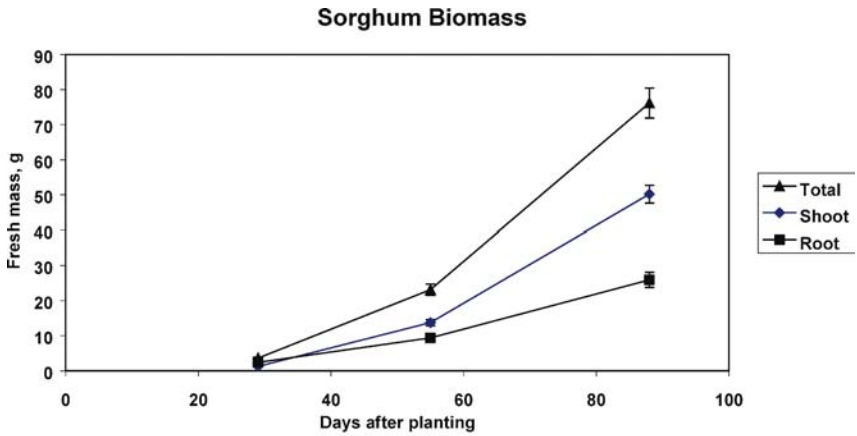

FIG. 3. Total, shoot and root fresh biomass of sorghum–sudangrass at each harvest. Bars indicate standard errors. If not shown, standard errors are too small to depict visually.

The volume estimates have been adjusted for the estimated 40% (v/v) of fumed silica found in PDMS tubing by Baltussen et al. (1999). The greater volume and surface area of the tubing may provide some integration of the presumably heterogeneous distribution of sorgoleone in the rhizosphere.

It is recognized that other forms of the non-PDMS materials—e.g., beads or resins mixed with soil and recovered by centrifugation (Thien and Myers, 1991; Lake et al., 1996; Yeom et al., 1996)—could provide usable results and avoid the problems with fine root penetration of capsules and disks. However, field placement in soil would be problematic, recovery would be destructive, and the extraction procedure would be much more difficult than the aqueous rinse followed by acetonitrile soak used for the PDMS materials. The importance of minimizing disturbance of roots is illustrated by the results of Paavolainen et al. (1998), who measured volatile organic compounds (VOCs) from soil in conifer forests by using microair passive diffusive samplers. These samplers use charcoal as an adsorbent for volatile compounds. The samplers were placed in 30 cm-deep holes (5 cm diam.) bored into the soil, and samplers were suspended within the borehole, which was capped. Significant quantities of monoterpene emissions were measured, but the authors noted that increased production of monoterpenes is a known result of root injury. Thus, there is no way of knowing whether the release rates measured by these researchers bear any relation to undisturbed forest.

Because PDMS rhizosphere sorbents do not extract a known volume of soil, the amounts of sorgoleone found cannot be directly translated into estimates of flux rates. The data provided by these sorbents estimate relative, rather than absolute, flux rates. However, even with this limitation, PDMS sorbents could

be useful in deepening our understanding of the qualitative and quantitative flux rates of allelochemicals in the rhizosphere. Ion-exchange resin capsules, used for the measurement of soil nutrients, have similar limitations and potential. Skogley (1992) buried ion exchange capsules in burned and unburned sites at three different locations. He found striking differences between burned and unburned sites in the amounts of nitrate, potassium, and sulfur recovered from capsules buried for 8–20 months. In addition to monitoring rhizosphere concentrations over time in response to external treatments or variables, PDMS materials could be used to map allelochemical distribution in the rhizosphere (Fan et al., 1992) or to follow microbial degradation of allelochemicals.

Existing methods for determination of allelochemical concentrations in soil have relied on aqueous or solvent extractions of the soil (e.g., Ponder and Tadros, 1985; Dalton et al., 1987) or direct analysis of soil solution (e.g., Gallet and Pellissier, 1997). The low concentrations of allelochemicals typically found in analyses of soils beneath allelopathic plants, and the often rapid degradation of allelochemicals by soil microorganisms, are commonly cited as evidence against these compounds playing a significant role in plant–plant interactions (Schmidt, 1988). However, static concentrations in the environment reflect the balance of input vs. output rates for a compound at a given point in time. As such, static concentrations do not provide information about these input and output rates any more than static concentrations of ammonium and nitrate in the soil indicate the magnitude of the processes of nitrogen mineralization, denitrification, and so on (Williamson and Weidenhamer, 1990). Seldom have attempts been made to quantify the input and output rates for suspected allelopathic compounds.

An additional problem with existing methodology is that bioassays of allelochemicals are normally conducted by one exposure of a test species to a known concentration of a compound, which then decreases in concentration over the length of the assay. This is a quite different situation than in nature, where small pulses of allelochemical inputs may disappear due to microbial degradation, plant uptake, and other processes, but be replenished by new inputs such as root exudation or leaching by precipitation throughfall. Chronic exposure to a low dose of allelochemical may inhibit plant growth significantly (Blum and Rebbeck, 1989), but this has seldom been examined. Weidenhamer et al. (1987) showed that in standard Petri dish germination assays using cucumber seeds and ferulic acid, the ferulic acid rapidly disappeared. When concentrations of ferulic acid were stabilized by either decreasing the number of cucumber seeds used per dish or by making a larger reservoir of ferulic acid available to the seeds, much greater inhibition of cucumber germination and growth resulted. If soil processes maintain the concentration of an allelochemical within a certain range, the potential toxicity of the compound will exceed that seen in bioassays where the concentrations available in soil are mimicked but decrease without replenishment over the course of the assay. Thus, data on the dynamic availability of

allelochemicals are essential to the serious evaluation of hypotheses of allelopathic activity.

The term "biomimetic" is proposed to describe the PDMS rhizosphere sorbents. Krauss and Wilcke (2001) used this term to describe the extraction of PAHs and PCBs from soil with C18 disks. Their intent was not to quantitatively extract all hydrophobic material from the sample, but an amount corresponding to what might be taken up by an organism. Allelochemical extraction procedures seek to recover all of a certain fraction of a compound present at a certain point in time (e.g., soil solution, reversibly/irreversibly adsorbed) (Blum et al., 1999). In contrast, the PDMS materials function in a manner more analogous to plant roots in sorbing compounds from soil solution or through direct contact with root exudates. In principle, allelochemicals sorbed by PDMS traps should be potentially available to roots of other plants. For this reason, the term "biomimetic extraction" is proposed to describe this technique.

Further research is needed to clarify the potential of biomimetic PDMS extractants for analysis of the rhizosphere. Questions that need to be addressed include: (a) How stable are adsorbed allelochemicals on PDMS over time? Do the amounts of sorgoleone found reflect the cumulative amount trapped by PDMS, or is some sorgoleone sorbed and subsequently desorbed over time? Should PDMS be found to desorb allelochemicals over time, such materials might also be used to provide controlled release of known doses of allelochemicals to measure their effects in the absence of the source plant. (b) How broad a range of allelochemicals can be effectively sorbed by PDMS? PDMS is a nonpolar material, and analyte recoveries have been correlated with $K_{o/w}$ (octanol–water partition coefficient) values. Higher recoveries are found for nonpolar compounds (Baltussen et al., 1999). However, SBSE has been used successfully to quantify the polar benzoic acid in beverages (Tredoux et al., 2000). Quantitation was linear for concentrations of 1–1000 ppm in both water and diluted soft drinks. Thus, PDMS would seem to have potential to measure both nonpolar and polar allelochemicals in the rhizosphere. (c) Do other forms of PDMS, e.g., membranes (Bruheim et al., 2003) or rods (Montero et al., 2004) have potential advantages for rhizosphere extraction? What proportion of PDMS volume to surface area is optimal? (d) Can relative flux rates of rhizosphere allelochemicals be correlated with the results of soil bioassays? Szmigielska et al. (1998) and Szmigielska and Schonenau (1999) applied such an approach to the analysis of herbicide residues. The utility of PDMS soil probes for the evaluation of hypotheses of allelopathic effects would be enhanced if the results obtained by soil analyses with the probes could be correlated to bioassays of soil activity, and (e) How effective is solvent extraction of PDMS materials compared to thermal desorption and analysis by GC? Solvent desorption is advantageous in that it does not require additional instrumentation beyond liquid or gas chromatographs that are available in many laboratories. However, in solvent desorption, only a portion of the desorbed sample is injected, so the

sensitivity is inherently less than thermal desorption, where the PDMS material is heated in a closed chamber and all of the volatized analytes transferred into the GC. Most applications of PDMS as a sorbent for anthropogenic pollutants use thermal desorption because of the greater sensitivity, although some compounds are unstable during the thermal desorption process or at high temperatures in the GC.

The demonstration that fluxes of allelochemicals can be measured in the rhizosphere will not prove that allelopathic interactions are occurring. Yet, it is also true that demonstration of allelopathic interactions without data on allelochemical dynamics in soil will remain problematic. PDMS-based materials provide a new tool for obtaining this information, and helping to assess the importance of allelopathic processes in plant communities. In principle, PDMS should also be an effective, low-cost sorbent for large-scale collections of rhizosphere exudates.

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INVOLVEMENT OF REACTIVE OXYGEN SPECIES GENERATED FROM MELANIN SYNTHESIS PATHWAY IN PHYTOTOXICTY OF L-DOPA

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Abstract—L-DOPA is an active allelochemical that inhibits plant growth. To determine whether the phytotoxicity is due to the reactive oxygen species generated during its oxidation to melanin, oxidative damage, melanin accumulation, and the effect of antioxidants on its phytotoxicity were examined in L-DOPA-tolerant (barnyard grass) and -susceptible (lettuce) plants, and in suspension-cultured carrot cells. L-DOPA suppressed root elongation in lettuce compared to barnyard grass. Levels of melanin and thiobarbituric acid reactive substances (TBARS) increased remarkably in L-DOPA-treated lettuce roots, but not in barnyard grass. L-DOPA also suppressed carrot cell growth to 60% of the control at 1 mM. Melanin content in 1 mM L-DOPA-treated carrot cells increased continuously; however, ascorbic acid and α-tocopherol suppressed accumulation. When melanin formation was inhibited by ascorbic acid and α-tocopherol, growth of L-DOPA-treated cells was restored. TBARS levels were higher in 1 mM L-DOPA-treated carrot cells than in untreated control cells 2 d after treatment, but not at 4 or 6 d. Ascorbic acid and α-tocopherol suppressed the production of lipid peroxide during the initial 2 d. These results suggest that the phytotoxicity of L-DOPA is due to oxidative stress caused by reactive oxygen species from the melanin synthesis pathway.

Key Words—L-DOPA, inhibition of root elongation, melanin synthesis pathway, lipid peroxidation, reactive oxygen species.

INTRODUCTION

L-DOPA (L-3, 4-dihydroxyphenylalanine), a precursor for alkaloids, phenylpropanoids, flavonoids, lignin, and melanin (Halbrock and Scheel, 1989) is an active allelochemical. Velvet bean (*Mucuna pruriens*), a leguminous species, exudes a

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large amount of L-DOPA from its root and inhibits the growth of nearby species (Fujii et al., 1991). Previous studies have revealed that L-DOPA suppresses the growth of roots more significantly than shoots, and the inhibitory effect is selective among plant species (Fujii, 1994; Fujii et al., 1991; Nakajima et al., 1999; Hachinohe et al., 2004).

In the past few decades, natural toxins, including allelochemicals that suppress or eliminate competing plant species near the source plant, have received special attention due to their agricultural potential as herbicides (Dayan et al., 2000). Although hundreds of allelochemicals have been identified, the mode of action of many of these is not yet clear. This study focuses on the phytotoxic mechanism of L-DOPA, which is synthesized via oxidation of tyrosine in the presence of the copper-containing enzyme tyrosinase and molecular O_2. In plant cells, L-DOPA is metabolized to several chatecholamines, phenylpropanoids, and melanin (Pattison et al., 2002). Nakajima et al. (1999) reported that cucumber plants detoxify L-DOPA to nontoxic amino acids such as phenylalanine or tyrosine. We previously compared the metabolic activity of L-DOPA in barnyard grass (*Echinochloa crus-galli* L.: tolerant) and lettuce (*Lactuca sativa* L. cv. Great lakes 366: susceptible) (Hachinohe et al., 2004). There was no significant difference in the composition of metabolites between the species; however, L-DOPA accumulated solely in lettuce suggesting that it is an active principle of the phytotoxic action.

There are numerous studies on the effect of L-DOPA in animal cells. It is a potential therapeutic agent for Parkinson's disease; however, there is considerable concern about the long-term effects caused by L-DOPA-induced cell apotosis and cytotoxicity (Basma et al., 1995; Lai and Yu, 1997; Melamed et al., 1998; Haque et al., 2003). L-DOPA can be oxidized non-enzymatically to form melanin (Riley, 1997); a process that generates reactive oxygen species (Kruk et al., 1999). The cytotoxicity of L-DOPA in nerve cells is due to oxidative damage from reactive oxygen species and can be suppressed with antioxidative enzymes or antioxidants in many cases.

This study was conducted to determine whether the phytotoxicity of L-DOPA is due to reactive oxygen species formed during oxidation to melanin. Oxidative damage and the effect of antioxidants on phytotoxicity were examined in L-DOPA-tolerant (barnyard grass) and -susceptible (lettuce) plants, and in suspension-cultured carrot cells.

METHODS AND MATERIALS

Plant Materials. Seeds of barnyard grass and lettuce were sown on aluminum mesh trays covered with cheesecloth and set on plastic boxes ($310 \times 220 \times 5$ mm^3) containing distilled water. They were germinated in a growth chamber

under 25°C/20°C (day/night, 12 hr each, 80–100 μmol m^{-2} s^{-1}). The uniformly germinated seeds, 1-d-old lettuce and 4-d-old barnyard grass were used for the experiments.

Effect on Root Elongation. Ten germinated seeds of barnyardgrass and lettuce were placed in a plastic box (60 × 60 × 100 mm^3) containing 200 ml of a 0.5% agar culture medium (pH 6.0) with L-DOPA (0.1 mM). They were kept in the chamber, and their root lengths were measured at 1, 2, 3, and 5 d. Each experiment was conducted using five boxes, and the experiment was repeated three times.

Cell Culture and Effect on Plant Growth. Suspension cultures of carrot (*Daucus carota* L. cv. Harumakigosun) cells were grown in Murashige and Skoog medium (MS medium), pH 6.0, containing 30 g L^{-1} of sucrose and 2 mg L^{-1} of 2,4-dichlorophenoxyacetic acid (Matusmoto et al., 2002). Cultures were maintained on a gyratory shaker (NR-20; TATITEC Corporation, Saitama, Japan) agitated at 100 rpm under dim light at 25°C, and subcultured at 2-wk intervals by transferring 20 ml of cell culture into 100 ml of fresh medium. Cell cultures were grown with medium containing L-DOPA with/without ascorbic acid or α-tocopherol for 6 or 8 d. The effect on growth was determined by measuring packed cell volume (Warabi et al., 2001) at 2, 4, and 6 d after treatment.

Spectrophotometric Assay of Melanin. Total melanin was determined according to Wakamatsu and Ito (2002). Roots of lettuce and barnyard grass (30 mg FW) were homogenized in 3 ml of a 90% (v/v) Soluene-350 solution, and solubilized completely by incubation for 30 min at 100°C. After cooling, absorbance at 500 nm was determined by using synthetic melanin as a standard. In carrot cells, the yellow pigments were removed with methanol before the cells, 0.2 ml, were homogenated in 3 ml of Soluene-350.

Determination of Lipid Peroxidation. Lipid peroxidation was determined with the thiobarbituric acid (TBA) test (Velikova et al., 2000). Roots of lettuce and barnyard grass (500 mg) were homogenized in a 0.1% (w/v) TCA solution (5 ml). The homogenate was centrifuged at 10,000 *g* for 20 min, and 0.5 ml of the supernatant were added to 1 ml of 0.5% (w/v) TBA in a 20% TCA solution. The mixture was incubated for 30 min at 98°C, and the reaction was stopped by cooling in an ice bath. The samples were centrifuged at 10,000 *g* for 5 min, and the absorbance of supernatants was analyzed at 532 nm. The value for nonspecific absorbance at 600 nm was subtracted. The amount of thiobarbituric acid reactive substances (TBARS) was calculated with an extinction coefficient of 155 mM^{-1} cm^{-1}.

Statistical Analysis. Data were analyzed with Statcel (OMS, Saitama, Japan), and tested for normality and homogeneity of variances (F-test or Bartlett test) followed by ANOVA or simple linear regression analysis. Differences between treatments were determined by Fisher's PLSD test, Student's t-test, or Welch's t-test.

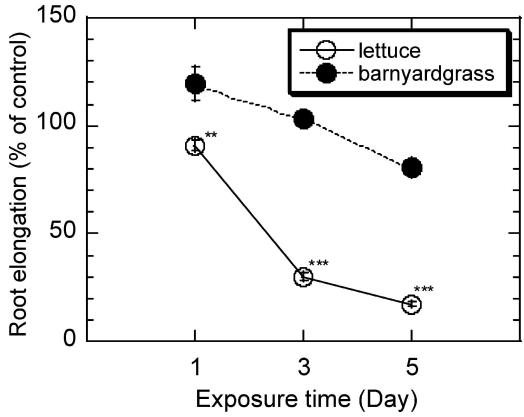

FIG. 1. Effect of 0.1 mM L-DOPA on growth of barnyard grass and lettuce roots. Bars indicate ±SE of the mean ($N = 5$). Asterisks indicate a significant preference between control and L-DOPA treatment ($^{**}P < 0.01$; $^{***}P < 0.001$).

RESULTS

L-DOPA caused selective suppression of root elongation in barnyardgrass and lettuce (Figure 1). The root lengths of both L-DOPA-treated species were approximately 80% and 20% of untreated control root lengths, respectively.

Although L-DOPA induced accumulation of melanin in the roots of both species, the increase was more remarkable in lettuce (Figure 2). Melanin content was positively correlated with growth inhibition of L-DOPA-treated roots

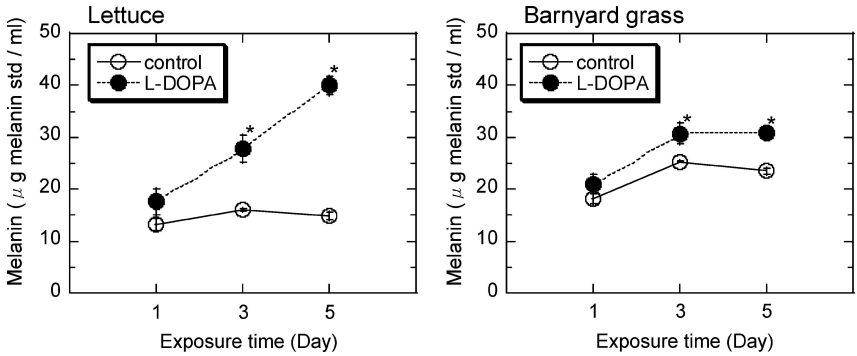

FIG. 2. Melanin levels in roots of barnyard grass and lettuce treated with L-DOPA. Bars indicate ±SE of the mean ($N = 3$). Asterisks indicate a significant preference between control and L-DOPA treatment ($^{*}P < 0.05$; $^{**}P < 0.01$).

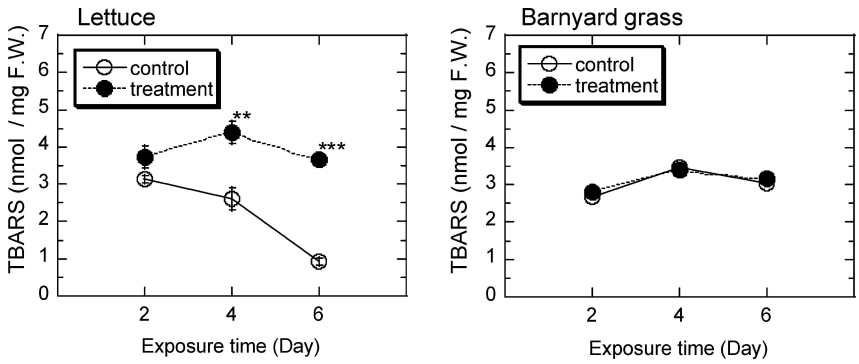

FIG. 3. Effects of L-DOPA on TBARS levels in roots of barnyard grass and lettuce. Bars indicate ±SE of the mean ($N = 3$). Asterisks indicate a significant preference between control and L-DOPA treatment (**$P < 0.01$; ***$P < 0.001$).

($r^2 = 0.85$, $F_{1,28} = 152.8$, $P < 0.001$). Levels of TBARS were higher in L-DOPA-treated lettuce roots, but not in barnyard grass (Figure 3). Lipid peroxidation also correlated with growth inhibition ($r^2 = 0.67$, $F_{1,10} = 20.4$, $P < 0.05$) in the regression analysis.

Toxicity of L-DOPA in nerve cells is caused by the reactive oxygen species, H_2O_2, $^1O_2$, O_2^-, and $OH^\bullet$, generated from its oxidative metabolism to melanin (Graham et al., 1978; Rosenberg, 1988; Kruk et al., 1999; Pattison et al., 2002). Cytotoxicity was restored with the addition of antioxidative enzymes or antioxidants (Parsons, 1985; Basma et al., 1995; Lai and Yu, 1997; Haque et al., 2003). To investigate the involvement of this oxidative process in L-DOPA's action, melanin content and TBARS content were determined in carrot cells cultured with MS medium. Suspension-cultured carrot cells were used for the experiments because it is easy to regulate concentrations of chemicals in the medium.

At 6 d after treatment, L-DOPA suppressed cell growth to 85% and 60% of the control level at 0.1 mM and 1 mM, respectively (Figure 4). The melanin content increased continuously in 1 mM L-DOPA-treated cells for 6 d (Figure 4). The amount of melanin accumulated in cells depended on the concentration of L-DOPA (data not shown). Ascorbic acid and α-tocopherol suppressed melanin formation in L-DOPA-treated carrot cells (Figure 5). When melanin production was inhibited by ascorbic acid and α-tocopherol, growth of L-DOPA-treated cells was restored (Figure 6). TBARS levels were higher in 1 mM L-DOPA-treated carrot cells than untreated control cells 2 d after treatment, but not at 4 and 6 d (Figure 7). Ascorbic acid and α-tocopherol suppressed this initial increase in the formation of the lipid peroxide.

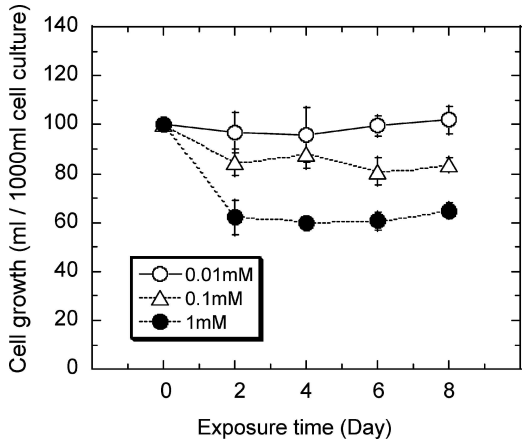

FIG. 4. Effect of L-DOPA on growth of carrot cells. Bars indicate ±SE of the mean ($N = 3$). A one-way ANOVA was performed ($F_{11,24} = 7.22$, $P < 0.001$).

DISCUSSION

L-DOPA is a strong neurotoxic agent owing to its ability to induce apotosis (Melamed et al., 1998; Haque et al., 2003) and adverse effects on neurological degenerative diseases in animals (Enochs et al., 1994; Bowling and Beal, 1995;

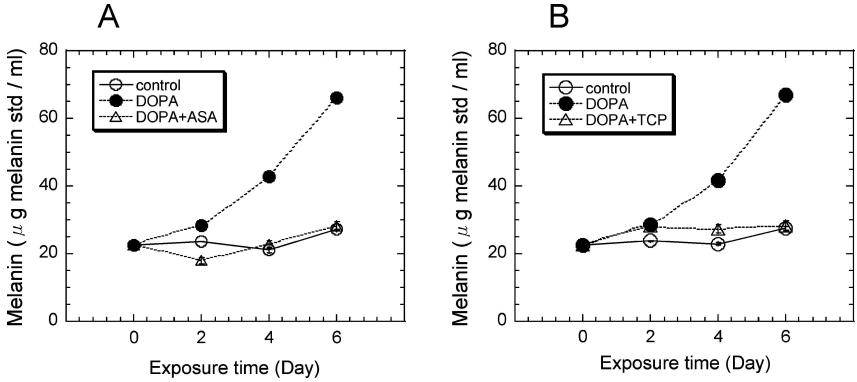

FIG. 5. Effect of ascorbic acid (A) and α-tocopherol (B) on melanin levels in carrot cells treated with L-DOPA. Concentrations of L-DOPA and antioxidants are 1 mM. Bars indicate ±SE of the mean ($N = 3$). A one-way ANOVA was performed: ascorbic acid at 4 d ($F_{2,6} = 2476.7$, $P < 0.001$); ascorbic acid at 6 d ($F_{2,6} = 980.2$, $P < 0.001$); α-tocopherol at 4 d ($F_{2,6} = 1221.2$, $P < 0.001$); α-tocopherol at 6 d ($F_{2,6} = 1223.3$, $P < 0.001$).

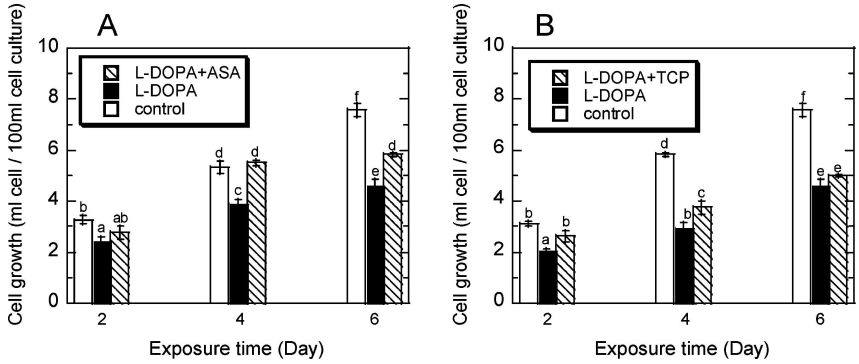

FIG. 6. Effect of ascorbic acid (A) and α-tocopherol (B) on growth of carrot cells treated with L-DOPA. Concentrations of L-DOPA and antioxidants are 1 mM. Bars indicate $\pm$SE of the mean ($N = 3$). A one-way ANOVA was performed: ascorbic acid ($F_{8,18} = 67.72$, $P < 0.001$); α-tocopherol ($F_{8,18} = 86.56$, $P < 0.001$). Bars labeled with same letters are not significantly different ($P = 0.05$).

Smythies, 1997). Several studies have proposed that the cytotoxicity is attributable to reactive oxygen species generated during its oxidation to melanin. The purpose of this study was to examine whether L-DOPA acts on plant cells as it does on animal cells.

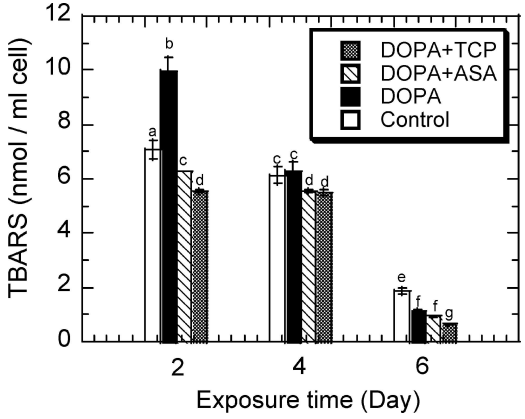

FIG. 7. Effect of antioxidants on TBARS levels in carrot cells treated with L-DOPA. Bars indicate $\pm$SE of the mean ($N = 3$). A one-way ANOVA was performed: ($F_{11,24} = 8376.87$, $P < 0.001$). Bars labeled with same letters are not significantly different ($P = 0.05$).

Based on our previous study, barnyard grass and lettuce were selected as L-DOPA-tolerant and -susceptible species, respectively (Hachinohe et al., 2004). First, we confirmed the difference in sensitivity between them (Figure 1). A greater melanin accumulation was observed in L-DOPA-treated lettuce (Figure 2). Our results indicate that the melanin synthesis pathway in plants metabolizes exogenously applied L-DOPA. From a regression analysis, the amount of accumulated melanin correlated with the growth inhibitory activity of L-DOPA. Therefore, the melanin synthesis pathway might be closely linked to the L-DOPA phytotoxic mechanism. We checked the effect of exogenously applied melanin on lettuce root. Elongation was not inhibited (92.9% of the untreated control) when plants were grown with 60 μg ml^{-1} melanin containing agar medium formed by polyphenol oxidase-catalyzed oxidation of medium containing 0.1 mM L-DOPA. Moreover, melanin itself has not been reported to be a cytotoxic substance, but is a free radical sink that protects cells from oxidative damage (Peters and Schraermeyer, 2001). Some intermediate(s) or by-product(s) of the melanin synthesis pathway may be involved in the phytotoxicity.

In neuroblastoma SH-SY5Y cells, Lai and Yu (1997) showed a correlation between L-DOPA cytotoxicity and formation of melanin. The cells suffered oxidative damage from reactive oxygen species that disrupted cell membrane integrity. This ultimately resulted in a reduction in growth or cell death. To estimate the oxidative damage due to L-DOPA in plants, amounts of lipid peroxides were determined. Lipid peroxides levels were much greater compared with the untreated lettuce control, but not in barnyard grass (Figure 3). This suggests the involvement of reactive oxygen species and a mechanism similar to cytotoxicity in animal cells.

Cytotoxic effects in animal cells can be completely prevented by antioxidants or antioxidative enzymes, particularly catalase, superoxide dismutase (SOD), ascorbic acid, and α-tocopherol (Parsons, 1985; Basma et al., 1995; Haque et al., 2003). We confirmed that the phytotoxicity of L-DOPA in carrot cells was stopped with ascorbic acid and α-tocopherol. However, the antioxidative enzymes catalase and SOD had less of a protective effect (data not shown). Macromolecules such as catalase and SOD may not easily penetrate cell membranes (Buckley et al., 1987; Beckman et al., 1988; Clement et al., 2002).

Growth of carrot cells was suppressed by L-DOPA in a concentration-dependent manner (Figure 4), although the cells were more tolerant than lettuce. A significant accumulation of melanin was observed in carrot cells (Figure 5), indicating that the melanin pathway functions in the metabolism of L-DOPA. Ascorbic acid and α-tocopherol suppressed the formation of melanin (Figure 5). Levels of TBARS in carrot cells decreased at 4 and 6 d in the untreated control (Figure 7). This is probably due to increased cell division in the new medium. In L-DOPA-treated cells, great peroxidation was observed at 2 d, but not at 4 or 6 d. When the synthesis of melanin was inhibited by the antioxidant chemicals, the levels of TBARS decreased at 2 d. These results suggest that the melanin

pathway is involved in oxidative damage, and that lipid peroxidation occurs in the early phase of the culture. Rapid recovery of carrot cells from lipid peroxidation might be due to greater levels of carotenoids, anitioxidants, and/or the rapid metabolism of L-DOPA to nontoxic metabolites other than melanin. Although melanin formation and lipid peroxidation were suppressed during the exposure period, the effect of α-tocopherol on cell growth was not remarkable compared to ascorbic acid. The reason for this difference is not clear, but, both antioxidants effectively reduced the phytotoxicity of L-DOPA in the early stages of exposure (Figure 6).

This study suggests that the phytotoxicity of L-DOPA is due to oxidative stress, and that reactive oxygen species generated by the synthesis of melanin are involved. This is the first report suggesting such involvement in the allelopathic activity of L-DOPA. Our future goals are to clarify the mechanisms behind the production of reactive oxygen species in the melanin synthesis pathway, and the selectivity difference between barnyard grass and lettuce.

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ISOLATION AND CHARACTERIZATION OF ALLELOPATHIC VOLATILES FROM MUGWORT (*Artemisia vulgaris*)

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Abstract—Several volatile allelochemicals were identified and characterized from fresh leaf tissue of three distinct populations of the invasive perennial weed, mugwort (*Artemisia vulgaris*). A unique bioassay was used to demonstrate the release of volatile allelochemicals from leaf tissues. Leaf volatiles were trapped and analyzed via gas chromatography coupled with mass spectrometry. Some of the components identified were terpenes, including camphor, eucalyptol, α-pinene, and β-pinene. Those commercially available were tested individually to determine their phytotoxicity. Concentrations of detectable volatiles differed in both absolute and relative proportions among the mugwort populations. The three mugwort populations consisted of a taller, highly branched population (ITH-1); a shorter, lesser-branched population (ITH-2) (both grown from rhizome fragments from managed landscapes); and a population grown from seed with lobed leaves (VT). Considerable interspecific variation existed in leaf morphology and leaf surface chemistry. Bioassays revealed that none of the individual monoterpenes could account for the observed phytotoxicity imparted by total leaf volatiles, suggesting a synergistic effect or activity of a component not tested. Despite inability to detect a single dominant phytotoxic compound, decreases in total terpene concentration with increase in leaf age correlated with decreases in phytotoxicity. The presence of bioactive terpenoids in leaf surface chemistry of younger mugwort tissue suggests a potential role for terpenoids in mugwort establishment and proliferation in introduced habitats.

Key Words—*Artemisia vulgaris*, mugwort, allelopathy, monoterpenes, volatiles, invasive weed, volatile bioassay, glands.

INTRODUCTION

Mugwort (*Artemisia vulgaris* L.) is a rhizomatous perennial weed that commonly infests roadsides, waste areas, and landscapes. The US nursery industry considers

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mugwort as one of the ten worst weed problems impacting nursery production (Henderson and Weller, 1985; Holm et al., 1997). Mugwort is a Eurasian dicot that spreads quickly upon introduction via an extensive rhizome system, and is difficult, to control chemically or culturally (Bing, 1983; Henderson and Weller, 1985; Foy, 2001; Neal and Adkins, 2001; Barney and DiTommaso, 2003). With few effective control strategies, this aggressive weed has rapidly colonized new areas in the eastern United States. Mugwort is most troublesome, often forming dense monospecific stands, along roadsides, in turfgrass and rights-of-way, and is a lesser threat in agronomic settings (Barney and DiTommaso, 2003). Not surprisingly, diversity of native flora, namely early successional species, in these habitats has declined following mugwort colonization (Holm et al., 1997; Barney and DiTommaso, 2003). However, the exact mechanism(s) of interference (e.g., allelopathy and/or competition) is unknown.

Allelopathy has been reported in an increasing number of plant species in recent years, several of which are classified as invasive (Qasem and Foy, 2001; Bais et al., 2003; Hierro and Callaway, 2003). The invasive ability of certain vigorous and often nonnative plants was thought to be associated with greater competitive ability of the invasive species, or a release from natural enemies (Keene and Crawley, 2002; Mitchell and Power, 2003). Recently, allelopathic activity of invasive species also has been reported as a significant factor that negatively influences species biodiversity and ecosystem succession, while enhancing nonnative species establishment and proliferation (Ridenour and Callaway, 2001; Hierro and Callaway, 2003).

The presence of volatile allelochemicals in aromatic shrubs was first established in the early 1960s in the semiarid chaparral regions of California (Muller et al., 1964; Muller, 1965). Characteristic volatiles or essential oils of species in *Artemisia* and many other taxa have since been explored for their inhibitory effects on plant growth in both field and laboratory assays (Halligan, 1975; del Amo and Anaya, 1978; Kim and Kil, 1989; Abraham et al., 2000). Major inhibitory components of the California chaparral shrubs are terpenes. Several monoterpenes inhibit seedling root and shoot growth (Penuelas et al., 1996), with specific cytotoxic effects that include the reduction of intracellular mitochondrial and Golgi body populations, inhibition of respiration and photosynthesis, decreasing cell wall permeability, as well as accelerating the oxidative destruction of chloroplast pigments (Charlwood and Charlwood, 1991; Loreto et al., 1996; Abraham et al., 2000). The antimalarial drug artemisinin, a sesquiterpenoid from annual wormwood (*A. annua* L.), is inhibitory to several broadleaf weeds and crops, but its mode of action is unknown (Duke et al., 1987; Lydon et al., 1997). Other *Artemisia* species with terpenoid allelochemics include *A. absinthium* L. (Funke, 1943), *A. californica* Less. (Muller et al., 1964; Muller, 1966), *A. princeps* var. *orientalis* (Yun and Kil, 1992), and *A. tridentata* Nutt. ssp. *vaseyana* (McCahon et al., 1973; Weaver and Klarich, 1976; Weaver and Klarich, 1977). Most *Artemisia* species

produce predominately monoterpenes, which suggests that the mugwort might as well. Previous studies have suggested that mugwort has allelopathic potential, but the source of its inhibitory chemicals is largely unknown (Hale, 1982; Melkania et al., 1982; Inderjit and Foy, 1999). Inderjit and Foy (1999) suggested that decomposing mugwort foliage and rhizomes were highly suppressive to red clover (*Trifolium pratense* L.) seedling growth.

More than 80 compounds have been isolated from the foliage of mugwort populations around the world, many of these being terpenes (Misra and Singh, 1986; Banthorpe and Brown, 1989; Milhau et al., 1997; Pino et al., 1999). To date, none of these has been examined for suppressive potential. Therefore, the objectives of this research were to evaluate the allelopathic potential of several populations of mugwort, determine which terpenes are implicated in allelopathic activity, and establish the potential for soil activity of these volatiles.

METHODS AND MATERIALS

Plant Material. Two separate mugwort populations were collected from two geographically isolated areas in Ithaca, NY; specifically from a cemetery site (labeled ITH-1) and the Cornell Test Gardens (labeled ITH-2). Both populations were managed in a turfgrass setting that received regular mowing to a height of approximately 6 cm, and no additional irrigation or fertilization for at least 5 yr previous to this study. Plant material was collected in August of 2000, and subsequently maintained in a greenhouse at 30°C day/24°C night, under high-pressure sodium lighting (Sylvania LV 400/EC) with a 12/12 L/D photoperiod and an average of 300 μmol m^{-2} s^{-1} of overhead irradiance. Both biotypes were transplanted and potted in a Cornell 1:2:1 media mix (soil:peat:perlite) from 10 cm rhizome pieces and watered as needed with no fertilizer applied throughout the experiment. An additional biotype was obtained from seed, and is referred to as VT (Vermont, USA) for the location of the seed company from which it was purchased. On average, the ITH-1 population was taller (>1 m), exhibited densely pubescent stems and light green, and deeply dissected leaves; ITH-2 was shorter (0.75–1 m) with hairless stems and dark green, and deeply dissected leaves; and VT had a prostrate-like growth habit (>1 m in length) with moderate pubescent stems and light green-lobed leaves.

Species evaluated in the bioassays included curly cress (*Lepidium sativum* L.), foxtail millet (*Setaria italica* (L.) Beauv.), large crabgrass (*Digitaria sanguinalis* (L.) Scop.), white clover (*T. repens* L.), and mugwort (Le Jardin seed company, VT).

Chemical Standards. Terpenoids identified from mugwort foliage via gas chromatography coupled with mass spectrometry (GC-MS) that were commercially available were purchased from Sigma-Aldrich, including camphene,

camphor, eucalyptol, D-limonene, β-myrcene, α-pinene, and β-pinene. However, not all of the terpenes identified were available.

Allelopathic Potential of Mugwort Foliage: Volatile Bioassay. Freshly harvested leaves were evaluated for bioactive volatile activity in a manner that allowed only atmospheric contact between the test species and the mugwort foliage. Foliage was harvested from the three populations, ITH-1, ITH-2, and VT, at 60 days after planting (DAP). At harvest, stock plants were nearly 0.75 m tall, and were trimmed to the soil surface. All leaves were stripped from shoots, including small petioles. Sampled foliage was randomly mixed, to obtain leaves of various maturities. Foliage (2, 5, or 10 g f.w.) was placed into a single layer of Grade 50 cheese cloth. Ten seeds of the bioassay indicator species, curly cress, were placed onto a single layer of Whatman #1 filter paper moistened with 2.5 ml Milli-Q water in a 500 ml Erlenmeyer flask. To maximize volatile release, mugwort foliage was hand-crushed in cheese cloth before placement into the flask, which was immediately sealed with a rubber stopper. The negative control contained only cheese cloth and the receptor species with no mugwort foliage, while the positive control contained the receptor species plus 10 g tall fescue shoots (*Festuca arundinacea* Schreb.), in an effort to simulate other commonly released plant volatiles. Sealed flasks were incubated at 27°C for 72 hr in the dark, after which test species root and shoot lengths were recorded. All experiments contained three replicates and were arranged in a completely randomized design, repeated once in time. Data were pooled after homogeneity analysis, and analyzed using a two-sample t-test for comparison to the control.

To examine the effect of foliage age on phytotoxicity, the experiment was repeated with 60 and 120 DAP foliage collected from the VT population, as this mugwort population exhibited the greatest range in test species response in the previous experiment. Flasks were arranged in a completely randomized design, with three replicates of 60 and 120 DAP foliage using 2, 5, or 10 g fresh leaf biomass, in addition to a control (no mugwort biomass). Ten g of fresh mugwort foliage is the equivalent of approximately 15 mature leaves, which represents one plant, 0.3 m in height. Cress radicle elongation was recorded after 72 hr. The experiment was repeated once in time and data were analyzed as above.

Identification of Volatile Compounds. GC-MS was utilized to identify and quantify volatiles collected from the atmosphere surrounding mugwort foliage. One leaf from the top, middle, and bottom of the shoot of each population was selected at 60 and 120 DAP. After being crushed by hand to maximize the release of volatiles, three leaves from each plant were placed together in a 5 ml conical vial with a Teflon septum. The VT population was not available for examination at 60 DAP.

After 30 min of equilibration, a hypodermic needle was used to withdraw 10 μl of headspace from each vial, which were then injected into an Hewlett Packard 6890 GC equipped with a 30 m $\times$ 250 μm $\times$ 0.25 μm HP-5MS (5% phenyl

methyl siloxane) column and an Hewlett Packard 5973 Mass Selective Detector. The oven temperature gradient used was as follows: 35°C (1 min); 200°C at 15°C/min for 10 min; and 300°C at 40°C/min for 5 min. The quadrapole and source settings were 150°C and 230°C, respectively. Two replicates were analyzed for each sample, and peak values were averaged. Individual peaks were identified by comparison of their mass spectrum with published spectra, as well as by comparisons with both the retention times and mass spectra of authentic samples when available.

Response factors for the seven terpenes readily available as standards (camphene, camphor, eucalyptol, D-limonene, β-myrcene, α-pinene, and β-pinene) were determined using standard curves formulated with concentrations of 0.1, 1, 10, 100, and 1,000 mg/l in chloroform. Concentrations of Santolina triene, the only other identified compound in the headspace that was not available as a standard, were estimated using an average of the response factors of the other seven standards.

Phytotoxicity of Identified Terpene Components. Individual terpenes identified above, except Santolina triene, were tested for inhibitory potential, utilizing the volatile bioassay described above. Test tubes (13×100 mm^2) containing pure terpenes dissolved in methanol (30 μl aliquots) were placed inside closed 500 ml Erlenmeyer flasks, which contained the seeds of test species sown on moist filter paper. Activity of each terpene was examined at 0, 1, 10, 50, 100, 250, 500, and 1,000 mg/m^3, plus a methanol control, with a curly cress indicator. In addition, mugwort, white clover, large crabgrass, and foxtail millet indicators were examined at 1,600 mg/m^3 (chosen to exaggerate toxicity effects). Flasks were arranged as a completely randomized design with three replicates, and were incubated for 72 hr at 27°C in the dark, after which root and shoot lengths were recorded. Tests were conducted on multiple days, with two terpenes evaluated per analysis plus a water and methanol control. Control means were significantly different among days as shown by ANOVA analysis. Therefore, data was not pooled. Data for each day were analyzed using ANOVA and means were compared using Dunnetts tests for comparison to the methanol control (SAS ver. 8).

Test for Soil Activity. In order to determine if the mugwort volatiles could impart a residual effect in soils, an assay was developed to examine the germination and establishment of test species in soil that had previously been exposed to mugwort volatiles. ITH-1, ITH-2, and VT greenhouse grown plant material was harvested at 60 DAP, and the foliage was removed, including petioles. Harvested tissue within each population was mixed for uniformity, and 20 g of the foliage was used for each treatment, after hand-crushing. A fine sandy loam soil was dried for 96 hr at 90°C, and subsequently sifted through a 2-mm soil sieve. In a sealed 11.4-l plastic box, 75 g of the soil were placed into a separate 0.71-l plastic dish, adjacent to, but not touching 20 g of the hand-crushed mugwort foliage. An aquarium pump was included inside the box to aid in circulation of the volatiles. The chamber

equilibrated for 24 hr at room temperature before the soil was removed. The soil box was removed and subsequently wetted with 35 ml Milli-Q water, and sown with 25 curly cress seeds. The control consisted of the soil inside the sealed chamber in the absence of mugwort foliage. The soil box was then sealed and incubated for 72 hr at 27°C in the dark, after which time test species root and shoot lengths were recorded. Boxes were arranged in a completely randomized design with three replicates. The experiment was repeated once in time and data were pooled, with means analyzed using a two-sample t-test for comparison to the control.

RESULTS

Phytotoxicity of Mugwort Foliage via Volatile Bioassay. Curly cress radicle elongation is an excellent indicator of plant growth inhibition due to its uniformity of growth and overall sensitivity, and was used as the assay of choice for bioassay-directed identification of unknown volatiles. Of the three mugwort populations evaluated, ITH-1 and ITH-2 significantly inhibited cress radicle elongation at 2 g f.w. whereas the VT population showed minor cress stimulation (Figure 1). Inhibition ($P < 0.05$) of both curly cress root and shoot elongation was observed with 10 g fresh foliage of ITH-1, ITH-2, and VT populations. Only VT, the seed

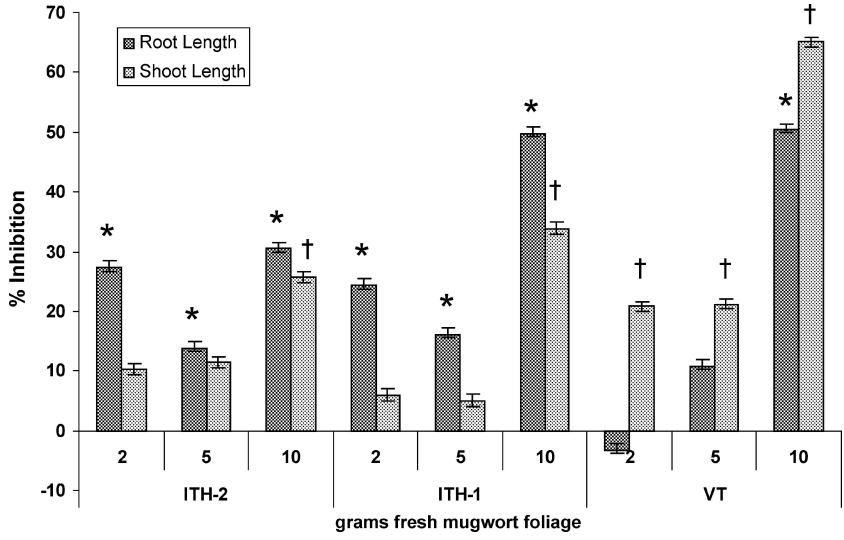

FIG. 1. Percent inhibition of root and shoot elongation of curly cress (*L. sativum*) compared with controls following 72-hr exposure to different amounts of fresh mugwort foliage. *Indicates significant radicle length inhibition ($P < 0.05$) and † indicates significant shoot inhibition compared to the control.

grown population, produced significant shoot growth inhibition of cress at 2, 5, and 10 g f.w. of foliage. Greater than 50% root growth inhibition and 65% shoot growth inhibition were observed with 10 g VT foliage, the most suppressive of all populations evaluated at this tissue concentration. Our findings show that population-level differences exist with respect to mugwort's potential phytotoxicity. Interestingly, percent inhibition of curly cress radicle elongation did not appear to follow a typical dose response relationship. Greatest tissue weight (10 g), however, did result in the greatest inhibition of both radicle and shoot length (Figure 1).

Harvest date, or plant age, also had an impact on phytotoxicity of foliar tissue (Figure 2). Foliar tissue (VT) was evaluated at 60 or 120 d after planting (DAP). The 60 DAP foliage significantly inhibited root elongation at 5 and 10 g, and shoot elongation at 2, 5, and 10 g. Conversely, the 120 DAP foliage significantly reduced cress shoot elongation at 10 g only, while cress radicle length was unaffected.

Test for Soil Activity. To determine if residual phytotoxicity was present in soils previously exposed to mugwort leaf volatiles, a soil bioassay was performed in an enclosed environment. Even after removal of the mugwort leaves, seeds that were germinated in soil that had 24 hr of prior exposure to leaf volatiles were inhibited with respect to root and shoot elongation (Figure 6). Root growth was inhibited up to 27%, whereas shoot growth was inhibited up to 50%. Differences

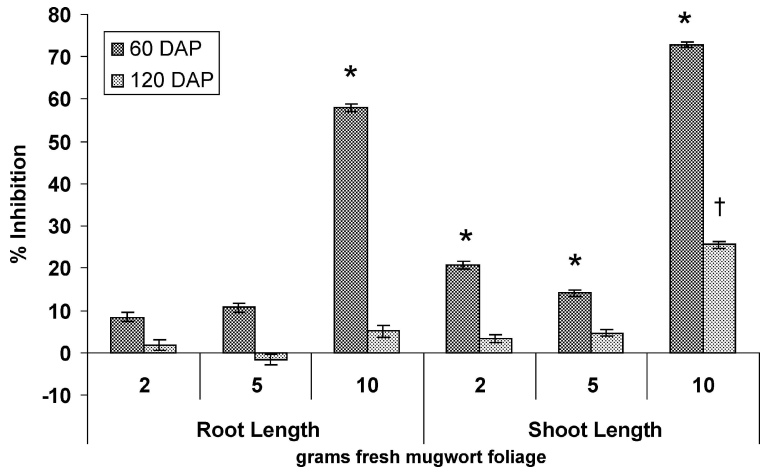

FIG. 2. Percent inhibition of root and shoot elongation of curly cress (*L. sativum*) compared with controls following 72-hr exposure to different ages (60 and 120 d after planting) and weights of fresh mugwort foliage from the VT population. *Indicates significant radicle length inhibition ($P < 0.05$) and † indicates significant shoot inhibition as compared to the control.

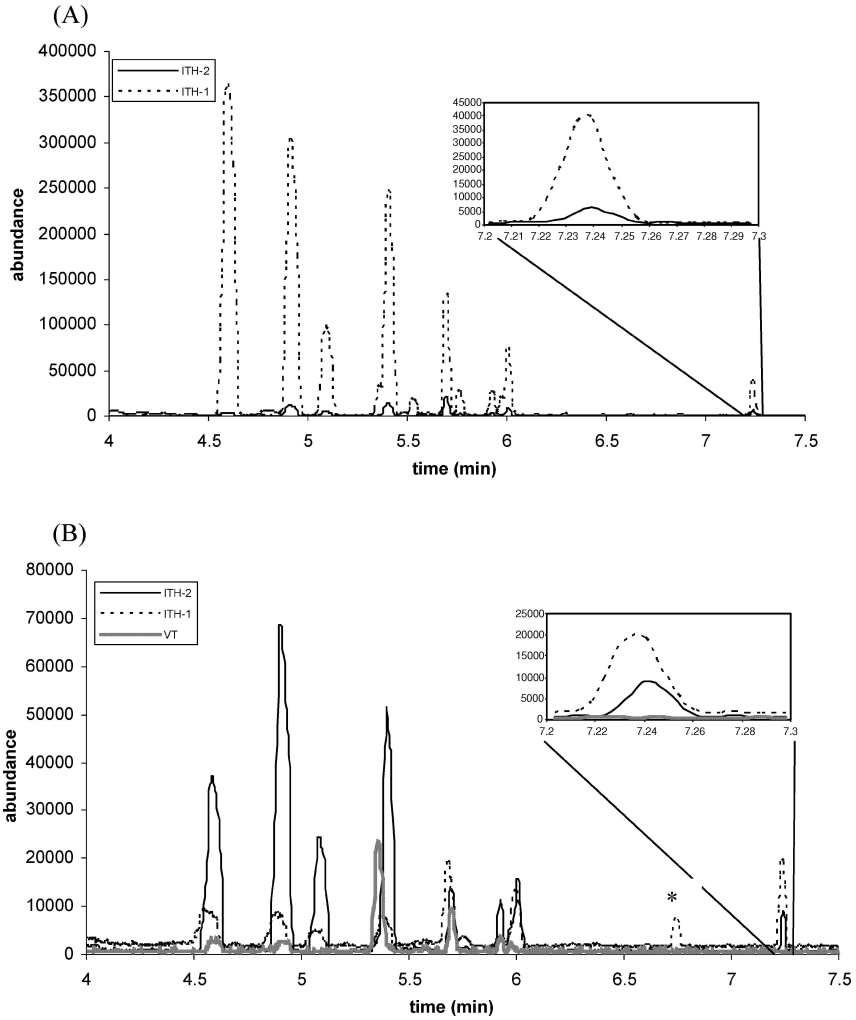

FIG. 3. GC-MS analysis of (A) 60-d-old and (B) 120-d-old plant material. Peaks labeled with "*" did not match any known compounds in the database.

among populations were observed with respect to shoot growth inhibition, with ITH-2 foliage causing greater phytotoxicity than ITH-1 and VT foliage.

Identification of Volatile Compounds. The components of the volatile atmosphere were collected and analyzed via GC-MS. Of the 10 identified peaks (Figure 3), 8 were monoterpenes, including Santolina triene, α-pinene, camphene, β-pinene, β-myrcene, limonene, eucalyptol (1,8-cineole), and camphor (Figure 4).

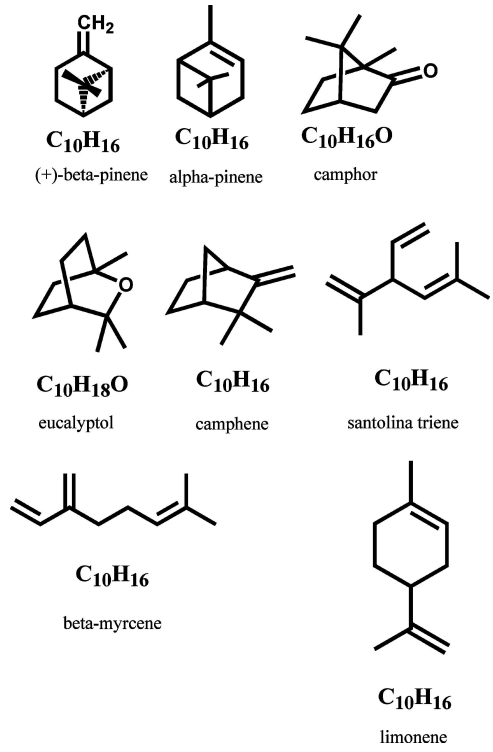

FIG. 4. Terpene structures identified in mugwort foliage.

The 60-d-old ITH-1 foliage contained between 1- and 28-fold higher concentrations of each of the compounds in the leaf headspace compared to older 120-d-old foliage (Table 1). ITH-2 60 DAP foliage contained between 1- and 12-fold greater concentrations of the identified compounds than did the 120 DAP foliage. The major component of ITH-1 foliage at 60 DAP was α-pinene (119.5 mg/m^3), with the second most abundant component being Santolina triene (86.9 mg/m^3). At 120 DAP, the most abundant volatiles were α-pinene and camphene (18.7 and 17.2 mg/m^3, respectively), followed by camphor (13.5 mg/m^3). Volatiles produced by the ITH-2 population included α-pinene (43.5 mg/m^3), followed by camphene (22.1 mg/m^3) at 60 DAP. At 120 DAP, the ITH-2 population contained α-pinene and camphene (19.8 and 17.0 mg/m^3, respectively) followed by camphor (12.0 mg/m^3). At 120 DAP, the VT foliage exhibited slightly different concentrations of volatiles than did ITH-1 and ITH-2 (Table 1). VT foliage at 120 DAP contained α-pinene (16.3 mg/m^3), followed by camphene (15.9 mg/m^3), while camphor and β-pinene were found at concentrations near 10 mg/m^3.

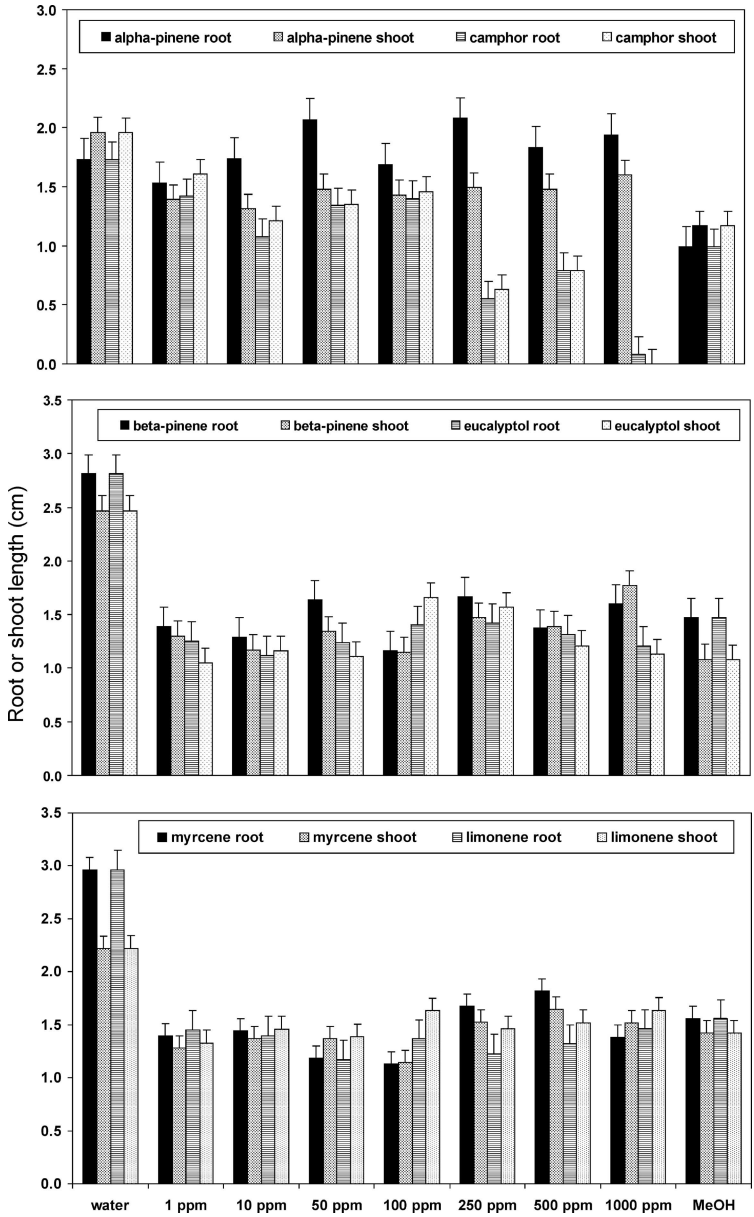

FIG. 5. Response of curly cress (*L. sativum*), to various terpenes identified from mugwort foliage as compared to a methanol and water control, as measured by radicle or shoot elongation. See Table 2 for significance values.

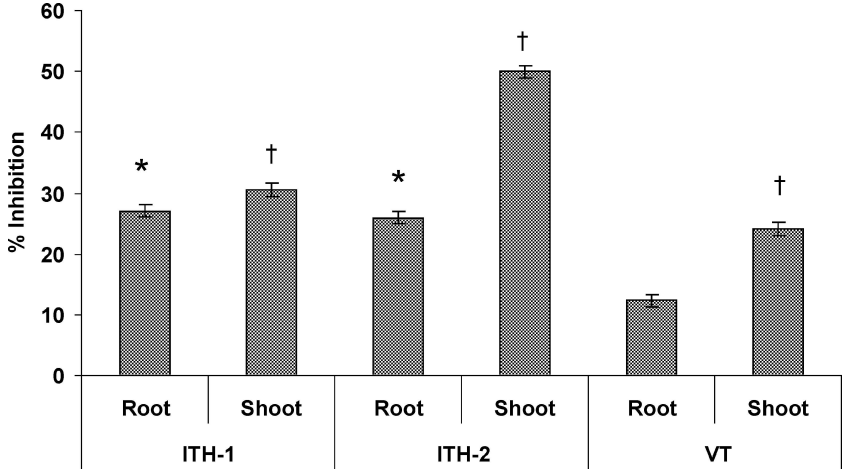

FIG. 6. Percent inhibition of curly cress (*L. sativum*) root and shoot elongation compared with controls after indirect exposure of soil to fresh mugwort foliage of different populations. *Indicates significant radicle length inhibition ($P < 0.05$) and † indicates significant shoot inhibition as compared to the control.

TABLE 1. INFLUENCE OF MUGWORT POPULATION AND TISSUE AGE, 60 AND 120 DAP, ON RELATIVE AMOUNTS OF TERPENES (MG/M^3) AS MEASURED BY GC/MS, AND ESTIMATED LOG Pk_{oc} AND WATER SOLUBILITIES OF COMPONENTS

	Retention times (min)	Log pk_{oc}^a	Water solubility[a] (mg/l)	60 DAP[b]		120 DAP		
				ITH-1	ITH-2	ITH-1	ITH-2	VT
Santolina triene	4.59	3.06	1.99	86.9[c]	10.3[c]	3.1[c]	0.82[c]	0.63[c]
α-Pinene	4.91	3.08	1.89	119.5	43.5	18.7	19.8	16.3
Camphene	5.08	3.08	4.89	37.2	22.1	17.2	17.0	15.9
β-Pinene	5.40	3.08	4.89	74.0	19.4	5.9	7.0	9.7
β-Myrcene	5.53	3.10	6.92	2.8[c]	0	0	0	0
3-Hexen-1-ol, acetate (Z)	5.69							
Hexyl ester acetic acid	5.76							
Limonene	5.93	3.12	3.15	2.9[c]	0.3[c]	0	1.0[c]	0.6[c]
Eucalyptol	6.01	2.03	532.4	5.9	1.4	1.3	0.6	0.1
Camphor	7.24	2.02	186.3	15.0	12.2	13.5	12.0	11.4

[a]Log pk_{oc} values and water solubilities estimated using Epi Suite v 3.10.
[b]Terpene concentrations calculated from response factor equations as determined by standard curves.
[c]Terpene concentrations estimated by average response factor of known standards.

TABLE 2. SIGNIFICANCE TABLE FOR INDIVIDUAL MONOTERPENES ASSESSED IN VOLATILE BIOASSAY

	Camphor		α-Pinene		β-Pinene	
[Terpene] (mg/m^3)	Root length	Shoot length	Root length	Shoot length	Root length	Shoot length
1	$*\uparrow$	$*\uparrow$	$*\uparrow$	ns	ns	ns
10	ns	ns	$*\uparrow$	ns	ns	ns
50	ns	ns	$*\uparrow$	ns	ns	ns
100	ns	ns	$*\uparrow$	ns	ns	ns
250	$*\downarrow$	$*\downarrow$	$*\uparrow$	ns	ns	ns
500	ns	$*\downarrow$	$*\uparrow$	ns	ns	ns
1,000	$*\downarrow$	$*\downarrow$	$*\uparrow$	$*\uparrow$	ns	$*\uparrow$

ns = Nonsignificant ($P > 0.05$), $\uparrow$= mean greater than MeOH control (i.e., stimulation), $\downarrow$= mean lower than MeOH control (i.e., inhibition). Terpenes not shown, myrcene and limonene, are all nonsignificant ($P > 0.05$).
$^*P < 0.05$.

Phytotoxicity of Terpene Components. Terpenes were tested at concentrations bracketing those calculated from the unknown injections (0–1,000 mg/m^3). Overall, myrcene, limonene, and β-pinene had no effect on the test species compared to the methanol control (Figure 5). Camphene had a pattern similar to both limonene and myrcene (data not shown). Eucalyptol had no phytotoxicity to radicle elongation, but did cause shoot length stimulation at 50 and 100 mg/m^3, while α-pinene stimulated cress radicle growth at all concentrations (Table 2). Only camphor suppressed radicle and shoot growth, showing a nonlinear trend (Figure 5 and Table 2). Methanol significantly reduced both radicle and shoot length compared to water ($P < 0.001$).

The terpenes were also evaluated on mugwort, crabgrass, and foxtail millet germination at 1,600 mg/m^3 to determine their phytotoxicity toward a variety of test species at high concentration (Table 3). Mugwort root and shoot inhibition was observed with all terpenes except camphene. Fewer inhibited monocotyledonous test species, suggest that broadleaf species may be more sensitive to the phytotoxic effects.

DISCUSSION

Plant species succession, whether in a natural or agroecosystem, is often disrupted by the introduction of a nonnative species that rapidly displaces native flora or interferes with crop production (Turner, 1988; Sax et al., 2002). Most often this phenomenon is attributed to the greater competitive ability of the introduced plant compared to the native flora or crops in a particular setting

TABLE 3. PERCENT GROWTH INHIBITION OF RADICLE ELONGATION BY MUGWORT TERPENES AT 1,600 MG/M^3

	Curly cress				Mugwort				Large crabgrass				Foxtail millet			
	Roots		Shoots		Roots		Shoots		Roots		Shoots		Roots		Shoots	
α-Pinene	21.0	ns	21.3	*	32.7	**	29.9	**	17.4	ns	−13.7	ns	−6.8	ns	−19.9	ns
β-Pinene	40.5	**	46.0	**	41.5	**	43.2	**	31.5	**	16.22	ns	−19.7	ns	−13.6	ns
Camphor	95.1	**	98.6	**	96.5	**	95.8	**	85.7	**	54.7	**	95.3	**	59.2	**
Camphene	−3.2	ns	16.0	n/s	29.8	**	11.0	ns	3.6	ns	−13.7	ns	35.1	*	−10.2	ns
Eucalyptol	59.5	**	76.6	**	35.0	**	43.2	**	35.9	**	31.6	**	−6.3	ns	3.1	ns
Caryophyllene oxide	1.7	ns	29.4	**	−1.8	n/s	10.8	ns	10.5	ns	13.7	ns	−10.1	ns	5.4	ns

[a] Percent inhibition based on comparison to the control.
[b] n/s = Nonsignificant ($P > 0.05$).
* $P < 0.05$; ** $P < 0.001$.

(Williamson, 1996; Williamson and Fitter, 1996; Lockwood et al., 2001). However, in the case of mugwort and about 80 other weedy species (Singh et al., 2001), habitat invasion and loss of biodiversity may be partially accomplished through the release of allelochemicals that can reduce seedling establishment and overall plant fitness (Ridenour and Callaway, 2001; Bais et al., 2003). A greater comprehension of the growth and ecology of nonnative species would assist in furthering our understanding of the mechanisms that contribute to invasiveness (Whittaker and Feeny, 1971; Wardle et al., 1998). A recent study by Callaway and Aschehoug (2000), suggested the importance of allelopathic interference in the ability of diffuse knapweed (*Centaurea diffusa* Lam.) to invade crop and rangelands across North America. The identified toxin in a related *Centaurea* species (*C. maculosa*), (–)-catechin, is believed to cause the formation of reactive oxygen species and lead to eventual root necrosis (Bais et al., 2003). This allelopathic response, deemed the "novel weapons hypothesis," states that nonnative species exude/emit compounds foreign to the native plant community, resulting in enhanced invasiveness (Bais et al., 2003). We attempted to test the "novel weapons hypothesis" in controlled laboratory studies using the invasive perennial weed mugwort, which belongs to a family known for their production of volatile aromatics.

We know that mugwort tends to establish in dense monospecific stands, gradually resulting in a decrease of plant biodiversity, while increasing its radius of expansion from the nucleus of introduction (Barney, 2003). Related *Artemisia* spp. are also reported to be invasive, and many are considered to be noxious weeds, and also known to contain volatile bioactive compounds, primarily terpenoids (Funke, 1943; Muller et al., 1964). Literature states that mugwort contains similar chemicals (LeFevre, 1964; Dung et al., 1992), although a thorough analysis of foliar chemistry has not been performed. Based on the hypothesis that many of the allelochemicals present in mugwort foliage would be volatile terpenoids, we developed a specific assay to assess this potential toxicity.

The assay allowed only indirect (atmospheric) contact between fresh mugwort leaves (donor) and the test species (receptor) in an enclosed environment. We observed significant inhibition of curly cress radicle elongation by volatiles released from as few as two mature mugwort leaves. Assays performed with leaves of various ages from identical mugwort populations demonstrated that the inhibitory potential of foliage, or quantity of volatiles produced, was leaf-age dependent. Similarly, monoterpene concentrations in peppermint (*Mentha* × *piperita* L.) leaves exhibited an exponential decrease with increase in leaf age (Rohloff, 1999; McConkey et al., 2000). This finding is consistent with the decrease of limonene concentrations in peppermint as the plant ages (Gershenzon et al., 2000), showing that terpene concentrations are not static throughout a plant's life cycle. GC–MS results obtained with the ITH-1 population indicate that younger foliar tissue contained up to 28-fold greater concentrations

of monoterpenes. This increase in total volatiles correlates with increased phytotoxicity, as younger tissue is significantly more inhibitory to seedling growth.

Volatile assays were conducted in enclosed receptacles using hand-crushed leaves, which may have resulted in greater volatile concentrations compared to actual field settings (Fehsenfeld et al., 1992). While this assay cannot mimic field conditions, our intention was to evaluate the potential impact of maximal terpene concentrations. If no phytotoxicity were observed with all possible monoterpenes (null hypothesis), one could assume that monoterpenes were not likely influencing the surrounding plant community structure.

Several studies have suggested the role of volatile compounds in suppression of neighboring vegetation. These include the chaparral species *Salvia* and *Eucalyptus*, and the rangeland weed *A. tridentata* (Muller, 1965; del Moral and Muller, 1970; Klarich and Weaver, 1973; Kohli and Singh, 1991), all of which create "monocultural islands" with no surrounding vegetation. These present studies suggest that foliar terpenes can influence the germinators of competing species. Interestingly, in field settings mugwort produces large quantities of foliage resulting in noticeable quantities of released aromatics (Barney, personal observation). Foliar tissues likely generate volatiles continuously over the course of the season, with environmental conditions influencing emission quantities and the association of potential allelochemicals with soil particulates, as observed in many forest and shrubby ecosystems (Lerdau et al., 1995; Guenther et al., 1996; Hayward et al., 2001). In addition, if terpene storage structures are ruptured, their contents are immediately released into atmosphere, with some terpenes likely partitioning into soil organic matter. Soils in a Sitka spruce forest (*Picea sitchensis* Bong.) re-emit monoterpenes accumulated throughout the growing season from the general emission of the spruce trees (Hayward et al., 2001).

Despite their volatility, these compounds have relatively high log pk_{oc} values, with lipophyllic monoterpenes having a 10-fold greater affinity for organic matter than the more polar terpenes. This suggests a potential for accumulation in soil organic matter over time. Association of phytotoxic terpenes with soil organic matter presents a possible mechanism for allelopathic interactions in natural settings. In addition, the solubility of terpenoids identified in mugwort foliage varies greatly (see Table 1), and toxicity is dependent upon concentration. Weidenhamer et al. (1993) showed that camphor in aqueous solution significantly inhibited germination of *Lactuca sativa* L. and *Rudbeckia hirta* L. with concentrations as low as 25 mg/l. Therefore, our results suggest that the affinity of various terpenoids for soil organic matter, coupled with their relatively high water solubility, may lead to accumulation in multiple environmental compartments.

It is possible that the terpenoids produced by mugwort foliage may be active in the environment. Mugwort tissue incorporation, or decomposition over time reduces neighboring species germination (Inderjit and Foy, 1999). The action

of rainfall or dew may also result in the transport of these compounds to the soil surface, resulting in reduced seed germination. Studies performed by Muller (1965) showed that *Salvia* spp. produced volatiles that bind to the soil surface underneath *Salvia* infestations, and subsequently reduce the growth of surrounding weedy species.

Our results indicate that volatile compounds are produced and released by the foliage of three separate mugwort populations. However, potential phytotoxicity varied with population. Numerous studies have shown similar variations in the production of allelochemicals with genotype and plant age (Weston et al., 1987; Fajer et al., 1992). In addition, Inderjit et al. (2001) found site variation, and likely concomitant mugwort population variation between sites, with respect to the effect of mugwort-infestation on soil characteristics. Population variation in phytotoxic chemicals likely explains some of the inconsistencies reported in field studies examining allelopathy (Muller, 1966; Fuerst and Putnam, 1983).

We attempted to identify the specific monoterpenes associated with the phytotoxicity observed in the presence of mugwort foliage, or soil associated volatiles. No one terpenoid could account for the phytotoxicity observed in foliar assays. Most of the monoterpenes produced no inhibition of either root or shoot growth, while some stimulated growth at low concentrations. Camphor treatments, however, did result in reductions to root and shoot growth of various indicator species. This finding is consistent with Abraham et al. (2000) who examined the effect of four monoterpenes on maize seed germination and found the following order of activity: camphor > eucalyptol > α-pinene > limonene.

It is possible that the toxicity observed in our initial experiments with mugwort foliage is due to synergistic combinations of phytotoxic terpenes. Due to the difficulty involved in accurately mimicking terpene mixtures in the lab, neither we nor any other researchers have examined terpene mixtures for allelopathic activity. Therefore, activity observed with mugwort foliage might include combinations of those tested in this study, as well as those untested, such as santolina triene, which occurs in substantial quantities in the foliage, but which was unavailable for evaluation. It is possible that Santolina triene, or unidentified components may influence the overall phytotoxic potential of mugwort volatiles emitted over time in an additive fashion.

Of the parameters assessed, shoot growth was generally slightly more sensitive than radicle elongation to the presence of mugwort volatiles. The quantity of the terpenes appears to be inversely proportional to plant tissue age, and is also population dependent. However none of the individual monoterpenes tested was highly suppressive. Further research on the production, release, and activity of volatiles produced by populations under field conditions are needed. In addition, the potential role of rhizome exudates should be

evaluated to elucidate the contribution of other allelochemicals involved in mugwort interference.

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EFFECTS OF ELEVATED CO_2 ON FOLIAR QUALITY AND HERBIVORE DAMAGE IN A SCRUB OAK ECOSYSTEM

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Abstract—Atmospheric CO_2 concentrations have increased exponentially over the last century and continuing increases are expected to have significant effects on ecosystems. We investigated the interactions among atmospheric CO_2, foliar quality, and herbivory within a scrub oak community at the Kennedy Space Center, Florida. Sixteen plots of open-top chambers were followed; eight of which were exposed to ambient levels of CO_2 (350 ppm), and eight of which were exposed to elevated levels of CO_2 (700 ppm). We focused on three oak species, *Quercus geminata, Quercus myrtifolia, Quercus chapmanii*, and one nitrogen fixing legume, *Galactia elliottii*. There were declines in overall nitrogen and increases in C:N ratios under elevated CO_2. Total carbon, phenolics (condensed tannins, hydrolyzable tannins, total phenolics) and fiber (cellulose, hemicellulose, lignin) did not change under elevated CO_2 across plant species. Plant species differed in their relative foliar chemistries over time, however, the only consistent differences were higher nitrogen concentrations and lower C:N ratios in the nitrogen fixer when compared to the oak species. Under elevated CO_2, damage by herbivores decreased for four of the six insect groups investigated. The overall declines in both foliar quality and herbivory under elevated CO_2 treatments suggest that damage to plants may decline as atmospheric CO_2 levels continue to rise.

Key Words—Elevated CO_2, *Quercus myrtifolia*, *Quercus chapmanii*, *Quercus geminata*, *Galactia elliottii*, herbivory, nitrogen fixer, Kennedy Space Center.

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INTRODUCTION

Atmospheric carbon dioxide (CO_2) concentrations began increasing with the advent of the industrial revolution and are continuing to increase at a rate of approximately 4 ppm annually. At the current rate of increase, CO_2 levels are expected to double by the end of this century to 750 ppm. The increase is expected to have significant effects on ecosystems, including short-term physiological changes in plants and long-term changes in ecosystem structure and function. Numerous studies have been conducted to determine the effects of elevated CO_2 on plants and their associated communities. Plant responses to elevated CO_2 concentrations are idiosyncratic within and among species (Williams et al., 1986; Lindroth et al., 1993; Curtis et al., 1996; Johnson et al., 1996; Mousseau et al., 1996; Cook et al., 1997; Van Gardingen et al., 1997; Woodward and Beerling, 1997). Some general trends, however, have emerged.

In the short term, elevated CO_2 increases photosynthesis across various ecosystems (Drake et al., 1997; Norby et al., 1999), including arctic tundra (Oberbauer et al., 1986; Tissue and Oechel, 1987), grasslands (Smith et al., 1987), and deciduous forests (Williams et al., 1986). After a period of time, some species adjust their photosynthetic rates whereas others show little or no adjustment (Williams et al., 1986; Smith et al., 1987; Tissue and Oechel 1987). Root to shoot ratio (Ceulemans and Mousseau 1994; Mousseau et al., 1996) and biomass tend to increase (Leadley et al., 1999; Owensby et al., 1999). Consistent plant chemical changes include increases in foliar C:N ratios and decreases in foliar nitrogen concentrations (Bezemer and Jones, 1998). According to some plant defense hypotheses, increases in carbon availability should result in increases in carbon based secondary metabolites (Bryant et al., 1983; Tuomi et al., 1984) though there have been no consistent affects of CO_2 on secondary metabolite concentrations (Bazzaz, 1990).

Resource availability appears to influence the degree to which plant species adjust to elevated CO_2 (Bazzaz, 1990; Lindroth et al., 1993). The effect of elevated CO_2 on plants is enhanced when other resources such as water, light, and nutrients are abundant (Bloom et al., 1985; Chapin et al., 1987), and the effects are mitigated when nitrogen or phosphorus is limiting (Zangerl and Bazzaz, 1984; Brown and Higginbotham, 1986). Plant responses appear to be linked to the ability of species to store carbohydrates by increasing the size and/or number of leaves, length of roots, and fine root turnover (Ceulemans and Mousseau, 1994; Johnson et al., 1996; Mousseau et al., 1996). Changes in the nutritional and defensive characteristics of host plants may result in behavioral and/or physiological changes in herbivores (Lindroth, 1996; Bezemer and Jones, 1998; Stiling et al., 1999). Lower levels of nitrogen and higher C:N ratios in plants under elevated CO_2 have generally been associated with compensatory feeding and subsequent increases in levels of damage or defoliation (Lincoln et al., 1984, 1993; Fajer et al., 1989; Lindroth

et al., 1993, 1995; Salt et al., 1995; Docherty et al., 1997; Kinney et al., 1997; Williams et al., 1997; but see Hamilton et al., 2004).

Leaf-chewing insects such as grasshoppers (Johnson and Lincoln, 1990) and lepidopteran larvae (Lindroth et al., 1993, 1995) generally consume more leaf area when fed plants that have been grown under elevated CO_2. Likewise, the area damaged by leaf-mining insects may also increase (Salt et al., 1995). Lepidopteran larvae exhibit increased mortality and slower growth rates when feeding on elevated CO_2 plants (Akey and Kimball, 1989; Fajer, 1989; Fajer et al., 1989, 1991). Consequently, herbivores may become more susceptible to pathogens, parasitoids, and predators (Price et al., 1980; Lindroth, 1996; Stiling et al., 1999). For example, increases in mortality of leaf miners feeding on elevated CO_2 plants has been linked to increases in parasitism (Stiling et al., 1999). In the longest field study to date, some insect herbivore populations have been shown to decline markedly under elevated CO_2 (Stiling et al., 1999, 2002, 2003).

Our study used open top chambers in a field-based experiment to examine the effects of elevated CO_2 on foliar chemistry and herbivore damage in a scrub oak community. We focused on four dominant plants species within the community; three oaks and a nitrogen-fixing legume, and hypothesized that the oak species would respond similarly with decreases in nitrogen and increases in C:N ratios and secondary metabolites under elevated CO_2. We further hypothesized that the nitrogen-fixing legume would exhibit little change in nitrogen concentrations since the ability to fix nitrogen may result in less nitrogen dilution. Additionally, we measured foliar damage by six different herbivore-feeding guilds on the four plant species. We hypothesized that herbivore damage would decline on the oaks under elevated CO_2 because we expected that herbivores would be unable to compensate for decreases in foliar nitrogen under the rigors of field conditions. We predicted that there would be little change in herbivore damage on the nitrogen-fixing legume.

METHODS AND MATERIALS

Study Site. Our study site lies within a 2-hectare native scrub-oak community located at Kennedy Space Center, Florida. This woody ecosystem is controlled by a natural fire return cycle of 8–12 yr and the mature canopy is 3–5 m high. The last burn cycle was in 1996 prior to site set up. Sixteen 3.6-m diam plots, each enclosed with a clear polyester film open-top chamber 3.4 m in height, were utilized to control CO_2 levels. Chambers were overlaid on an octagonal framework of PVC pipe with a removable access door and frustrum to reduce dilution of air within the chamber by outside wind. All re-growth was cut to ground level in May 1996 and, since that time, the vegetation in eight of the chambers has been exposed to almost twice ambient CO_2 (700 ppm), while the other eight have been

exposed to ambient levels (350 ppm). The CO_2 is supplied 24 hr a day. Monitoring and control of CO_2 injection into each chamber is done by infrared gas analyzer in conjunction with manually adjusted needle valves. In ambient CO_2 chambers, the airflow is identical to that of the elevated CO_2 chambers but is not supplemented with CO_2. Four species dominate this community and are present in every chamber: three oak species, *Quercus myrtifolia* Willd; *Q. chapmanii* Sargent; *Q. geminata* Small; and the nitrogen fixing legume, *Galactia elliottii* Nuthall. Chambers were originally established to investigate effects of elevated CO_2 on plant productivity and nutrient cycling. Studies of herbivores and leaf chemistry were a later addition to the project.

Foliar Chemistry. Samples of fresh leaves from each of the four study species were collected for chemical analysis. We haphazardly removed four undamaged leaves from each of three individuals per chamber from each of the three oak species every 3 mo (May 2001–May 2003). While in the field, a hole punch was used to remove two disks of leaf tissue from each leaf. One disk was used to obtain the dry weight of the disk, while the other was placed into 70/30 acetone/water with 1 mM ascorbic acid and used for subsequent phenolic analysis. The remaining portion of the leaf was returned to the lab on ice, dried, and used to measure C, N, and fiber (cellulose, hemicellulose, lignin). Because of its small leaf size, the collection method for *G. elliottii* differed slightly. Two opposite leaflets, each from three individuals were collected from each chamber. One leaflet was placed in acetone for phenolic analysis; the opposite leaflet in a bag and used to obtain leaf weights and, subsequently, C, N, and fiber content. Samples from different individual plants of a given species were pooled within chambers so that chambers (8 per treatment) acted as replicates. Dried leaves were ground to a fine powder and stored at -80°C prior to analysis.

Percent dry weight nitrogen and carbon were estimated from leaf powder on a Carlo-Erba NA1500 model C/N analyzer (Milan, Italy). These data also provided estimates of foliar C:N ratios. Subsamples of leaf powder (above) were also used to assess the effects of elevated CO_2 on foliar concentrations of cellulose, hemicellulose, and lignin by sequential neutral detergent/acid detergent digestion on an Ankom fiber analyzer (Abrahamson et al., 2003). Phenolic analysis was conducted using the leaf disks collected into 70% acetone in the field. Proanthocyanidins, an estimate of condensed tannin, were assayed by using N-butanol:HCL methods described in Rossiter et al. (1988). Total phenolics were estimated by using the Folin-Denis assay (Swain, 1979), and gallotannins (hydrolyzable tannins) were estimated with a potassium iodate technique developed by Bate-Smith (1977) and modified by Schultz and Baldwin (1982). Standards for tannin analysis were generated by multiple sequential washes of bulk samples (one for each species) by acetone extraction. All tannin assays produced colorimetric readings, in proportion to tannin concentration, which were quantified by using a BioRad microplate reader.

Damage Estimates on Green Leaves. We counted 200 randomly selected leaves from each of the species from each chamber every 3 mo starting in May 2001 and ending in May 2002. Each leaf was scored for the presence of six types of herbivore damage; leaf gall, eye spot gall, leaf tier, chewed leaf, mined leaf, and (*G. elliottii* only) leaf mite.

Statistical Analysis. All data met the assumptions of normality (Kolmogorov-Smirnov test, $\alpha = 0.05$) and were analyzed using parametric statistics. Foliar chemistry was analyzed over a 2-yr period (May 2001–May 2003). This represents 9 sampling dates for the oak species and 8 for *G. elliottii*, which was absent from all chambers during February 2003. Variation in foliar quality (carbon, nitrogen, fiber, and phenolics) under elevated and ambient CO_2 treatments across time was analyzed using repeated measures GLM with Tukey's HSD test for significant differences ($\alpha = 0.01$). Damage estimates were analyzed for a single year (May 2001–May 2002). Variation in herbivore damage under elevated and ambient CO_2 treatments across time was analyzed using repeated measures GLM with Tukey's HSD test for significant differences ($\alpha = 0.05$).

RESULTS

Foliar Chemistry. As predicted, foliar nitrogen concentrations declined and C:N ratios increased under elevated CO_2 treatments. The mean percent dry weight of nitrogen was 1.37 (SE $\pm$ 0.02) under ambient CO_2 conditions and 1.25 (SE $\pm$ 0.02) under elevated CO_2 conditions ($F_{1,38} = 9.20$, $P = 0.004$). The mean C:N ratio was 38.57 (SE = 0.56) in the ambient treatments and 41.74 (SE = 0.58) in the elevated treatments ($F_{1,38} = 13.39$, $P < 0.001$). Contrary to our predictions, foliar phenolics, total carbon, and fiber content were unaffected by elevated CO_2 (Table 1). There were no significant treatment (CO_2) by date or species interactions, suggesting that elevated CO_2 also caused reductions in foliar nitrogen of the nitrogen-fixer, *G. elliottii* comparable to those observed in the three oaks. Hydrolyzable tannin concentrations were greatest in *Q. chapmanii* and lowest in *Q. geminata*, while total phenolic concentrations were greatest in *G. elliottii* and lowest in *Q. geminata* (hydrolyzable tannins, $F_{3,33} = 4.94$, $P = 0.006$; total phenolics $F_{3,21} = 9.63$, $P < 0.001$). Nitrogen concentrations were highest and carbon and C:N ratios lowest for the nitrogen fixer *G. elliottii*, while *Q. myrtifolia* had the lowest levels of nitrogen and highest levels of carbon and C:N ratio (statistical values for species effects on nitrogen, carbon, and C:N ratio, respectively; $F_{3,38} = 9.74$, $P < 0.001$; $F_{3,38} = 7.09$, $P < 0.001$, $P < 0.001$, $F_{3,38} = 12.16$, $P < 0.001$. Q. geminata had the highest percentage of fiber (cellulose, hemicellulose). G. elliottii had the lowest levels of cellulose and hemicellulose (cellulose, $F_{3,37} = 9.18$, $P < 0.001$; hemicellulose, $F_{3,37} = 7.49$, $P < 0.001$). Species differed in their relative foliar chemistries over time (Figure 1). The only consistent

TABLE 1. FOLIAR CHEMISTRIES OF FOUR PLANT SPECIES UNDER ELEVATED AND AMBIENT LEVELS OF CO_2

Species	Condensed tannins		Hydrolyzable tannins		Total phenolics	
	Ambient	Elevated	Ambient	Elevated	Ambient	Elevated
Q. myrtifolia	33.56	35.11	35.31	36.23	42.94	43.50
	(1.50)	(1.76)	(1.68)	(2.12)	(2.60)	(2.70)
Q. chapmanii	41.50	44.66	38.97	39.49	42.21	44.04
	(1.89)	(1.59)	(2.50)	(2.43)	(2.35)	(2.31)
Q. geminata	25.76	28.65	28.15	30.34	33.10	35.60
	(1.43)	(1.47)	(1.20)	(1.39)	(2.13)	(2.23)
G. elliottii	37.37	38.84	32.32	36.40	48.88	52.38
	(2.38)	(2.62)	(2.87)	(3.02)	(2.95)	(3.03)
	Nitrogen		Carbon		C:N Ratio	
Q. myrtifolia	**1.12**	**1.07**	50.73	50.78	**45.83**	**48.44**
	(0.02)	(0.02)	(0.17)	(0.27)	(0.62)	(0.77)
Q. chapmanii	**1.29**	**1.21**	49.00	49.25	**38.77**	**41.79**
	(0.02)	(0.02)	(0–17)	(0.37)	(0.71)	(0.92)
Q. geminata	**1.19**	**1.12**	49.23	49.66	**42.11**	**45.29**
	(0.02)	(0.02)	(0.22)	(0.37)	(0.62)	(0.72)
G. elliottii	**2.03**	**1.71**	48.47	48.78	**24.66**	**28.95**
	(0.04)	(0.04)	(0.29)	(0.44)	(0.65)	(0.66)
	Cellulose		Hemicellulose		Lignin	
Q. myrtifolia	20.55	20.69	13.26	13.48	9.18	9.48
	(0.46)	(0.45)	(0.31)	(0.26)	(0.50)	(0.47)
Q. chapmanii	18.37	17.52	13.02	12.84	8.09	8.74
	(0.43)	(0.42)	(0.31)	(0.30)	(0.44)	(0.56)
Q. geminata	26.11	25.91	15.37	15.19	10.32	10.49
	(0.51)	(0.46)	(0.29)	(0.27)	(0.47)	(0.48)
G. elliottii	15.35	14.05	12.29	11.22	9.14	9.52
	(0.39)	(0.53)	(0.40)	(0.51)	(1.47)	(1.51)

Note. Data represent % dry weights (except C:N ratio) and are the means of 72 samples except *G. elliottii* (64 samples). Standard errors are in parentheses. Significant teatment differences are in bold.

differences among species were higher nitrogen concentrations and lower C:N ratios in *G. elliottii* compared with the three oak species (Figure 1a and c).

Damage Estimates on Green Leaves. Herbivore damage by chewers, miners, eye spot galls, and leaf tiers declined under elevated CO_2 (chewed, $F_{1,52} = 29.01$, $P < 0.001$, mined $F_{1,52} = 17.06$, $P < 0.001$; eye spot galls, $F_{1,38} = 14.91$, $P < 0.001$, leaf tier $F_{1,38} = 6.92$, $P = 0.012$) (Figure 2). Additionally, there was a weak trend for leaf mite and leaf gall damage to decline under elevated CO_2 (Figure 2). Chewers and miners occurred on all species, while leaf galls, eye spot galls, and leaf tiers occurred exclusively on the *Quercus* species and leaf mites

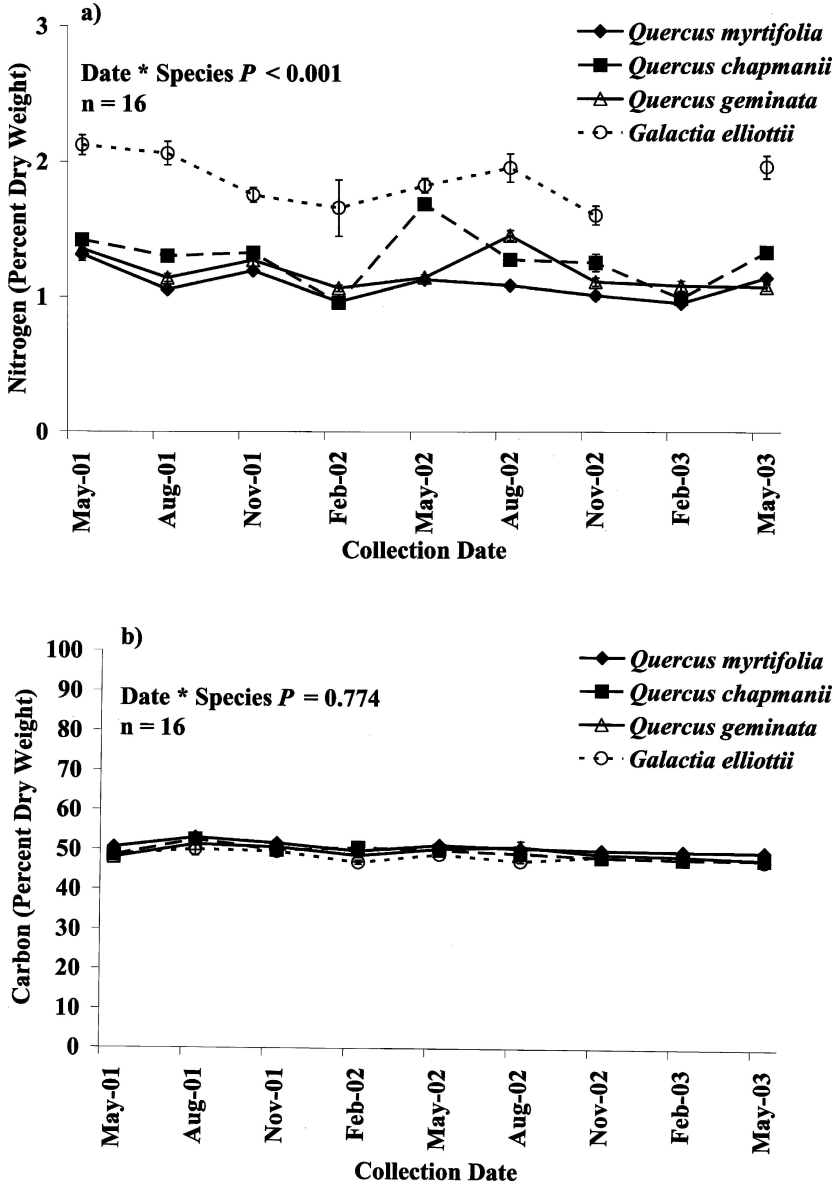
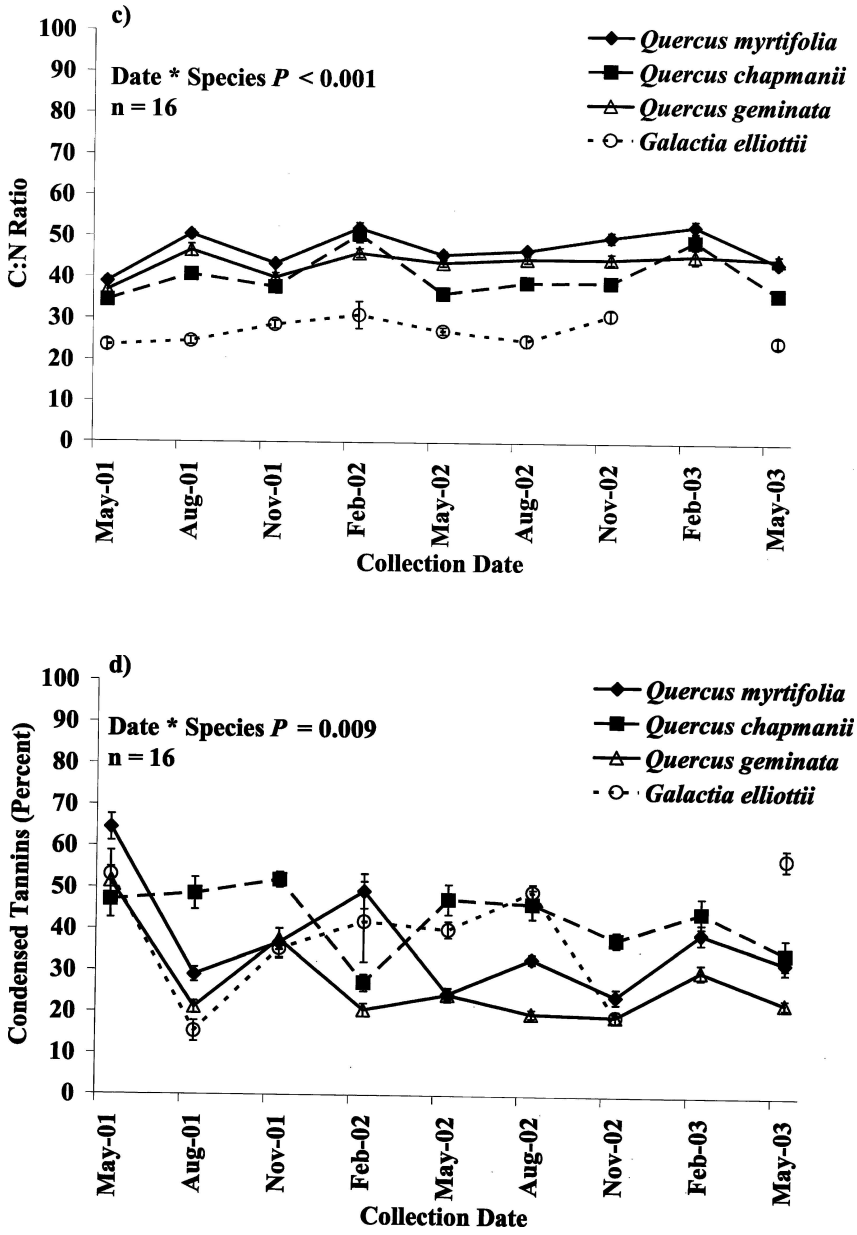


FIG. 1. Foliar chemistry of plant species over time. Nitrogen = a, carbon = b, C:N ratios = c, condensed tannins = d, hydrolyzable tannins = e, total phenolics = f, cellulose = g, hemicellulose = h, lignin = i. Data are the means of 16 samples (chambers). Bars represent standard errors. Note that G. elliottii was absent from all chambers in February 2003.



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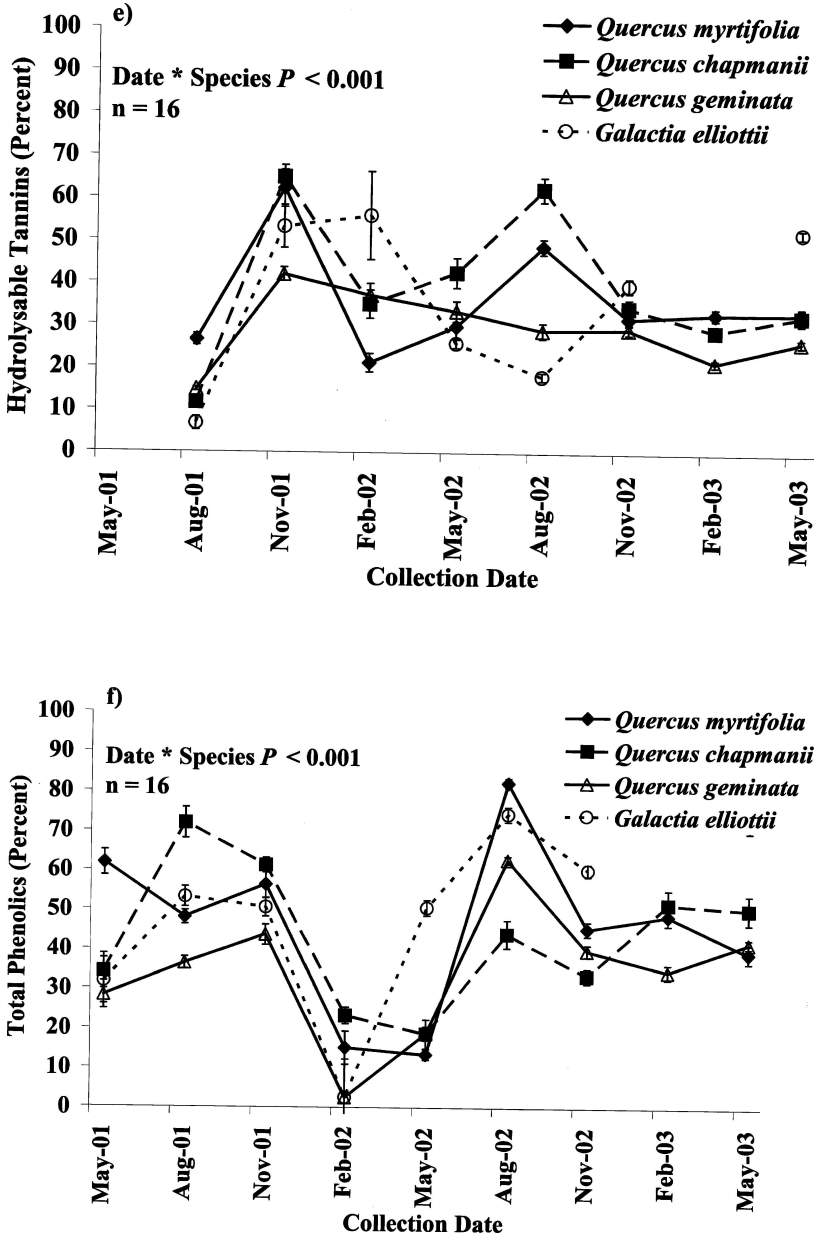

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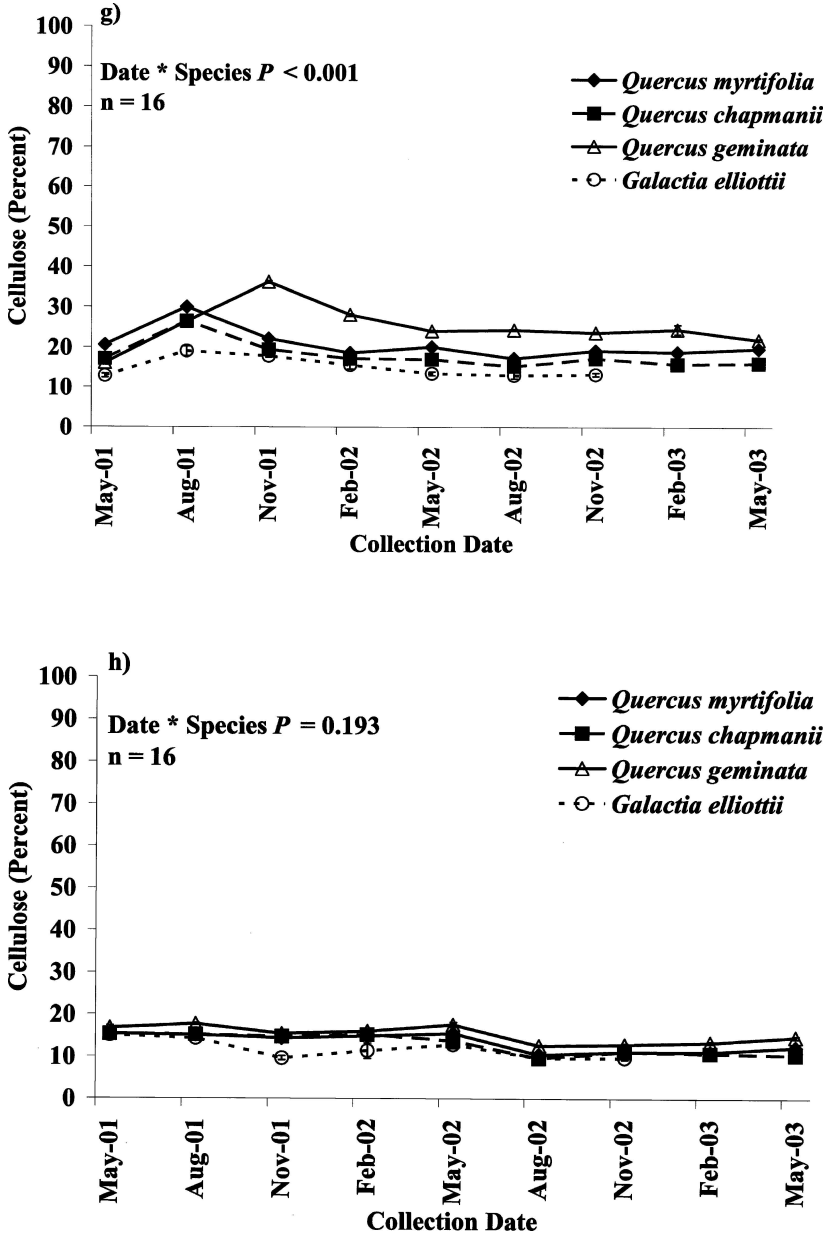

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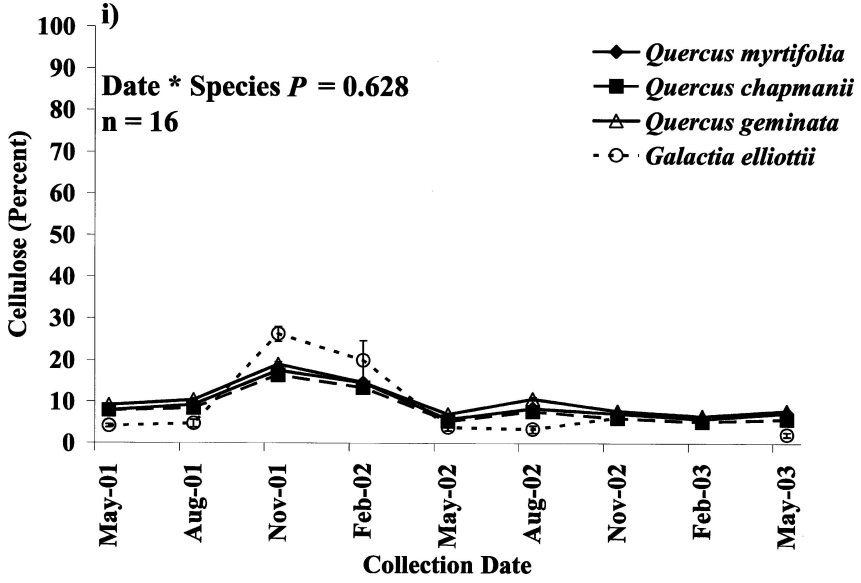

FIG. 1. Continued

occurred exclusively on *G. elliottii* (Table 2). Chewing damage was higher under ambient than elevated CO_2 during all months except November (date* CO_2 interaction $F_{4,208} = 7.18$, $P < 0.001$, Figure 3a). Leaf tier damage was higher under ambient than elevated CO_2 during all months except May 2001 and February 2002 (Date* CO_2 interaction $F_{4,152} = 2.98$, $P = 0.021$ Figure 3b). Effects of elevated CO_2 on chewer damage were particularly pronounced on *Q. chapmanii* ($F_{3,52} = 4.30$; $P = 0.009$, Figure 4a), while CO_2 effects on eye spot galls were pronounced on *Q. myrtifolia* and *Q. geminata* ($F_{2,38} = 7.31$, $P = 0.002$, Figure 4b).

DISCUSSION

Though not all plants respond identically to increased concentrations of CO_2 (Bezemer and Jones, 1998; Hunter, 2001), all four of our study species responded in a similar fashion. While there were differences in overall chemical composition among the four species (Table 1), all exhibited declines in foliar nitrogen concentrations and increases in C:N ratios under elevated CO_2, while polyphenolics and fiber were unaffected. Changes in nitrogen levels and C:N ratios in the oak species are not surprising given the consistency with which this has been found in previous studies (Cipollini et al., 1993; Ceulemans and Mousseau, 1994; Luo et al., 1994; Curtis et al., 1996; Wilsey, 1996; Stiling et al., 1999; Reich et al., 2001).

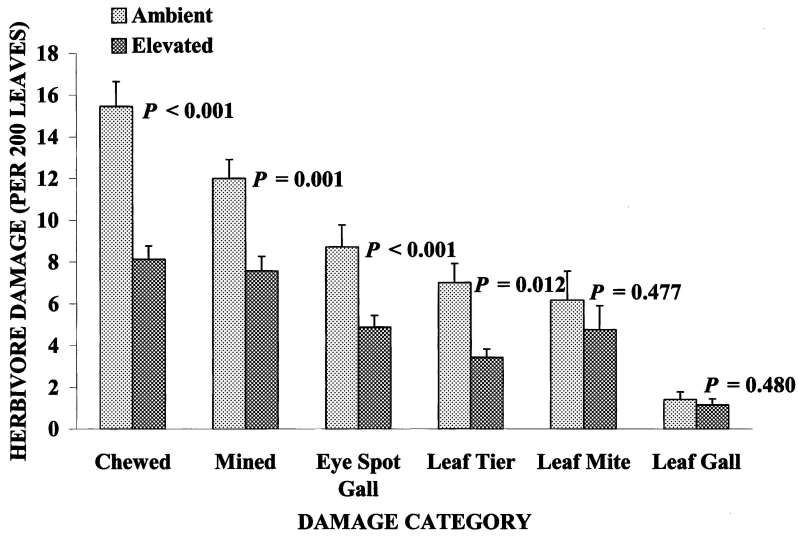

FIG. 2. Herbivore damage across all plant species. Data are the means of 160 samples for miner and chewer damage (occurred on all plant species), 120 samples for eye spot gall, leaf gall, and leaf tier damage (occurred only on oak species) and 40 samples for leaf mite damage (occurred only on the nitrogen fixer). Bars represent standard errors.

TABLE 2. HERBIVORE DAMAGE ON FOUR PLANT SPECIES AT THE KENNEDY SPACE CENTER, FLORIDA

Species	Chewing damage	Mined damage	Eye spot gall damage	Leaf tier damage	Leaf gall damage
Q. myrtifolia	11.61	13.35	12.56	5.56	2.17
	(1.07)	(1.01)	(1.40)	(0.76)	(0.55)
Q. chapmanii	18.75	12.49	0.43	5.60	0.33
	(2.14)	(1.34)	(0.15)	(1.13)	(0.13)
Q. geminata	9.84	7.60	7.12	4.59	1.27
	(0.96)	(0.88)	(0.70)	(0.73)	(0.37)
G. elliottii	7.45	5.89	—	—	—
	(0.86)	(1.00)	(·)	(·)	(·)
P value	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.807$	$P = 0.018$

Note. Not all herbivore damage occurred on all plant species, therefore data are the means of 80 samples for chewers and miners and 60 samples for eye spot galls, leaf tiers, and leaf galls. Standard errors are in parentheses and *P* values represent the significance of species effects on herbivore damage.

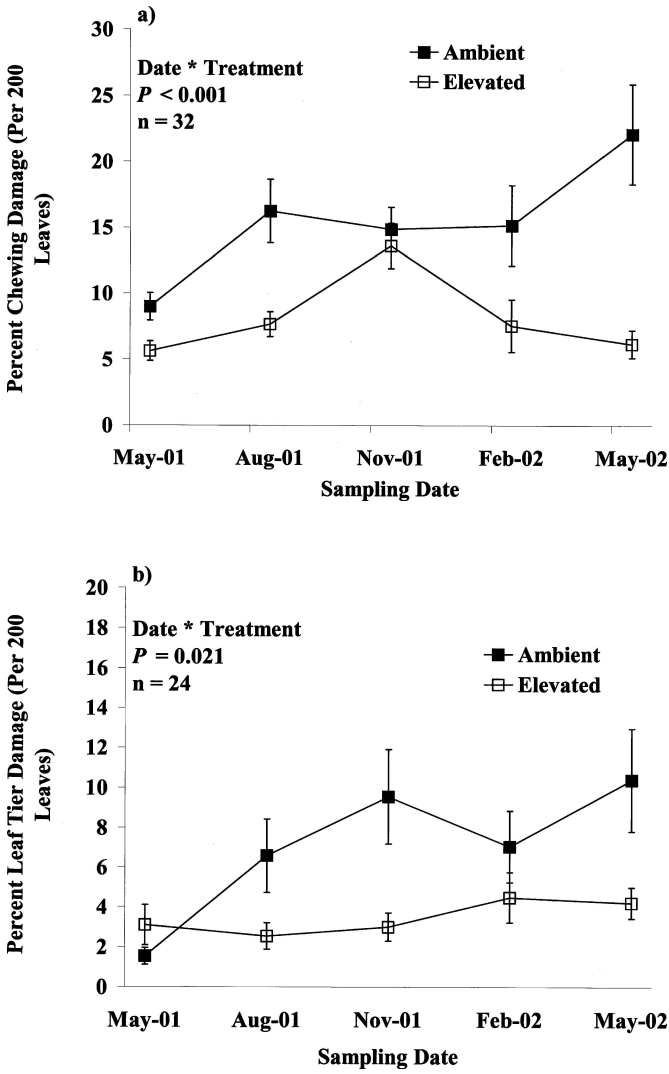

FIG. 3. Damage by chewing herbivores (a) and leaf tiers (b) under elevated CO_2. Data are the means of 32 samples for chewing herbivores and 24 samples for leaf tiers and bars represent standard errors.

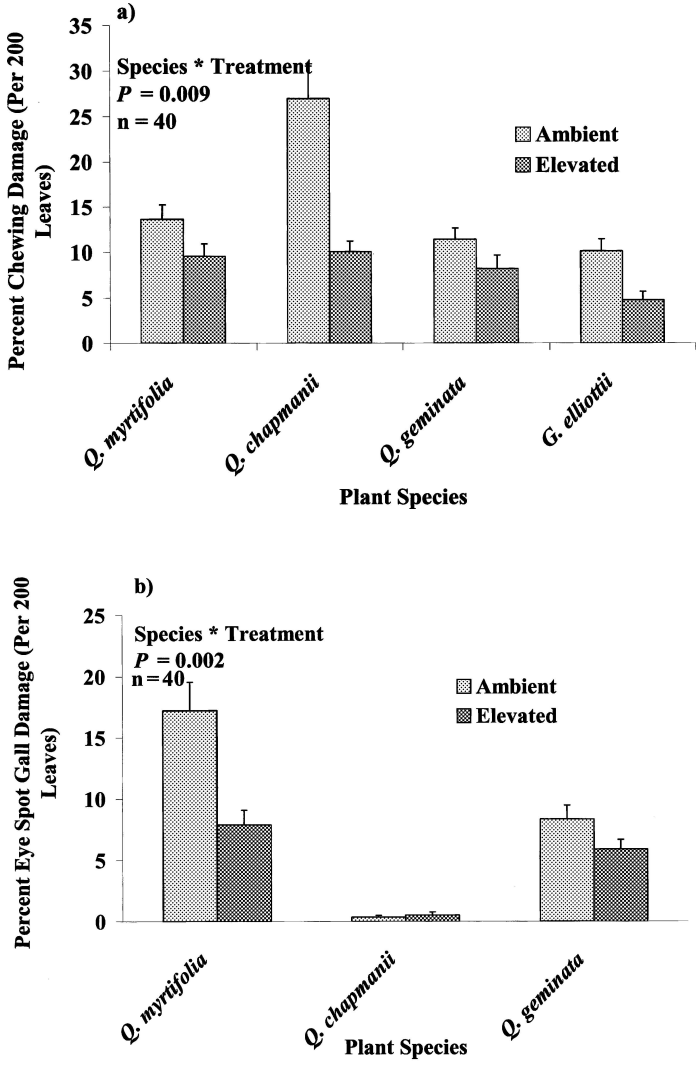

FIG. 4. Treatment effects by plant species for a) chewing damage and b) eye spot gall damage. Data are the means of 40 samples and bars represent standard errors.

Finding the same response in the nitrogen fixer, however, was not expected. Reich et al. (2001) did report that aboveground (foliar) nitrogen levels declined in four legumes (*Amorpha canescens, Lespedeza capitata, Lupinus perennis, Petalostemum villosuni*), while below ground (root) nitrogen levels remained unchanged

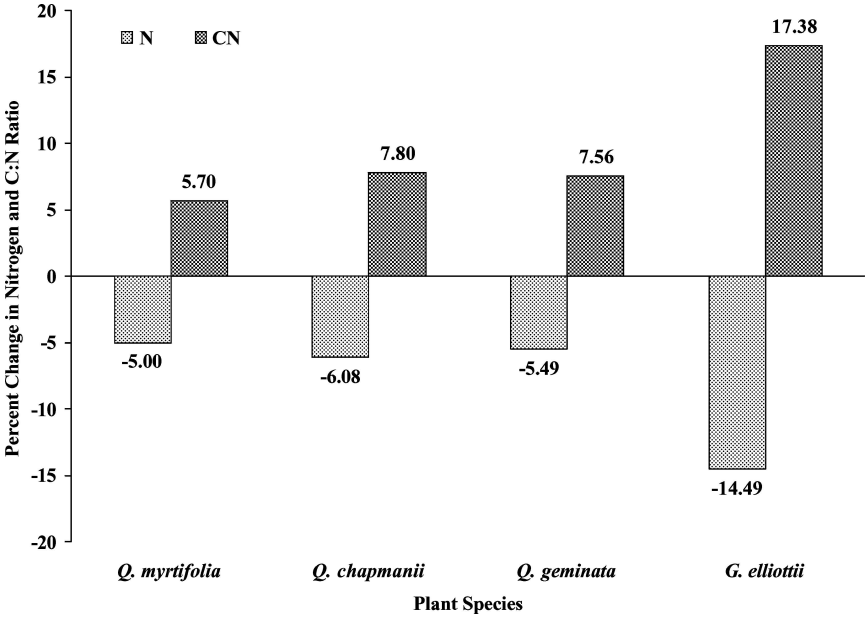

FIG. 5. The percent change in nitrogen and C:N ratio under elevated CO_2 treatments for *Q. myrtifolia*, *Q. chapmanii*, *Q. geminata*, and *G. elliottii*. Percent change values are shown.

under elevated CO_2. This indicates that even nitrogen fixers exhibit modified leaf nitrogen under elevated CO_2. More surprising, however, was the magnitude of change in nitrogen levels and C:N ratios of the nitrogen fixer when compared to the three oaks. The percent decrease in nitrogen and increase in C:N ratio of the nitrogen fixer under elevated CO_2 was more than twice the percent change seen in the three oak species (Figure 5). Given that lower nitrogen levels appear to be the primary driver affecting herbivores under elevated CO_2, the decrease in foliar nitrogen and increases in C:N ratios may be expected to have strong effects on herbivores that depend on nitrogen fixing plant species.

Though nitrogen levels were lower and C:N ratios higher under elevated CO_2 conditions, there were no treatment effects on the compounds measured (condensed tannins, hydrolyzable tannins, total phenolics, cellulose, hemicellulose, and lignin). It is expected that increased availability in carbon via increased levels of CO_2 would result in changes in carbon-based compounds in plants. However, studies thus far have been inconclusive. Some species increase in levels of phenolics, while others decrease or remain the same (Bezemer and Jones, 1998, Hamilton et al., 2004). Dury et al. (1998) found short term increases in phenolics in *Quercus robur*. Lindroth et al. (1993) examined seedlings of three

trees (*Populus tremuloides*, *Quercus rubra*, *Acer saccarum*) and found varying responses in phenolic concentrations with increases, decreases, and no changes being recorded. Phenolic concentrations also varied with leaf flush (i.e., time of CO_2 exposure). Williams et al. (1998) found that young leaves of *Quercus alba* had significantly lower leaf nitrogen content and significantly higher total nonstructural C:N ratio as plant CO_2 concentrations rose, while nonstructural carbohydrates and total carbon-based phenolics were unaffected. In some instances, total nonstructural carbohydrates (TNC), especially starch, show large increases in elevated CO_2 conditions (Saxe et al., 1998). Also, in woody species, available carbon might be allocated to woody tissue rather than foliar tissue. Therefore, it is possible that changes in carbon concentrations occurred in our species, but were not found because we did not measure TNC or woody tissue.

In our study, herbivore damage by a number of feeding guilds was lower under elevated CO_2. Additionally, there were no treatment by plant species interactions, indicating that the response to elevated CO_2 was the same across all species. The nutritional quality of plant foliage can have profound effects on herbivores (Feeny, 1968, 1992; Coley and Aide, 1991; Dury et al., 1998). Low nitrogen and high concentrations of polyphenolics and lignin indicate low nutritional quality. Nitrogen is often a limiting resource for herbivores, and limited nitrogen levels can lead to decreased growth rates, development, and fecundity (Scriber and Slansky, 1981; Schultz and Baldwin, 1982; White, 1984; Bazzaz, 1990). High concentrations of polyphenolics, such as tannins, may inhibit digestion. Increased fiber can lead to tougher and/or indigestible leaves (Feeny, 1968, 1992; Schultz and Baldwin, 1982; Schowalter et al., 1986; Coley and Aide, 1991; Dury et al., 1998; Williams et al., 1998).

Studies showing compensatory feeding by herbivores on high C:N foliage have occurred exclusively under laboratory conditions (Lincoln et al., 1986; Fajer, 1989; Johnson and Lincoln, 1990). Low nitrogen foliage may increase development time and under field conditions increase the risks posed to herbivores by natural enemies (Stiling et al., 1999, 2002, 2003) or abiotic factors.

The decline in foliar nitrogen concentrations and increase in C:N ratios in our study are the only indication of low foliar quality under elevated CO_2. Previous studies have shown that low quality foliage has negative impacts on leafmining insects (Stiling et al., 1999, 2002, 2003). These impacts appear to result from the combined effects of nutrient limitation and increases in parasitism and predation. Stiling et al. (1999) found that decreases in plant quality due to elevated CO_2 doubled leafminer mortality, while increases in parasitism due to elevated CO_2 quadrupled leafminer mortality. Insect herbivores often compensate for low foliar quality by increasing their food intake (Karban and Baldwin, 1997; Agrawal et al., 1999), yet such compensation did not act to increase the proportion of leaves damaged under elevated CO_2 in our study.

Herbivore damage can influence foliar and litter quality (Findlay et al., 1996; Agrawal et al., 1999) by a number of mechanisms including chemical induction and premature leaf abscission. These changes can affect vital ecosystem processes, such as decomposition and nutrient cycling. Therefore, elevated CO_2 can potentially influence these ecosystem processes via direct effects through changes in foliar quality and indirect effects through herbivores that change foliar and litter quality. Future research will investigate some of these ecosystem level effects.

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EFFECTS OF QUANTITATIVE VARIATION IN ALLELOCHEMICALS IN *Plantago lanceolata* ON DEVELOPMENT OF A GENERALIST AND A SPECIALIST HERBIVORE AND THEIR ENDOPARASITOIDS

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Abstract—Studies in crop species show that the effect of plant allelochemicals is not necessarily restricted to herbivores, but can extend to (positive as well as negative) effects on performance at higher trophic levels, including the predators and parasitoids of herbivores. We examined how quantitative variation in allelochemicals (iridoid glycosides) in ribwort plantain, *Plantago lanceolata*, affects the development of a specialist and a generalist herbivore and their respective specialist and generalist endoparasitoids. Plants were grown from two selection lines that differed ca. 5-fold in the concentration of leaf iridoid glycosides. Development time of the specialist herbivore, *Melitaea cinxia*, and its solitary endoparasitoid, *Hyposoter horticola*, proceeded most rapidly when reared on the high iridoid line, whereas pupal mass in *M. cinxia* and adult mass in *H. horticola* were unaffected by plant line. *Cotesia melitaearum*, a gregarious endoparasitoid of *M. cinxia*, performed equally well on hosts feeding on the two lines of *P. lanceolata*. In contrast, the pupal mass of the generalist herbivore, *Spodoptera exigua*, and the emerging adult mass of its solitary endoparasitoid, *C. marginiventris*, were significantly lower when reared on the high line, whereas development time was unaffected. The results are discussed with regards to (1) differences between specialist and generalist herbivores and

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their natural enemies to quantitative variation in plant secondary chemistry, and (2) potentially differing selection pressures on plant defense.

Key Words—Chemical defense, iridoid glycosides, *Melitaea cinxia*, multitrophic interactions, *Plantago lanceolata*, *Spodoptera exigua*.

INTRODUCTION

Many plants produce a range of toxic secondary compounds (allelochemicals) that are either constitutively expressed, or induced in response to herbivory (Karban and Baldwin, 1997). These chemicals may act as feeding deterrents or alter the physiology and development of herbivores, resulting in reduced rates of growth, smaller adult size, and increased mortality. However, plant allelochemicals not only affect the behavior and performance of herbivores feeding on the plant, but also the behavior and performance of organisms at higher trophic levels (Hare, 2002). Such effects have implications for the evolution of plant chemical defense. In some cases, higher-trophic level effects are beneficial for the plant and enhance the selective advantage of producing high levels of an allelochemical in the presence of parasitoids or predators of herbivores. For instance, allelochemicals that slow down the rate of herbivore development increase the exposure time or window of their vulnerability to parasitoids and predators (Turlings and Benrey, 1998). In other cases, these effects may be detrimental, especially if herbivores use plant allelochemicals for their own defense against parasitoids and predators (e.g., Campbell and Duffey, 1979). Many insect herbivores are specialized on plants producing particular groups of allelochemicals. For example, larvae of many species of checkerspot (fritillary) butterflies (Nymphalidae) are restricted to plants producing iridoid glycosides (Wahlberg, 2001). Specialized herbivores can often deal with high levels of specific phytotoxins in their diet (Nishida, 2002), even though their performance may be lower on plants containing high rather than low amounts of these phytotoxins (Adler et al., 1995). Although some herbivore species break down and excrete ingested allelochemicals during development, many others use them to their own advantage by sequestering them in hemolymph and other tissues, or concentrating them in the gut (Rimpler, 1991; Nishida, 2002).

A number of studies have reported that allelochemicals in herbivores reduce the performance of less well-adapted predators and parasitoids (e.g., Duffey et al., 1986; Gunasena et al., 1990; Barbosa et al., 1991). In contrast, in the handful of studies addressing the performance of specialized natural enemies, such as parasitoids that attack one or only a few related hosts in nature, development appears to be less affected by differences in allelochemicals (Barbosa et al., 1986; Sznajder and Harvey, 2003). The higher-tropic-level effects of allelochemicals are, thus, likely to depend on the level of specialization of the herbivores and parasitoids or predators involved.

We examined the developmental responses of a generalist and a specialist insect herbivore and their endoparasitoids to quantitative variation in iridoid glycosides (IGs) in ribwort plantain, *Plantago lanceolata* L. (Plantaginaceae), a perennial plant species with a worldwide distribution and a large ecological amplitude. IGs are a group of monoterpene-derived compounds that have been recorded in over 50 plant families (Jensen, 1991). The main IGs found in *P. lanceolata* are catalpol and its precursor aucubin (Bowers, 1991). Concentrations of IGs in natural populations of *P. lanceolata* range from undetectable to ca. 9% of plant dry weight, and vary both among populations and individuals within populations (Bowers, 1991; Nieminen et al., 2003). IG concentrations in *P. lanceolata* are partly under genetic control (Adler et al., 1995; Marak et al., 2000), but also vary in response to plant attributes such as leaf and plant age (Bowers and Stamp, 1993) and abiotic factors such as light and nutrient levels (Marak et al., 2003), and can be induced by both herbivores and pathogens (Darrow and Bowers, 1999; Marak et al., 2002a). In artificial diet studies, these compounds reduce the growth of generalist, but not specialist, insect herbivores (Bowers and Puttick, 1988; Puttick and Bowers, 1988). Specialist insects use these compounds as oviposition and feeding stimulants (Pereyra and Bowers, 1988; Nieminen et al., 2003) and sequester them for their own defense (Bowers and Collinge, 1992; Camara, 1997; Suomi et al., 2001).

The insect species used in this study were (i) the specialist herbivore, *Melitaea cinxia* L. (Lepidoptera: Nymphalidae) and its endoparasitoids, *Cotesia melitaearum* Wilkinson (Hymenoptera: Braconidae) and *Hyposoter horticola* Gravenhorst (Hymenoptera: Ichneumonidae) and (ii) the generalist herbivore, *Spodoptera exigua* Hubner (Lepidoptera: Noctuidae) and its endoparasitoid, *C. marginiventris* L. (Hymenoptera: Bracondiae).

M. cinxia, the Glanville fritillary, is a specialist feeder of plants containing iridoid glycosides. Larvae use IGs as feeding stimulants, whereas adults use them as oviposition cues (Nieminen et al., 2003; van Nouhuys and Kumar, unpublished data). The first five instars develop within a large silken web during the first year. The following spring, after diapause, larvae complete the final two instars. In Northern Europe, *M. cinxia* is univoltine. A detailed description of the life cycle is provided by Kuussaari et al. (2004). *C. melitaearum* is a specialist parasitoid of butterfly larvae in the genus *Melitaea* (van Nouhuys and Hanski, 2004). It attacks early and late instars of *M. cinxia*, ovipositing into the host hemocoel. Larvae feed primarily on host hemolymph and fat body. It may complete up to three generations in a single year. *H. horticola* is also a specialist parasitoid of *M. cinxia*. It produces only one generation per year and its development is closely synchronized with that of its host. It lays a single egg in first instar host larvae just before they hatch from the egg (van Nouhuys and Hanski, 2004; van Nouhuys and Ehrnsten, unpublished results). Although the eggs hatch within a few days, development is suspended as a first instar until the host has reached its fifth instar

and has broken diapause. Once the host has entered its final instar, the parasitoid larva starts attacking all tissues, ultimately consuming the entire host except for its cuticle.

S. exigua, the southern beet armyworm, is a highly polyphagous insect herbivore (Greenberg et al., 2001). It is endemic to South East Asia, but has been introduced over much of the world. Larvae complete five instars during development. In warm regions, it produces several generations per year. *C. marginiventris* parasitizes larvae of several species in the family Noctuidae. It parasitizes first to fourth instars of *S. exigua* and oviposits a single egg in the host hemocoel. Like the closely related species *C. melitaearum*, larvae of *C. marginiventris* feed primarily on host hemolymph and fat body.

In this study, we compare fitness correlates (development time and pupal or adult body mass) of the herbivores and their parasitoids reared on two lines of *P. lanceolata* containing different levels of iridoid glycosides. The results are discussed in terms of the role that generalist and specialist herbivores and their natural enemies play in selection for direct chemical defense.

METHODS AND MATERIALS

Plants. P. lanceolata used in this experiment was derived from an artificial selection experiment (for details see Marak et al., 2000), in which plants were selected on the basis of high and low concentrations of total leaf iridoid glycosides for four generations. Selection resulted in an average 3-fold difference in leaf iridoid glycoside concentration between upward and downward selected lines (Marak et al., 2000, 2003). Within each of these selection lines, iridoid glycoside concentrations vary considerably among maternal half-sib families, so that much larger, up to 20-fold, differences are present between extreme families from these selection lines. For the current experiment, seeds from the six most extreme female half-sib families from each selection line were used. Ten seeds from each family were germinated in a growth cabinet (14/10 hr and 25/15°C L/D), and transplanted individually into plastic 2.2 l pots with a mixture of potting soil and sand (4:1 v:v). Plants were grown in a greenhouse (16/8 hr and 22/18°C L/D) and regularly fertilized with half strength Hoagland's nutrient solution. Two extra plants per family were grown and harvested 5 wk after transplantation for chemical analysis. Of each plant, 50 mg from the freeze-dried and fine-ground leaf material were extracted overnight in 10 ml of 70% methanol for analysis of iridoid glycosides (aucubin and catalpol) using HPLC (methods in Marak et al., 2002b). Another 300 mg of each plant were used for analysis of total nitrogen and phosphorus using a Technicon Traacs 800 autoanalyzer (Technicon Instruments Corp., Tarrytown, NY) following methods described in Novozamsky et al. (1983).

Specialist Insects. Unless indicated otherwise, cultures of insect species were maintained in climate rooms under 16:8 hr L/D photoperiod under a constant temperature of 25 ± 0.5°C and 50 ± 2% RH. *M. cinxia* used in the experiments all originated from the Åland islands of SW Finland. Two different groups of *M. cinxia* larvae were used, one group (reared in 2000–2001) of which a subset was parasitized by *C. melitaearum* and another group (reared in 2001–2002) of which a subset was parasitized by *H. horticola*.

The first group of *M. cinxia* larvae consisted of approximately 200 offspring of field-caught butterflies that were reared at ambient temperatures in the laboratory from June to August 2000 (diapause as L5) on *P. lanceolata* plants that had been collected randomly from natural populations in the Åland islands. Larvae were maintained in diapause at 2°C under 6:16 hr L/D photoperiod until April 2001. At this time, diapause was broken by gently spraying water on the larvae. Upon resuming activity, larvae were randomly separated into two cohorts of approximately 100 individuals and placed into plastic boxes containing moistened paper towel and excised leaves of *P. lanceolata* containing either high or low levels of iridoid glycosides (see below for a quantitative measure). Leaves were selected from both plant lines, which were of approximately the same age and location on the plant, and were refreshed on a daily basis.

Approximately 100 cocoons of *C. melitaearum* were obtained from overwintering Åland laboratory populations in April 2001. Upon emergence, wasps were housed collectively in plastic Petri dishes (20 cm diam) at 10°C and were constantly supplied with honey and water. The remaining insects were reared according to standard protocol for cultures (top). Approximately half of the *M. cinxia* larvae reared on each line of *P. lanceolata* were randomly selected from culture as L6, and were presented individually to an adult female of *C. melitaearum* in small plastic vials. Oviposition was verified by a single insertion and removal of the ovipositor. Parasitized caterpillars were reared separately on leaves of *P. lanceolata* from either the high or low IG line, continuing the treatment prior to parasitism. Upon larval egression from the host, parasitoid brood sizes were determined, and following adult parasitoid emergence, wasps were killed by freezing. Egg-to-adult development time in days was determined, and adult (wet weight) body mass was measured using a Sartorius microbalance (accuracy 1 μg).

The second group of *M. cinxia* larvae was directly collected from the field. In contrast to *C. melitaearum*, experimental parasitation of *M. cinxia* by *H. horticola*, which parasitizes larvae before hatching, is extremely difficult. Therefore, eight larval groups of *M. cinxia* that had been observed being naturally parasitized by *H. horticola* (S. van Nouhuys, personal observation) were obtained from Åland populations in July 2001. *H. horticola* parasitizes 1/4 to 1/3 of the larvae in each larval group, so approximately 200 of the 800 larvae were parasitized. From L3, each larval group was split into two, and one half was reared (under standard rearing conditions, top) on intact plants from the high IG line and the other half on

plants from the low IG line. At the end of August, L5 larvae were removed from food-plants and maintained under diapause conditions at 2°C under 6:16 hr L/D photoperiod until April 2002. Thereafter, the experimental protocol was the same as for *C. melitaearum*.

The parasitized and the unparasitized larvae were reared together because they are indistinguishable from each other until just prior to pupation of the parasitoid. The development of healthy (unparasitized) larvae of *M. cinxia* reared on each line of *P. lanceolata* was monitored along with that of the parasitized larvae. At pupation, the development time (calculated in days from the breaking of disapause in L5 to adult butterfly emergence) and pupal wet weight (mg) of *M. cinxia* were measured from larvae reared on each line of *P. lanceolata*.

Generalist Insects. The host moth *S. exigua* was maintained on artificial diet as described by Vickerman and Trumble (1999). Moths were housed in 1 l plastic beakers containing a vermiculite base and were provided *ad libitum* with 20% sugar solution absorbed into cotton wool. Adult females oviposited onto filter paper placed inside the beaker. Newly hatched eggs were placed onto artificial diet. In rearing *C. marginiventris*, approximately 50 L1 larvae were placed into Petri dishes (20 cm diam) in which five mated female wasps were added. Wasps were allowed to parasitize hosts for several hours, after which they were returned to separate Petri dishes containing drops of honey and water. Cocoons of *C. marginiventris* were collected periodically, and wasps were allowed to emerge in Petri dishes (above).

For experiments, newly hatched larvae of *S. exigua* were reared on the two lines of *P. lanceolata* in April 2002. Newly molted L2 larvae of *S. exigua* were individually presented to female *C. marginiventris* in small plastic vials. Parasitism was verified by a single insertion and removal of the ovipositor. Parasitized larvae were reared using the same methods described for *M. cinxia*. At adult eclosion, wasps were killed by freezing, and wasps were weighed on a Sartorius microbalance. Egg-to-adult development time in days was also measured. The development of control (unparasitized) larvae was measured as for *M. cinxia*.

Statistical Analyses. Differences in weight and development time between hosts reared on high and low iridoid glycoside plants and between parasitoids developing on these two types of hosts were analyzed with independent t-tests if conditions for parametric analyses were met (Levene's test for homogeneity of variances and Kolmogorov–Smirnov's test for normality) or with Mann–Whitney U-tests otherwise. Differences in leaf concentrations of nitrogen, phosphorus, and the iridoid glycosides aucubin and catalpol between selection lines were analyzed with ANOVA. Line effects were tested over effects of maternal half-sib family within selection lines. Data for iridoid glycosides were square-root transformed prior to analysis to meet assumptions for parametric analysis. All analyses were performed using Statistica version 6.1 (StatSoft, Inc., Tulsa OK, USA).

RESULTS

Differences in Iridoid Glycosides, Nitrogen, and Phosphorus Among P. lanceolata Lines. Leaf iridoid glycoside levels differed 5-fold between plants from the low and from the high selection line (Table 1). The higher total iridoid glycoside level in the leaves was due to both higher levels of aucubin and of catalpol (Table 1). Nitrogen concentrations did not differ between selection lines (Table 1, $P = 0.10$). Phosphorus concentration varied among families within lines, but no consistent difference between selection lines was observed (Table 1, $P = 0.56$).

Development of Specialist Insects. Development time of *M. cinxia* postdiapause larvae until adult in 2001 was affected by plant line ($t_{17} = 2.49$, $P = 0.024$); butterflies developed more rapidly on plants containing high levels of iridoid glycosides (Figure 1a). However, host pupal weight was not affected by plant line ($t_{19} = 1.29$, $P = 0.21$), although there was a tendency for pupae to be slightly larger when reared on lines containing higher levels of iridoid glycosides (Figure 1b).

In line with 2001 results, development time in *M. cinxia* in 2002 was affected by plant line (Mann–Whitney U-test, $U_{30,24} = 502.5$, $P = 0.006$). Development proceeded more rapidly when larvae were reared on lines containing high levels of iridoid glycosides (Figure 1c). In both years, development was completed about 2 days earlier for cohorts reared on the line of *P. lanceolata* containing high levels of iridoid glycosides. As in 2001, the pupal weight of unparasitized individuals was not affected by plant line ($t_{68} = 0.70$, $P = 0.49$) although pupae tended to be somewhat larger than in the previous year (Figure 1d).

Development time ($t_{46} = 1.45$, $P = 0.15$) and adult body mass ($t_{46} = 0.97$, $P = 0.34$) of *C. melitaearum* were not affected by plant line on which their host had been reared (Figure 1e and f). Also, secondary clutch size of *C. melitaearum*

TABLE 1. LEAF CONCENTRATIONS OF THE IRIDOID GLYCOSIDES AUCUBIN AND CATALPOL, TOTAL *N*, AND TOTAL *P*, IN SIX MATERNAL HALF-SIB FAMILIES FROM EACH OF TWO *Plantago lanceolata* LINES SELECTED FOR LOW AND HIGH LEVELS OF IRIDOID GLYCOSIDES[a]

	Low line Mean (SE)	High line Mean (SE)	Line F[1,10]	Fam(Line) F[10,12]
Iridoid glycosides (% dw)	0.56 (0.09)	2.98 (0.33)	70.58***	1.26
Aucubin (% dw)	0.33 (0.07)	1.73 (0.16)	88.20***	1.31
Catalpol (% dw)	0.22 (0.03)	1.25 (0.33)	34.13***	1.03
Nitrogen (% dw)	4.02 (0.29)	3.41 (0.19)	3.23	1.08
Phosphorus (% dw)	0.49 (0.03)	0.48 (0.01)	0.36	7.72***

[a]Significance of differences between lines and between families nested within lines (ANOVA) are indicated (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$); sample size $N = 24$.

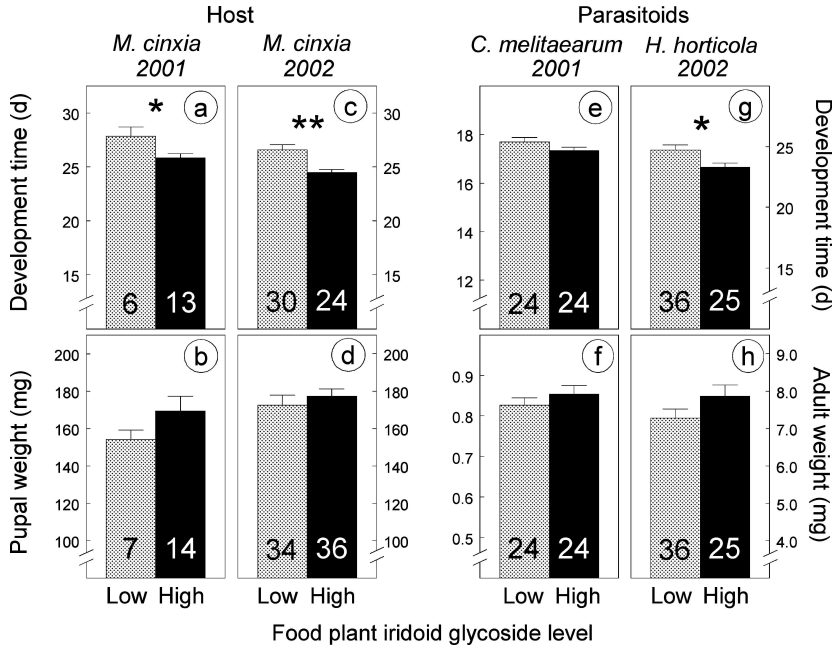

FIG. 1. Development of unparasitized caterpillars of the specialist herbivore *M. cinxia* feeding on *P. lanceolata* selected for low (gray bars) and high (black bars) levels of iridoid glycosides in 2001 (a and b) and 2002 (c and d) and development of its endoparasitoids *C. melitaearum* (e and f) and *H. horticola* (g and h) in parasitized caterpillars from the larval groups raised in 2001 and 2002, respectively. Numbers within bars represent sample sizes. Bars represent 1 SE of the mean. Asterisks indicate significant effects of plant selection line ($^{*}P < 0.05$; $^{**}P < 0.01$).

did not vary among wasps reared on *M. cinxia* that were fed *P. lanceolata* leaves containing high or low levels of iridoid glycosides ($t_{27} = 0.31$, $P = 0.76$). Brood sizes were on average only slightly higher for wasps reared from high (mean $\pm$ SE: 7.13 ± 0.69) than from low iridoid plants (6.71 ± 0.69).

As with the host *M. cinxia*, development time in *H. horticola* was significantly different when reared on hosts from the two lines of *P. lanceolata* ($t_{59} = 2.42$, $P = 0.019$). Parasitoids completed their development approximately 1–2 days earlier when originating from high iridoid lines (Figure 1g). However, plant line did not affect emerging adult parasitoid size ($t_{59} = 1.49$, $P = 0.14$) even though wasps tended be larger on high lines (Figure 1h).

Development of Generalist Insects. Egg-to-adult development time of *S. exigua* did not vary with the diet ($t_{19} = 0.78$, $P = 0.45$). Irrespective of iridoid glycoside content, larvae of *S. exigua* completed their development in

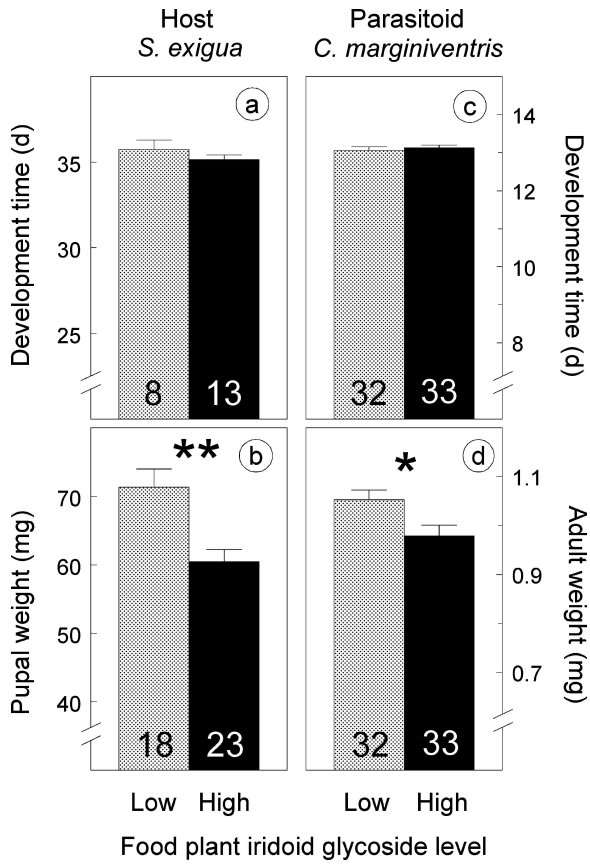

FIG. 2. Development of unparasitized caterpillars of the generalist herbivore *S. exigua* feeding on *P. lanceolata* selected for low (gray bars) and high (black bars) levels of iridoid glycosides (a and b), and development of its endoparasitoid *C. marginiventris* in parasitized caterpillars (c and d). Numbers within bars represent sample sizes. Bars represent 1 SE of the mean. Asterisks indicate significant effects of plant selection line ($^{*}P < 0.05$; $^{**}P < 0.01$).

approximately 35–36 days (Figure 2a). In contrast, pupal weight in *S. exigua* was strongly affected by the line of *P. lanceolata* upon which the larvae had developed ($t_{39} = 3.49$, $P < 0.001$). Pupae of *S. exigua* reared on the low iridoid line were some 15% larger than conspecifics reared on the high iridoid line (Figure 2b).

Similarly, development time in *C. marginiventris* was uniform when reared from *S. exigua* on both lines of *P. lanceolata* ($t_{63} = 0.51$, $P = 0.61$). Parasitoids typically took just over 13 days to complete their development in host caterpillars on the two iridoid lines (Figure 2c). However, like *S. exigua*, adult parasitoid size

differed with the plant line upon which their hosts had been reared ($t_{63} = 2.48$, $P = 0.016$). Wasps originating from the high iridoid line were 7% smaller than those originating from the low iridoid line (Figure 2d).

DISCUSSION

Our results show that the effect of genotypic variation in IG content in *P. lanceolata* on the development of herbivores differed between the generalist and specialist species used. High levels of IGs reduced the pupal weight of the generalist *S. exigua*. In contrast, IG levels did not affect the pupal weight of the specialist *M. cinxia*, and larvae of this specialist were able to complete their development more quickly on plants with higher levels of these allelochemicals. Levels of nitrogen and phosphorus did not consistently differ between the IG lines, suggesting that effects of allelochemical differences were not confounded by differences in primary metabolites. Our results are in agreement with previous studies on the effects of IGs from *P. lanceolata* on other generalist and specialist herbivores using artificial diet (Bowers and Puttick, 1988; Puttick and Bowers, 1988; Bowers, 1991); addition of aucubin and catalpol to artificial diet reduced larval growth rate of the generalists *Lymantria dispar* and *S. eridania*, but not of the specialist *Junonia coenia,* that developed more quickly on diets with higher amounts of IGs (but see Adler et al., 1995 for *in vivo* effects on *J. coenia*). This suggests that specialized mono- or oligophagous herbivores are better able to cope with plant allelochemicals than polyphagous herbivores. We note, however, that the generalist herbivore was derived from a lab-strain that did not necessarily have previous experience with *P. lanceolata*. As a consequence, it might have had limited opportunity to adapt to the chemistry of this host plant.

Recent studies provide insight into the effects of IGs on the performance of *S. exigua* and *M. cinxia*. Using the same selection lines, it was found (Biere et al., 2004) that the reduced growth of L4 *S. exigua* caterpillars on the high IG line was due to a reduced ingestion rate and not to a reduced digestibility or lower efficiency of conversion of ingested food. Thus, for the generalist, IGs act as feeding deterrents without additional post-ingestive effects, at least at this larval stage. In contrast, L4 caterpillars of *M. cinxia* had higher consumption rates on the high IG line (S. van Nouhuys and S. Kumar, unpublished results). Hence, for the specialist, IGs act as feeding stimulants. Moreover, L4 *M. cinxia* larvae ingested food from the high IG line more efficiently, and had a higher growth rate than caterpillars feeding on the low IG line. This contrasts with artificial diet studies of the specialist *J. coenia*, where lower efficiency of using high IG diets was observed due to reduced digestibility (Camara, 1997). As we did not observe a significantly higher pupal weight of *M. cinxia* reared on the high-IG plants in the present experiment, the higher growth rate of L4 *M. cinxia* may be instar-specific

or may not be sufficiently large to translate into a significant effect on weight at the pupal stage.

The development of endoparasitoids was also affected by the level of allelochemicals in the food plant of their herbivore hosts. For two of the parasitoids, developmental responses paralleled those of their hosts. High IG levels in host plants resulted in a more rapid development of both the specialist *M. cinxia* and its parasitoid *H. horticola* and in reduced weight in both the generalist herbivore *S. exigua* and its parasitoid *C. marginiventris*. In the only other study addressing effects of IGs on herbivore-parasitoid development (Mallampalli et al., 1996), a similar pattern, i.e., parallel responses of herbivore and parasitoid, was found. Early stages of the generalist herbivore *L. dispar* suffered reduced growth on diets with high levels of catalpol, but total larval development was not significantly affected, nor was that of the tachinid parasitoid *Compsilura concinnata* (Mallampalli et al., 1996). In the present experiment, only the parasitoid *C. melitaearum* was not affected by the IG level in the diet of its host, while its host showed a more rapid development.

A number of studies have reported detrimental effects of allelochemicals in the diet of host or prey on the growth and development of parasitoids and predators (Barbosa et al., 1986; Duffey et al., 1986; Gunasena et al., 1990; Havill and Raffa, 2000). In general, toxic phytochemicals in the plant tend to have more negative effects on the development of polyphagous herbivores and their natural enemies than on oligophages and their antagonists, presumably because the former are less-well adapted to cope with them (Gunasena et al., 1990; Barbosa et al., 1991; Sznajder and Harvey, 2003). Consequently, beneficial effects of the production of allelochemicals to the plant in terms of reduced plant damage by generalist herbivores may be partly mitigated by harmful effects of these allelochemicals on the natural enemies of the herbivore. On the other hand, slower development of generalist herbivores on more toxic host plants may increase their "window of vulnerability" to parasitoid or predator attack (the "slow-growth-high-mortality hypothesis," sensu Turlings and Benrey, 1998), enhancing the efficacy of natural enemies through higher parasitation rates, even if they have a slower development on these hosts. Since the development rate of neither *S. exigua* nor *C. marginiventris* was affected by IG level, such a mechanism does not seem to operate in the *P. lanceolata*—*S. exigua*—*C. marginiventris* system.

Positive responses (accelerated development while attaining similar size) to increased levels of IGs were observed for *M. cinxia* and the parasitoid *H. horticola*. This suggests adaptation of the parasitoid to the presence of IGs in its herbivorous host. Like other butterfly larvae that are restricted to feeding on IG producing host plants (Bowers and Collinge, 1992; Bowers, 2003), caterpillars of *M. cinxia* sequester IGs from their food plants (Suomi et al., 2001; Nieminen et al., 2003). Catalpol seems to be a more effective deterrent to predators than aucubin, and is also sequestered in higher proportions than aucubin in *J. coenia* (Bowers and

Collinge, 1992), *Ceratomia catalpae* (Bowers, 2003), and *M. cinxia* (Nieminen et al., 2003). Endoparasitoids must cope with sequestered or concentrated phytochemicals during their entire larval development because ingested allelochemicals cannot be excreted outside the host environment until after egression (Quicke, 1997). If we assume that *M. cinxia* caterpillars raised on food plants from the high-IG line sequester higher levels of IGs than caterpillars raised on the low line, then *H. horticola* does not appear to suffer from the higher levels of sequestered IGs in its host. On the contrary, like its host, *H. horticola* showed accelerated development while reaching similar weight, suggesting increased consumption or more efficient use of hemolymph, and later fat and other ingested tissues, from hosts raised on high-IG plants.

In contrast to *H. horticola*, the development of *C. melitaearum* was not affected by differences in IG levels in the diet of its host. In the field, parasitism of *M. cinxia* by *C. melitaearum* significantly decreased with increasing average level of catalpol in the *P. lanceolata* plant that larval groups of caterpillars had been feeding on (Nieminen et al., 2003). This trend could be the consequence of adult parasitoids avoiding oviposition into larvae that were feeding on plants with high levels of catalpol, or differential mortality of parasitoids developing in larvae feeding on high and low catalpol containing plants. Our results appear to support the first hypothesis.

The differential developmental responses of the two parasitoids may be due to the different host exploitation strategies they exhibit (Harvey and Strand, 2002). *C. melitaearum* is gregarious and passes through several generations during a single host generation. It completes development in a relatively short time, feeding primarily on host hemolymph until the last instar during which it feeds on a portion of the host fat body. In contrast, the development of *H. horticola* is more synchronized physiologically with that of the host. It inhabits hosts from early in the first host instar until just before the time that an unparasitized host would pupate. During most of this time, the parasitoid remains small and ingests little host hemolymph, but at the end of the final stage it consumes all host tissues except for the cuticle (E. Punju and S. van Nouhuys unpublished results; Lei et al., 1997). The positive effect of IGs on *H. horticola* development rate may be a direct response to an increase in host development rate mediated by allelochemicals ingested from its food plant.

Since the seminal paper by Price et al. (1980), many studies have assessed the effects of plant traits on the interaction between herbivores and their natural enemies (for reviews, see e.g., Bottrell et al., 1998; Turlings and Benrey, 1998; Cortesero et al., 2000), mostly by using agricultural, artificially assembled, biological control systems. Of great concern in classical biological control programs are cases where the combined effects of plant resistance and natural enemy impact on herbivores are less than the additive effect of each these components separately ("antagonistic" interactions sensu Hare, 2002). In

extreme cases, where natural enemies are more susceptible to plant resistance mechanisms than herbivores (e.g., Campbell and Duffey, 1979), chemical defense may prevent herbivore control by natural enemies ("disruptive interactions" sensu Hare, 2002). In agricultural systems, additive effects seem to predominate, and only few examples of disruptive interactions have been found (Hare, 2002).

In contrast to agricultural systems, knowledge of indirect effects of plant secondary metabolites on the natural enemies of herbivores in natural systems is scarce despite the fact that plant secondary chemistry has evolved under natural conditions. Non-additive effects might have a large impact on the evolution of levels of plant secondary metabolites ("direct defense") in natural populations. For instance, antagonistic effects may result in selection for lower levels of plant allelochemicals in the presence than in the absence of natural enemies, and we would not be able to understand patterns of selection in natural populations when studied in the traditional bitrophic plant-herbivore or plant-pathogen context (Hare, 1992, 2002).

Thus far, few studies have assessed the effects of quantitative allelochemical variation in non-agricultural plants on the development of higher trophic level organisms (e.g., Harvey et al., 2003; Sznajder and Harvey, 2003). As far as we know, our study is one of the first to document effects of genetic variation in secondary metabolites within a single natural plant species on the parasitoids of its herbivores. We suggest that there might be antagonistic effects of IGs on the generalist parasitoid of the generalist herbivore, but not on the specialist parasitoids of the specialist herbivore of *P. lanceolata*. Conclusive evidence for effects of natural enemies of herbivores on the evolution of concentrations of plant allelochemicals in natural populations still awaits studies that exploit natural study systems (i) in which there is evidence for top-down control of herbivores by parasitoids or predators, (ii) the pattern of selection on plant chemical defense differs in the presence and absence of natural enemies of the herbivores, and (iii) the evolutionary response to selection is investigated.

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EFFECT OF VOLATILE CONSTITUENTS FROM *Securidaca longepedunculata* ON INSECT PESTS OF STORED GRAIN

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Abstract—*Securidaca longepedunculata* Fers (Polygalaceae) is commonly used as a traditional medicine in many parts of Africa as well as against a number of invertebrate pests, including insects infesting stored grain. The present study showed that *S. longepedunculata* root powder, its methanol extract, and the main volatile component, methyl salicylate, exhibit repellent and toxic properties to *Sitophilus zeamais* adults. Adult *S. zeamais* that were given a choice between untreated maize and maize treated with root powder, extract, or synthetic methyl salicylate in a four-way choice olfactometer significantly preferred the control maize. Methyl salicylate vapor also had a dose-dependant fumigant effect against *S. zeamais*, *Rhyzopertha dominica,* and *Prostephanus truncates,* with a LD_{100} achieved with a 60 μl dose in a 1-l container against all three insect species after 24 hr of exposure. Probit analyses estimated LD_{50} values between 34 and 36 μl (95% CI) for all insect species. Furthermore, prolonged exposure for 6 days showed that lower amounts (30 μl) of methyl salicylate vapor were able to induce 100% adult mortality of the three insect species. The implications are discussed in the context of improving stored product pest control by small-scale subsistence farmers in Africa.

Key Words—*Securidaca longepedunculata*, Polygalaceae, methyl 2-hydroxybenzoate, methyl salicylate, olfactometer, fumigant toxicity, *Sitophilus zeamais*, *Rhyzopertha dominica*, *Prostephanus truncatus*.

INTRODUCTION

Interest in the discovery of new chemicals for the control of insect pests has continued to increase despite the commercial difficulties of bringing new products

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to market (Isman, 1997). Although much of this work is agrochemically driven to find novel compounds and modes of action, other aspects of bioprospecting such as providing low-cost "natural" alternatives to synthetic pesticides, which are safer and less polluting, have also continued. Poor rural farmers in developing countries particularly have difficulty in accessing good quality and affordable synthetic pesticides that are suited to their needs (Belmain and Stevenson, 2001). These farmers, however, have access to local ethnobotanicals and indigenous knowledge systems that could help increase agricultural productivity with minimal human and environmental health hazards that are often experienced when synthetic pesticides are used inappropriately (Belmain, 2002).

Securidaca longepedunculata Fers (Polygalaceae) is commonly used as a medicine in many parts of Africa for the treatment of rheumatic conditions, fever, headache, and various other inflammatory conditions (Oliver-Bever, 1986; Assi and Guinko, 1991; Iwu, 1993). Powdered dried roots are also used as a pest control agent and have potential as a protectant against insect pests in stored grain (Belmain et al., 2001; Boeke et al., 2001). During research to investigate the active principles in *S. longepedunculata*, we analyzed the volatile fraction and found a single major component, methyl salicylate (methyl 2-hydroxybenzoate), accounting for over 90% of volatile material together with two related minor components (Jayasekara et al., 2002). In this report, we evaluate the deterrent and toxic effects of methyl salicylate, the crude root material of *S. longepedunculata,* and a methanol extract of the root material against three major stored product pests, *Rhyzopertha dominica*, *Sitophilus zeamais*, and *Prostephanus truncatus* to determine if methyl salicylate is responsible for use of the plant material as a pest control agent. The implications of these results in storage pest management are discussed.

METHODS AND MATERIALS

Insects and Test Materials. Strains of *Sitophilus zeamais* Motschulsky, *Rhyzopertha dominica* (Fabricius), and *Prostephanus truncatus* (Horn) originally collected in Ghana were reared in the laboratory on organic maize, wheat, and maize, respectively, at 27 ± 5°C, 60 ± 5% RH, and a 12L:12D photoperiod. Roots of *S. longepedunculata* were collected from northern Ghana where they were immediately shade-dried for a period of 2 wk before transport to the UK. The plant roots were ground to a fine powder and extracted in methanol according to the methods described by Jayasekara et al. (2002). Methyl salicylate (Aldrich, Gillingham, Dorset, UK; 98%) was used for all bioassays.

Four-Way Choice Olfactometer Bioassay. Maize grain was treated with *S. longepedunculata* root powder, its methanol extract, methyl salicylate, and methanol only (as the control). The dried methanol extract (0.3 g), prepared according to Jayasekara et al. (2002), was re-dissolved in methanol (5 ml) and

mixed with maize (25 g) in a 100 ml glass jar for 1 min. Treated maize was dried for 15 min under a fume hood at room temperature (18°C). Another two sets of maize (25 g each) were treated separately with methyl salicylate (1.4 mg in 5 ml methanol) and methanol only (5 ml) by following the same procedure described above. Three glass tubes (15 cm × 2 cm diam) were filled separately with the maize samples and connected with polythene tubes to three of the four outlets of a four-way choice olfactometer arena as illustrated by Bashir et al. (2001). The fourth outlet of the arena was connected to a tube containing maize (25 g) mixed with *S. longepedunculata* root powder (1.25 g). A continuous stream of air was passed through each treatment and into the arena by using a vacuum pump (DA7C, Charles Austin Ltd, UK). The airflow rate through each treatment into the arena was set at 150 ml/min by using flow meters (D1X640, Meterate GPE, UK). Unsexed *S. zeamais* adults (7–14-days old; 200) that had been deprived of food for 4 days were introduced into the center of the olfactometer arena. The location of each insect was recorded after 12 hr. The bioassay was carried out in a controlled temperature and humidity room (27 ± 5°C and 60 ± 5% RH). Each experiment was repeated ×12, rotating the tubes containing the different maize commodity clockwise to the adjacent delivery pipe between trials to correct for any potential photoresponse behavior. The mean numbers of live adults in each treatment were compared by ANOVA, and means were separated by the LSD test at the 95% confidence level.

Fumigant Potential of Methyl Salicylate. A piece of cotton wool (8 mm × 3.5 mm height) was fixed to the center on the interior surface of a plastic lid to serve as a vapor diffuser inside a glass jar (1 l). Methyl salicylate (10, 20, 30, 40, 50, 60, and 70 μl) was applied with a syringe to the diffusers with 10 replicates per concentration per insect species. Fifty unsexed *S. zeamais*, *R. dominica*, and *P. truncatus* adults (7–14-days old) were introduced separately into glass jars (1 l), and the jars were capped with the lids holding the diffusers. After 24 hr, the dead adults in each jar were removed to separate clean jars and kept for a further 24 hr to confirm whether they were dead or alive, at which time the numbers of dead adults were recorded. The numbers of dead adults were subjected to Probit analysis to estimate the LD_{50} values of methyl salicylate.

To evaluate the effect of prolonged exposure of adult insects to low concentrations of methyl salicylate, the above experiment was repeated using 10 and 30 μl methyl salicylate and an untreated control. Dead adults in each jar were removed at 2-day intervals up to 6 days and collected in separate clean jars. As previously described, the total number of dead adults in each clean jar was recorded after a further 24 hr. The mean numbers of dead adults in each treatment were analyzed by ANOVA with the significant differences between means compared by using the LSD test. All statistical analyses were carried out using the program SPSS v.10 (SPSS Inc., Chicago, IL 60606).

RESULTS

The data from the choice olfactometer bioassay showed that the number of live *S. zeamais* adults found in maize treated with the *S. longepedunculata* root powder, methanol extract of the roots, and methyl salicylate was significantly lower than the number of insects present in the control (methanol only) maize (LSD, $P < 0.05$, Figure 1). Approximately half of all "non-participating" insects remaining in the arena were recorded as dead at the end of the trial. Living insects in the arena that had not made a choice at the time of data collection had usually settled along the edges of the arena. There were no significant differences in the number of insects present among the three treatments, suggesting the repellent effects of the treatments were similar.

The fumigant effects of methyl salicylate were evident and showed that insect mortality increased with increasing concentration (Figure 2). Doses of methyl salicylate above 10 μl in the 1-l jar increased insect mortality in all three insect species when compared to the untreated controls (LSD, $P < 0.05$). The observed mortality rates were dose-dependent, and 100% mortality occurred at 24 hr when there was 60 μl of methyl salicylate in the jars. Linear and nonlinear regression analyses of the data showed cubic polynomial relationships for each insect species with R^2 values of 0.98, 0.97, and 0.97 for *S. zeamais*, *R. dominica*, and *P. truncatus*, respectively. The LD_{50} and LD_{90} values, estimated by Probit analysis, showed similar values for all three insect species (Table 1).

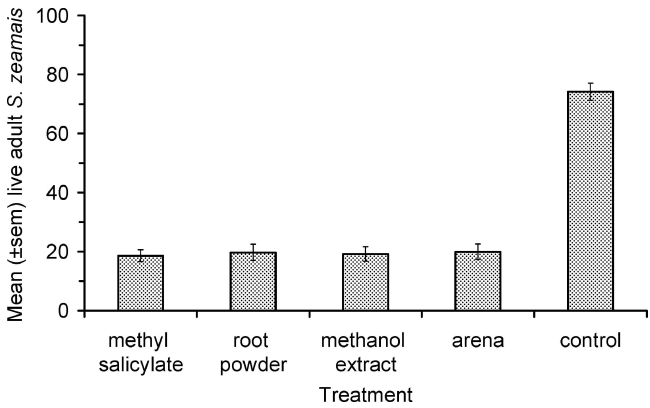

FIG. 1. Mean number of adult *S. zeamais* present in different treatments of maize grain after a period of 12 hr in a continuous airflow multiple choice olfactometer. Error bars represent the standard error of 12 means when 200 insects were initially introduced into the arena.

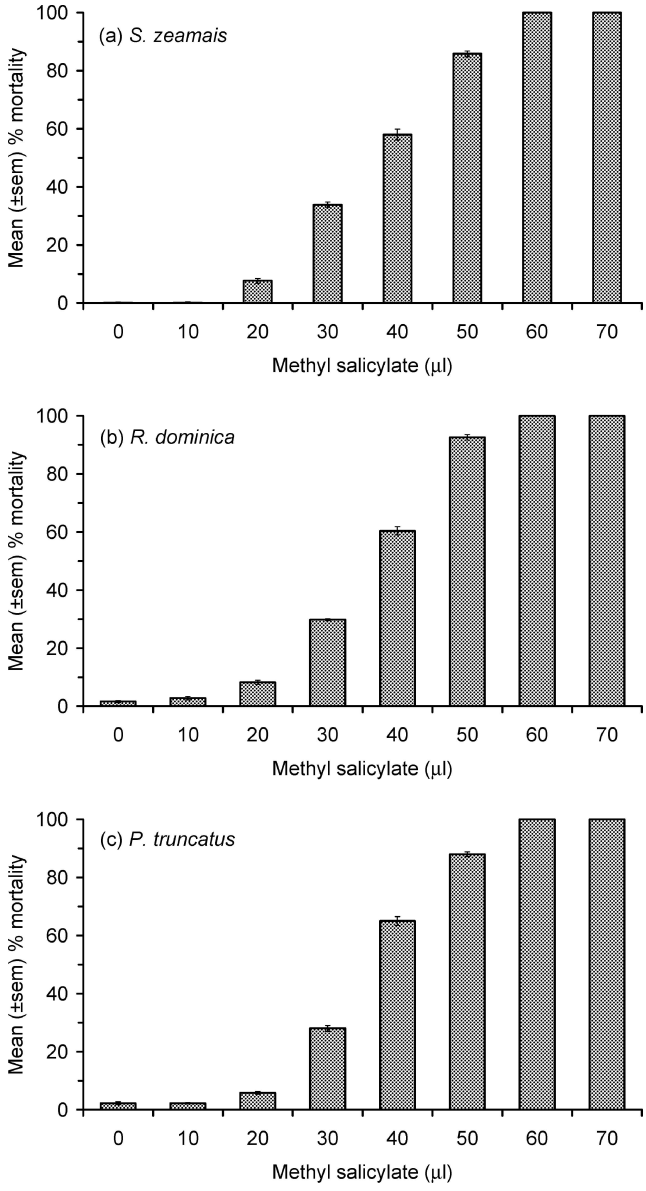

FIG. 2. The effect of dose (μl in 1-l jar) of methyl salicylate vapor on the adult mortality of (a) *S. zeamais*, (b) *R. dominica,* and (c) *P. truncatus* after a period of 24 hr. Error bars represent the standard error of 10 means when 50 insects were initially introduced into the fumigation chamber.

TABLE 1. PROBIT REGRESSION ANALYSIS ESTIMATES OF LETHAL DOSES OF METHYL SALICYLATE AGAINST *S. zeamais*, *R. dominica,* AND *P. truncatus*

Insect species	Probit Estimation	Dose (μl)		
		Lethal dose	Lower 95%	Upper 95%
S. zeamais	LD_{50}	36.17	35.46	36.88
	LD_{90}	51.27	50.23	52.41
R. dominica	LD_{50}	35.12	34.41	35.83
	LD_{90}	50.19	49.15	51.33
P. truncatus	LD_{50}	35.61	34.90	36.32
	LD_{90}	50.88	49.83	52.02

Amounts of methyl salicylate below the LD_{50} value resulted in high insect mortality with prolonged exposure times (Figure 3). After 2 days of exposure, 30 μl of methyl salicylate increased mortality compared to the control (LSD, $P < 0.05$), and 100% mortality was observed when insects were exposed to 30 μl of methyl salicylate vapor for 6 days. However, 10 μl of methyl salicylate did not show a strong effect on adult mortality even after 6 days of exposure.

DISCUSSION

Previous research has shown that methyl salicylate comprises more than 90% of the volatile components found in the roots of *S. longepedunculata* (Jayasekara et al., 2002). Methyl salicylate is a ubiquitous aromatic ester that is a well-known plant stress signal (Wees et al., 2000; Cardoza et al., 2002) acting as a semiochemical to recruit insect predators (Shimoda et al., 2002; James, 2003; De Boer and Dicke, 2004), is involved in host orientation and selection (Ninkovic et al., 2003), and has insect repellent properties (Hardie et al., 1994). Its antimicrobial and antifungal properties are well-established (Meiller et al., 2001; Papandreou et al., 2002), and these have been exploited in liniments, ointments, and toothpaste, and it has been widely exploited by the flavor and fragrance industry (Clark, 1999). The absorption of high concentrations of methyl salicylate in some traditional medicinal preparations can be fatal (Chan, 1996).

In this study, adult *S. zeamais* demonstrated clear orientation choices between the volatiles generated by untreated maize and maize that had been mixed with *S. longepedunculata* root powder, a methanol extract of the powder, or its main volatile component, methyl salicylate. This suggests that *S. zeamais* are able to detect methyl salicylate through olfaction and avoid it when given the choice. This could explain, at least in part, how the application of powdered roots of *S. longepedunculata* protects grain from insect infestation.

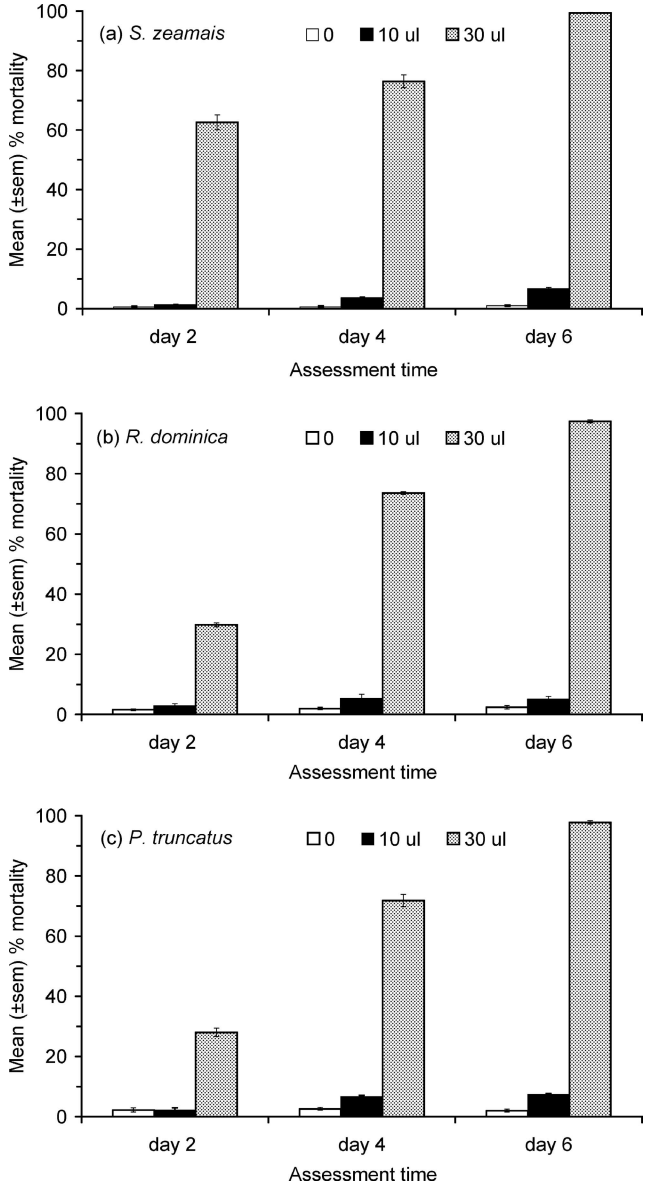

FIG. 3. The effect of prolonged exposure to methyl salicylate vapor on the adult mortality of (a) *S. zeamais*, (b) *R. dominica,* and (c) *P. truncatus* over a period of 6 days. Error bars represent the standard error of ten means when 50 insects were initially introduced into the fumigation chamber.

A number of stored product insects are attracted to host volatiles (Barrer, 1983; Phillips et al., 1993; Bashir et al., 2001). Pike et al. (1994) showed that *S. zeamais* is attracted to maize volatiles, and identified the main volatile components as hexanoic acid, nonanoic acid, nonanal, decanal, 2-phenylethanol, and vanillin. Methyl salicylate has not been previously reported to occur in the seeds of other grains or legumes, although it is emitted by damaged potato plants, and as a component of the blend attractive to the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera; Chrysomelidae) (Dickens, 2000). It would be interesting to test the effect of methyl salicylate on a species such as *P. truncatus* that does not orientate towards host volatiles and apparently relies on an aggregation pheromone (Hodges et al., 1998). However, the relative immobility of *R. dominica* and *P. truncatus* and their inability to walk through plastic tubing in comparison to *S. zeamais* prevented their inclusion in this experimental design. A different bioassay would be required to evaluate whether these species were similarly able to detect and avoid the volatile components of *S. longepedunculata* (Bashir et al., 2001). Methyl salicylate has been shown to induce plant defense responses (Ozawa et al., 2000; Wees et al., 2000), and it is possible that the avoidance of maize grains treated with methyl salicylate by *S. zeamais* is not due to the methyl salicylate itself but to other defense mechanisms induced by the methyl salicylate. This is considered to be unlikely due to the slow rate of metabolism of the grains, but the possibility cannot be excluded.

Avoidance of methyl salicylate vapors by *S. zeamais* was accompanied by a toxic effect on the insects. Dose- and time-dependant mortality effects were recorded when adult insects were in a sealed chamber containing the volatile compound. The low mortality recorded by 10 μl methyl salicylate in the 1-l container over 6 days may suggest that insects can tolerate low levels of methyl salicylate and potentially develop resistance to higher concentrations (Rajendran and Gunasekaran, 2002). However, this result may be an artifact of the experimental methodology because the fumigation chamber was opened every second day to remove dead insects, resulting in a partial loss of methyl salicylate from the chamber and diffuser.

Although there will be relatively minor volatile components present in the experimental treatments using the crude powder of *S. longepedunculata* roots and its methanol extract, our study suggests that it is the main volatile compound to which *S. zeamais* adults were responding. This is indicated by the similar numbers of insects found in the maize treated with the crude root powder containing approximately 4 mg of methyl salicylate in the experimental treatment (Jayasekara et al., 2002), the methanol extract of the root powder that also contained approximately 4 mg methyl salicylate, and the synthetic methyl salicylate at 1.4 mg per experimental treatment.

This research provides evidence that the indigenous strategy used by African farmers of mixing *S. longepedunculata* with their stored grain to protect it from

storage pests is valid. The study also helps elucidate the mode of action of the botanical through the effects of repelling and killing adult insects. For methyl salicylate to act as a true grain fumigant, the compound would have to possess sufficient grain penetrative properties to kill the insect developmental stages inside the grain (Shaaya et al., 1997; Lee et al., 2001). As with all fumigants, insect mortality will only be achieved if the material is applied within a sealed environment, ensuring the parameters of concentration and duration are sufficient to achieve 100% mortality (Rajendran, 2000; Reed and Pan, 2000). Using a volatile or essential oil compound to protect grain stocks may be more acceptable to some consumers than, for example, applying a dilute dust or powder that may result in higher residue levels (Shaaya et al., 1991; Isman, 2000). Nonvolatile, bioactive compounds present in *S. longepedunculata* have been recently characterized, and they also contribute to its observed efficacy in protecting stored grain (Jayasekara et al., 2003).

Evidence from other recent studies on *S. longepedunculata* indicates that there can be considerable variability in its bioactivity depending on the origin of the plant (Boeke et al., 2004). Therefore, potential phytochemical variability across African regions should be more adequately understood before the plant is promoted widely for pest control by small-scale African farmers. Although some preliminary evaluations of the potential vertebrate toxicity of *S. longepedunculata* have indicated that low concentrations of the material should be safe (Belmain et al., 2001), further investigations are essential to confirm the safety of mixing the botanical with stored grain.

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KINETICS OF THE NATURAL EVOLUTION OF HYDROGEN CYANIDE IN PLANTS IN NEOTROPICAL *Pteridium arachnoideum* AND ITS ECOLOGICAL SIGNIFICANCE

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Abstract—The time-dependent natural release of hydrogen cyanide (HCN) was studied quantitatively using young croziers of the neotropical bracken fern *Pteridium arachnoideum*. HCN production was quantified in crushed tissue using a flow reactor at 30.0 ± 0.1°C. Released HCN was carried into appropriate traps with a moist air flow. Aliquots were drawn from the traps at fixed time intervals, and the HCN concentration was evaluated spectroscopically. All available prunasin (Pru), the only cyanogenic glycoside present, underwent decomposition into HCN in less than 1200 min. Fiddleheads ($N = 76$) contained 1.84–107.70 mg Pru g^{-1} dw in a continuous fashion suggesting genetic polymorphism. Acyanogenic morphs were rare (1/77). From the kinetics of the samples with Pru content near the median histographic distribution ($N = 46$), accumulated HCN formation as a function of time, initial velocities, average HCN production rate, and corresponding rate equations were obtained. Initial and average velocities correlated well with total Pru content. The yield of cyanide liberation varied widely between 0.51 and 47.86 μg HCN min^{-1} g^{-1} dw and was a linear function of $[Pru]_t$. However, the β-glucosidase enzyme involved in this reaction was not rate limiting and occurs in excess in the natural system. Enzyme activity was found to be independent of $[Pru]_t$. The contribution of HCN as an allomone-upon-request against herbivores was assessed quantitatively. Bracken fiddleheads produced a pulse of HCN soon after tissue injury that waned rapidly, leaving a large portion of intact prunasin to decompose more slowly in the herbivore's lumen. The balance between the external

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and internal courses was found to depend on the concentration of prunasin in the plant, the amount of crozier eaten, and the time used to consume it.

Key Words—Cyanogenesis, kinetics, defense, herbivory, *Pteridium arachnoideum*.

Abbreviations: $[Pru]_t$, Total prunasin contained in a given sample; v_A, Average velocity of HCN formation; v_R, Average velocity of HCN evolution relative to $[Pru]_t$ in the sample; v_i, Initial velocity of HCN formation during the first few minutes after tissue crushing; $\Re$, The time-dependent accumulated formation of HCN g^{-1} dw of crozier relative to $[Pru]_t$; $\Delta v_A/\Delta\tau$, Time-dependent variation in the velocity of HCN evolution.

INTRODUCTION

Cyanogenesis, or the emanation of hydrogen cyanide (HCN), has long been recognized as an effective means of deterring predation (Ellis et al., 1977; Conn, 1981; Nahrstedt, 1988; Schappert and Shore, 1999a; Magalhaes et al., 2000). Plants, in particular, are capable of yielding HCN (Jones, 1998; Vetter, 2000) when their tissues are crushed during maceration by chewing herbivores (Vetter, 2000). In some tropical environments where insect pressure is high, as many as 4% of woody plants are cyanogenic and concentrate HCN precursors in reproductive parts (Thomsen and Brimer, 1997).

Since HCN is not only toxic to many animals, but also deleterious to the producing organism, it is generally stored as a stable precursor or phytoanticipin, glycosylated α-cyanohydrins of which many are known. Under the operation of an appropriate β-glucosidase, the glycoside portion can be excised. The cyanohydrin, which may itself be implicated in xenobiosis (Magalhaes et al., 2000), either decomposes on standing by way of β-elimination or under the auspices of a second enzyme, a hydroxynitrile lyase. Both routes yield HCN and a carbonyl component, generally an aldehyde or ketone that may have deterrent functions of its own (Peterson et al., 1987).

Gaseous HCN so produced is likely to be involved in trophic interactions (Jones, 1973; Jones et al., 1978; Conn, 1979). Thus, during feeding, HCN escapes into the atmosphere immediately surrounding the producer. However, for HCN to be an effective deterrent, a sufficiently high concentration of the gas must be attained (Nahrstedt, 1985; Jones, 1998). To achieve this goal, and in attention to the natural diffusion of this gas in air and its dispersion by wind, two ingredients must participate from the producer standpoint: the amount of HCN precursors available and the rate at which the cyanogenic glycoside-enzyme system is capable of releasing HCN.

Much attention has been paid to the first of these factors (e.g., Conn, 1981; Seigler, 1998; Gleadow and Woodrow, 2002; Goodger et al., 2002), in terms of

frequency of cyanogenic genotypes, actual content of cyanogenic glycosides in tissues at risk, and impact on associated biota (Zagrobelny et al., 2004). A great deal of variability has been observed. For example, cyanogenic species such as *Trifolium repens* contain enough cyanogenic glycosides to give between 3.2 and 350 μg of HCN g^{-1} dw, while others like *Linum usitatisimum* and *Dimorphoteca ecklonis* are capable of yielding a total of 910 and 1580 μg of HCN, respectively (Butler, 1965). The seasonal variability in cyanogenic glycoside content has also been recognized (Cooper-Driver et al., 1977; Gleadow and Woodrow, 2000; Gebrehiwot and Beuselinck, 2001). However, the amount of cyanogenic glycosides alone and their static potential to yield HCN have not satisfied all questions open to cyanogenesis as an effective defense strategy (Hruska, 1988; Gleadow and Woodrow, 2002).

With regard to the second component, the kinetics of the natural HCN gas formation from crushed plant tissue has rarely been investigated in cyanogenic plants. One recent report (Goodger et al., 2002) records the initial velocity of HCN release from foliage of cyanogenic genotypes of *Eucalyptus polyanthemos* in Southern Australia between 0.02 and 0.14 μg g^{-1} dw hr^{-1}. This is the result of the rate of the combined reactions leading to the formation of HCN. In a first approximation, we had estimated the pseudo first-order rate of prunasin decomposition in *Pteridium aquilinum* (L.) Kuhn as $2.20 \pm 0.01 \times 10^{-4}$ sec^{-1} (Alonso-Amelot and Oliveros, 2000). Earlier, the kinetics of the enzymatic transformation of the mandelonitrile was examined *in vitro* using *Hevea brasiliensis* enzymes (Jorns, 1980; Bauer et al., 1999). Both reaction products, HCN and benzaldehyde, were found to act as substrate competitors against mandelonitrile to inhibit the enzyme, indicating a degree of product self-regulation. These advances provide thrust to develop kinetic data of natural cyanogenesis in plants that might help to understand the ecological significance of this defense line and the toxic potential to animals and humans.

One of the cyanogenic plants in which a clear selection against the cyanogenic genotypes by vertebrates has been recognized is *P. aquilinum* (Cooper-Driver et al., 1977; Hadfield and Dyer, 1986; Low and Thomson, 1990). Few insects use this abundant resource as feed (Lawton, 1976; Jones, 1983; Salinas and Ortega, 1990). Cyanogenesis stemming from its only precursor, prunasin (Kofod and Eyjolfsson, 1966; Berti and Bottari, 1968), among other lines of chemical defense (Alonso-Amelot, 2002), is thought to be in part responsible (Schreiner et al., 1984).

Therefore, bracken fern stood as well-suited for examining its cyanogenic potential in terms of the rate at which HCN is produced upon crushing, in light of the hypothesis regarding its ecological role. Among the *Pteridium* taxon, the species *P. arachnoideum* (Kaulf.) Maxon (Pteridophyta: Dennstaedtiaceae) was selected because of its abundance and success in mountainous regions of the neotropics (Ortega, 1990) where (year round) pressure by herbivory is high and there is a high frequency of cyanogenic morphs (99%, personal observation). Thus,

the time-dependent evolution of gaseous HCN from this plant, the initial velocity of its formation, its correlation with the amount of prunasin present, the variation of the reaction rate with time, and the rate dependence on total prunasin content were investigated in the young bracken crozier. No efforts to derive a molecular mechanistic elucidation were attempted, and the results are only interpreted within the ecological context.

METHODS AND MATERIALS

Warning. Solid picric acid and sodium picrate may explode spontaneously when stored for long periods. It is advisable to maintain this material always as a supersaturated water solution or suspension at temperatures not exceeding 20°C and avoid all contact with skin to prevent long-term toxic effects. Sodium cyanide is highly toxic and should never be handled as solid, in neutral or acidic solutions without proper protection or outside a fume hood.

Reagents and Apparatus. Sodium bicarbonate (J. T. Baker Chemical Co.) and picric acid (BDH Laboratory Reagents) were used without further purification. Sodium cyanide was purified by recrystallization from ethanol–dichloromethane solutions and dissolved in degassed water. Almond emulsin (Sigma-Aldrich, Milwaukee, Wisconsin) was used as source of β-glucosidase. Absorbances of sodium picrate–cyanide complex were determined at 515 nm using an Hewlett-Packard (Palo Alto, California) Vectra UV-Vis 8453 spectrophotometer controlled by a Vectra-Pentium I workstation. Field temperature measurements were determined from mid-April to mid-May of 1999 and 2002 at sites A and B with the aid of StowAway XTI field logs (Onset Computer Corp., Bourne, Massachussetts).

Plant Sampling. *P. arachoideum* grows in dense thickets in open areas affected by fire or cattle husbandry, and in natural grasslands in the Andes mountain range of western Venezuela. Plants can persist for many years without further human intervention (Alonso-Amelot and Rodulfo, 1996). Voucher specimens were collected and stored in the laboratory herbarium, and also sent to the New South Wales Botanical Gardens in Sydney, Australia (NSW 361276). Four sites for the survey of cyanogenic morphs were selected in the 1880–2100 m altitudinal range. All sites were located between 8°30′ to 8°42′N and 71°04′ and 71°19′W in the environs of the city of Mérida, Venezuela and surrounding mountains. Sampling was performed at the onset of the rainy season, between late March and May of 2003. At each site, 25–35 croziers of *P. arachnoideum* (30–40 cm long) were cut at ground level, stored in plastic clip bags, and brought to the laboratory within 2 hr. Ten of the collected croziers were analyzed for moisture content, and the remaining were used for chemical analysis. As it was determined that the crozier apex contained the greater amount of prunasin, only this part was used for the kinetic measurements.

Temperature Determination of Kinetics Runs. To emulate in the laboratory the thermal conditions where cyanogenesis of croziers takes place naturally in the field, it was necessary to determine the temperature at which bracken stands grow in our geographical area. An average temperature of 29.9°C during the hours of maximum insect activity (10 A.M.–5 P.M.) was recorded within the bracken canopy during the April–May period with the aid of field logs. Therefore, the kinetic runs were standardized at 30.0 ± 0.1°C.

HCN Analysis. The sodium picrate gas flow quantitation method of Alonso-Amelot and Oliveros (2000) was employed as it provided the selective detection of HCN through its picrate complex at $\lambda = 515$ nm without interference from other carbonyl volatiles possibly stemming from the plant sample. Indeed, an acyanogenic morph of bracken yielded no response in the picrate absorption spectrum between 480 and 700 nm, confirming the lack of interfering components not associated with the process of cyanogenesis. Benzaldehyde and other carbonyl compounds were trapped in the first dinitrophenyl hydrazine solution through which the gas flow was passed (see below). A known amount (0.6–1.2 g) of intact, fresh bracken crozier heads were crushed with mortar and pestle using acid-washed sand (3–5 g) as abrasive (−15°C for 3 min). The cold mass was rapidly placed into a 12 ml flow reactor constructed from a disposable plastic syringe. The syringe was inserted into the gas-flow system and immersed into a circulating water bath at 30 ± 0.1°C at time zero when the gas flow was started. The gas flow was conducted through a first trap of dinitrophenyl hydrazine in acidic water–ethanol solution as previously described, and then through a saturated alkaline picrate solution (10.0 ml) where all HCN was trapped. With the aid of a precision syringe, a 1.0 ml aliquot was drawn every 20 min for 180 min, and then every 60 min. One last measurement was performed after 1200 min (20 hr) when no additional HCN evolution occurred. Immediately after every aliquot was drawn, 1.0 ml of fresh picrate solution was added to the gas trap. Therefore, the corresponding dilution factor was applied to calculate the actual amount of HCN incorporated into the picrate solution in every time interval, using the following equation

$$[\mathrm{HCN}]_i = [\mathrm{HCN}]_{m(i)} - 0.81 \times [\mathrm{HCN}]_{(i-1)} \tag{1}$$

where

$[\mathrm{HCN}]_i$ = increment in HCN concentration during the ith time period
$[\mathrm{HCN}]_{(i-1)}$ = increment in HCN concentration during the previous time period
$[\mathrm{HCN}]_{m(i)}$ = spectrally measured HCN concentration in each aliquot corresponding to the ith time period

The dilution factor (0.81) was derived from the combination of the remaining HCN in the picrate when an aliquot (1.0 ml) was drawn and the 9 ml remaining

solution was brought back to 10.0 ml by the addition of fresh picrate (1.0 ml). $[HCN]_{m(i)}$ was determined by contrasting the spectral absorbance at 515 nm of the HCN–picrate complex against a calibration regression ($r^2 = 0.99$, $P < 10^{-4}$) of NaCN solutions in the standard basic picrate reagent (range: 2.0–200.0 μg HCN equivalents).

All samples were allowed to stand in the gas flow reactor for a total of 20 hr, to ensure that all the prunasin was decomposed into HCN. To confirm that the measured HCN represented the total content of prunasin, a phosphate buffered (pH = 6.8) β-glucosidase solution (0.2% w/v) was added to the moist sand-crushed crozier mass in the flow reactor after the end of the kinetics experiment. The passing gas was examined for the presence of HCN. No additional hydrocyanic acid could be detected. Therefore, Eq. (2) conveyed the total amount of prunasin present.

$$[HCN]_{(1200\ min)} \times \left[\frac{MW(p)}{MW(HCN)}\right] = [Prunasin]_t \qquad (2)$$

Here MW is the molecular weight of species. A total of 76 croziers were studied kinetically.

Isolation of Prunasin. Modification of the method suggested by Brinker and Seigler (1992) was used. Freshly collected crozier heads (314 g) were dried under high vacuum (72 hr) yielding 43.5 g of material that was blended to a fine powder. This material was extracted in boiling methanol–water 4:1 (800 ml × 2) to counteract possible enzymatic decomposition of the cyanogenic glucoside. Each batch was then placed into an ultrasound bath for 30 min at 35°C. A 5% solution of lead acetate was added to the filtrate at room temperature to precipitate tannin and pigments. After filtration and centrifugation of solids, methanol in the supernatant was removed by rotary evaporation below 35°C. The remaining aqueous solution was frozen and freeze dried to a light yellowish powder (4.6 g) This material was purified by flash column chromatography using TLC-grade silica gel. Fractionation was conveniently monitored by β-glucosidase treatment of each fraction (5 μl) after solvent exchange to water at pH = 5.5, Sep-Pack (Millipore) pre-purification and HPLC determination of the formation of benzaldehyde (reverse-phase C_{18}, 10 cm Radial Pack column, MeOH–H_2O 70:30, 1 ml min^{-1}, 6.06 min ret. time, λ_{max} = 210 and 245 nm). This method requires only very small amounts of β-glucosidase and is more specific than the sodium picrate paper-TLC sandwich procedure (Brimer et al., 1983). The fraction eluting with ethyl acetate–acetone–methanol–water 16:3:2:2 gave the only HCN-yielding component. This fraction was further purified by thick layer chromatography (same solvent mixture, 8:1:1:1, $R_f = 0.3$–0.45) and crystallization from methanol–dichloromethane–hexane mixtures (187 mg, mp = 146–148°C).

Comparison of Enzyme Activity. Twelve field collected samples from the same sites above, each consisting of seven bracken fiddleheads were vacuum-dried,

reduced to a fine powder, and stored at -15°C until use. Each sample was subdivided in two subsamples. The kinetics of natural decomposition of prunasin to HCN in the first subsample (0.400 g dw) was determined as above except that, at the time of cold sand maceration, the original water content was reconstituted (1.6 ml corresponding to 80% moisture content). After 1200 min, the total amount of HCN collected in the picrate trap gave the prunasin content of the sample. At the time of cold sand maceration, to each one of the second subset of samples was added enough purified prunasin [as a cold (2°C) water solution, 16–231 μl, plus enough distilled water to complete 1.6 ml) to give a final concentration of 12.0 mg g^{-1} dw of the cyanogenic glucoside. The kinetics was then determined as above. In both sets, the initial velocities were calculated from the first degree coefficient of the 2° polynome to which the plot of accumulated HCN g^{-1} dw vs. time, up to 180 min, was best adjusted ($r^2 > 0.997$).

In a second series of experiments, the exact weight ($\pm 10^{-4}$ g) of approx. 0.35 g of fresh crozier head was determined, and the remaining material was used to estimate its dry weight. The sample was crushed with 10 g of fine sand at -15°C for 1 min adding 500 μl of phosphate buffer pH 6.8. The mixture was quickly placed inside the flow reactor, warmed to $30.0 \pm 0.1^{\circ}$C and the kinetics of HCN evolution determined during the first 180 min as described. A second sample excised from the same bracken plant was processed similarly except that 500 μl of β-glucosidase solution in phosphate buffer pH 6.8 was added during maceration. The enzyme solution was prepared from 1.0 mg of commercial almond emulsin dissolved in 1.0 ml of phosphate buffer, from which 50 μl was drawn and dissolved in 450 μl of buffer. The kinetics of HCN evolution was determined as before.

Statistical Calculations. Data were analyzed with Kruskal-Wallis and ANOVA tests of comparison of the means were performed using Statistix V 7.0 package (Analytical Software, St. Paul, MN). Curve fitting, plotting, and accompanying statistics were accomplished with Origin Professional software package V 5.0 (Microcal Software, Northampton, MA).

RESULTS

P. arachnoideum *Cyanogenic Potential.* The crozier phenological stage of bracken was selected as the best sample, because it contained the largest concentration of prunasin (Lawton, 1976). It was also the more fragile phase, with softer tissues, only one irreplaceable meristem, and close to ground-bound plant predators.

Table 1 shows the results of a homogeneity test for prunasin content of the four field populations. There were no statistically significant differences between the means (ANOVA within differences: $F = 3.28$, $P = 0.037$). Prunasin content ranged from 1.84 to 107.70 mg Pru g^{-1} dw (mean $= 18.62 \pm 20.82$ mg Pru g^{-1} dw) in a continuous fashion (Figure 1A) with 70% of the samples containing between

TABLE 1. COMPARISON OF PRUNASIN CONTENT (μg g^{-1} dw) IN CROZIERS OF FOUR ALLOPATRIC POPULATIONS OF NEOTROPICAL *Pteridium arachnoideum* ($N = 46$)

Population	A	B	C	D
Mean	21,661	11,596	31,687	23,614
SE	3,200.1	1,524.5	8,859.8	64,221.8
Minimum	7,613.2	4,725.0	12,827.2	24,20.1
Maximum	33,948.8	15,886.1	64,239.3	64,133.1

2 and 39 mg Pru g^{-1} dw (Figure 1B). Of the 77 samples collected, only one was acyanogenic. Efforts to find other acyanogenic genets among *P. arachnoideum* in the typical bracken grounds in the fields near Mérida failed. Squeezing young crozier heads between the fingers released enough benzaldehyde—the co-product of prunasin decomposition—to be clearly noticeable by smell, a quality that allowed for the fast identification of strongly cyanogenic populations of bracken in the field.

Kinetics of Cyanogenesis. In the kinetic runs of crushed croziers, the average rate of HCN formation v_A(μg HCN min^{-1}) was calculated from the increments per 20-min period of the evolution of HCN during the first 180 min of incubation at 30.0 $\pm$ 0.1°C. v_A of each independent sample was a linear function of

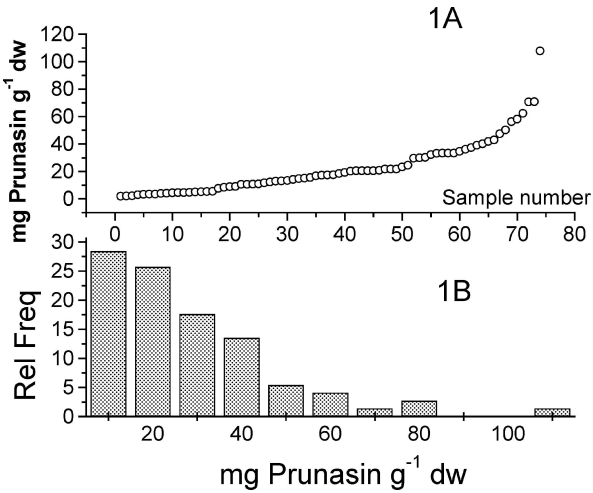

FIG. 1. Concentrations of prunasin in *P. arachnoideum* crozier head samples used in this study (A) ($N = 74$) and histogram of prunasin distribution in 10 mg g^{-1} dw intervals (B).

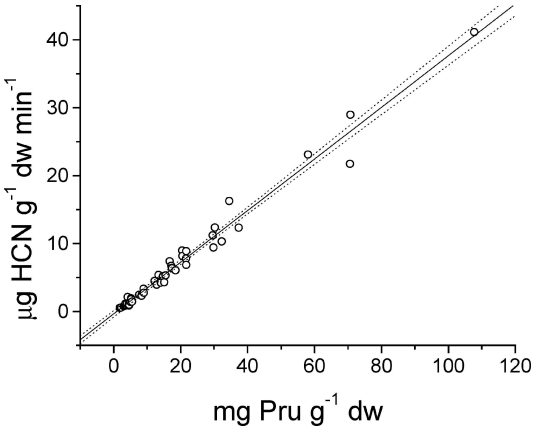

FIG. 2. Correlation between the increment of HCN production per unit time v_A (μg g^{-1} dw min^{-1}) and the total prunasin (Pru) (mg g^{-1} dw) contained in the crozier head. The linear regression followed the expression: HCN [μg g^{-1} dw min^{-1} $= -0.36$ ($\pm$0.23) + 0.38 ($\pm$0.008) $\times$ [Pru]$_t$. $N = 48$, $r = 0.989$, $SD = 1.180$, $P < 0.001$]. Dotted lines denote the 95% confidence limits of the regression.

the total prunasin [Pru]$_t$ available in the corresponding crozier (Figure 2). The slope of this equation gave v_R, which is v_A relative to [Pru]$_t$, 0.380 $\pm$ 0.008 μg HCN min^{-1} mg^{-1} [Pru]$_t$ during the first 180 min after tissue crushing. Based on this strong correlation, which suggests a first-order reaction on [Pru]$_t$, it was possible to examine collectively the whole set of kinetic experiments by normalizing all HCN g^{-1} dw of plant readings against [Pru]$_t$ in each individual crozier.

The time-dependent accumulated formation of HCN g^{-1} dw of crozier and relative to [Pru]$_t$ that emerged (Figure 3) followed a hyperbolic course whose mathematical expression [Eq. (3)] is the normalized rate equation of the HCN evolution in bracken croziers.

$$\Re(\mu\text{g HCN g}^{-1}\text{dw min}^{-1}) = \frac{P_1 t}{P_2 + t}[\text{Pru}]_t \tag{3}$$

where t = time (min)
$P_1 = 103.2 \pm 2.6$
$P_2 = 132.5 \pm 9.7$
$\chi^2 = 8.48$

In order to estimate the initial velocity v_i of the overall process, the accumulated formation of HCN (μg HCN g^{-1}dw) released during the first 180 min of individual bracken samples was adjusted mathematically to a 2° polynome. The

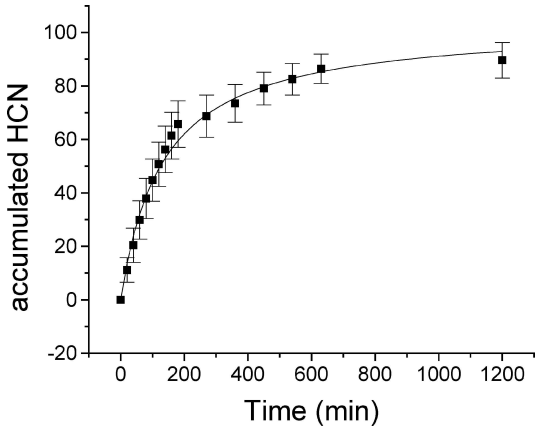

FIG. 3. $[Pru]_t$-standardized time-dependent accumulation of HCN [μg g^{-1} dw mg^{-1} $(Pru)_t$] produced by young croziers of *P. arachnoideum* until complete decomposition of the contained prunasin. Error bars are one standard deviation of the means ($N = 34$).

first degree coefficient, representing the slope at the onset of the data, gave v_i. It varied widely from 2.29 to 47.86 μg HCN g^{-1} dw min^{-1}, but it was also consistently positively associated with $[Pru]_t$ in each sample (data not shown) $v_i = 2.78\ (\pm 1.75) + 0.14\ (\pm 0.02) \times [Pru]_t$ g^{-1} dw; $r = 0.812$; SD $= 5.13$; $P < 0.001$).

Cyanide Evolution and Enzyme Activity. As the decomposition of prunasin not only depends on $[Pru]_t$ but also on β-glucosidase activity, there was the need to prove whether enzyme activity is tied to or is independent from $[Pru]_t$ in *P. arachnoideum*. To this end, the initial velocities of a set of 12 crozier samples were explored under two conditions: first, the kinetics of the accumulation of evolved HCN was monitored during the first 180 min, while $[Pru]_t$ was determined after 1200 min. To a second set of the same samples, enough purified prunasin was added to reach 12 mg g^{-1} dw and the kinetics was again determined. After 180 min, 57.24 $\pm$ 9.88% of $[Pru]_t$ had decomposed in the first set, whereas 49.86 $\pm$ 6.30% decomposed in the $[Pru]_t$ normalized group. Both figures were undifferentiated (ANOVA $F = 3.36$, $P = 0.062$), although the range of v_i was visibly narrowed by normalization of the prunasin content in the set of croziers studied (Figure 4).

In relation to the kinetics of prunasin decomposition in the presence of additional β-glucosidase, the initial velocities of HCN evolution with and without added enzyme were 45.36 $\pm$ 4.42 and 47.86 $\pm$ 3.52 μg HCN min^{-1} g^{-1}, respectively. After 100 min, the proportion of decomposed prunasin relative to $[Pru]_t$ was 0.242 and 0.250 with and without, added enzyme, respectively.

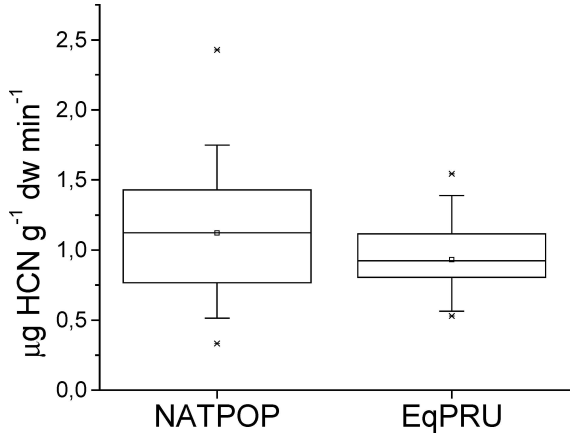

FIG. 4. Comparison of initial rates of HCN evolution (μg HCN g^{-1} dw min^{-1}) of a natural population (NATPOP) of *P. arachnoideum* croziers and the same samples whose prunasin content was corrected to 12.0 mg g^{-1} dw (EqPRU) by the addition of purified prunasin. The square symbol within the boxes denotes the means of the data, the horizontal lines in the box indicate the 25th, 50th, and 75th percentile values. Error bars denote 5th and 95th percentile values.

DISCUSSION

Tropical plants appear to be more stressed by invertebrate herbivory than those from temperate climates, due to greater insect species diversity and their year round presence. The ensuing pressure finds a response, for example, in the frequency of cyanogenesis (Thomsen and Brimer, 1997). Croziers of *P. arachnoideum* in the northern Andes appear to respond to this general pattern by their sole prunasin content, which collectively taken display a continuous trend (Figure 1A), and the elevated frequency of the cyanogenic genotype. Under the environmental constraints of these habitats, most of the population within the sampling universe of this study (>70%) synthesize and store 15–40 mg g^{-1} dw of prunasin, and some individual croziers contain in excess of 100 mg g^{-1} dw (Figure 1B). Upon complete conversion, this prunasin will turn into 1370–3660 μg HCN g^{-1} dw and occasionally up to 9150 μg g^{-1} dw. This quantity surpasses that found in some strongly cyanogenic strains of *Lotus corniculatus* such as the Aran variety from Ireland that can yield 2205 μg HCN g dm^{-1} and is selected against by voles (Viette et al., 2000). Within the sample range of $[Pru]_t$ found in *P. arachnoideum,* four populations (Table 1) that represented well the prunasin content of the majority of plants in the area were selected for the kinetic studies.

Prunasin content is insufficient to postulate a comprehensive cyanide-based defense in bracken. When the natural prunasin–HCN conversion rate is considered,

two defense strategies emerge. First, if the rate of conversion is sufficiently fast, the HCN evolved should have a deterrent effect on the plant predator as it feeds on its tissues and protection should take place in the brief feeding time span. As a result, damage to affected plant parts may be limited. Secondly, if the rate of HCN formation is much slower than the rate at which the plant tissue is devoured, little HCN will be available for immediate deterrence and a large proportion of prunasin will enter the gut of the predator and continue its decomposition in the digestive tract. Confined to the lumen, the HCN released would be bound to cause the greatest harm. This will depend on the size, eating strategy of the predator, amount of plant eaten, and the intervention of metabolic processes of detoxification such as the rhodanese sulfur transfer and the β-cyanoalanine pathway (Beesley et al., 1985). Inhibition of plant β-glucosidase activity by the herbivore mouth secretions is also conceivable. The frond itself would suffer the greatest damage, but the loss of fitness or even death of the predator—as it occurs in farm animals exposed to cyanogenic pastures—will eventually be to the advantage of the remaining plant fronds.

At the temperature profiles of the tropical mountains where bracken thrives, the conversion of prunasin into HCN in *P. arachnoideum* occurs relatively fast, as indicated by the average initial velocity of 0.418 ($\pm$0.014) μg HCN min^{-1} mg^{-1} [Pru]$_t$ which converts to a mean absolute velocity of 6.722 μg HCN min^{-1} g^{-1} dw of plant. When compared with the only available data (Goodger et al., 2002) for *E. polyanthemos* foliage for which the initial velocity was calculated as 0.02 to 0.14 μg HCN h^{-1} g^{-1}dw, meaning 0.33–2.3 $\times$ 10^{-3} μg HCN min^{-1} g^{-1} dw, it is apparent that *P. aquilinum* possesses a much more powerful cyanide response to the crushing of its fiddleheads. The standard plot in Figure 3 also gives an estimation of the variation of this velocity as time progresses that it was found to follow a rapidly decaying exponential function very closely represented by equation (4):

$$\frac{\Delta v_{\mathrm{A}}}{\Delta \tau} = \mathrm{A[Pru]}_t e^{-(t/b)} \tag{4}$$

where

$A = 0.703 \pm 0.024$
$b = 164.99 \pm 10.11$
$t =$ time (min)
$\chi^2 = 7.15 \times 10^{-4}$

The abrupt decay in the rate of HCN production indicates that in *P. arachnoideum* the overall decomposition of prunasin into HCN is modulated to yield a flash of defense material soon after injury of plant tissue. The initial rate v_i is a function of the activity of reaction components, prunasin, and lytic enzymes in bracken. The significant positive correlation illustrated in Figure 2 suggests

that the genes encoding for prunasin and β-glucosidase synthesis, though independent, may be activated in concert. Consistent with this is the conservative percentage of prunasin decomposed after 180 min that took place in croziers containing a natural quantity of prunasin ($57.24 \pm 9.88\%$) and the same croziers with added prunasin to yield the same amount of the cyanogenic glucoside ($49.86 \pm 6.30\%$). Namely, in both cases the same proportion of prunasin was broken down. This, in addition to the narrowing of the initial velocities (Figure 4) of the 12 samples used for this part of the study, is an indication that within this group of crosiers, β-glucosidase activity is not rate limiting and possibly exceeds the required activity for effective decomposition of the prunasin contained in these fiddleheads. A concurring result stems from the absence of statistical differentiation in the initial velocities of prunasin decomposition in croziers with and without added β-glucosidase (45.36 ± 4.42 and 47.86 ± 3.52 mg HCN min^{-1} g^{-1} dw). Hence, not only an excess of enzyme activity is contained in these crosiers, but also the activity of the enzyme is independent of $[\text{Pru}]_t$. A similar conclusion emerged from the work of Goodger et al. (2002) in *E. polyanthemos*, although other trees of this genus did not show such a correlation (Gleadow and Woodrow, 2000).

The application of Eq. (3) gives a more accurate estimate to these considerations. When the necessary variables are introduced [Eqs. (4) and (5)], it is possible to calculate the amount of HCN evolved into the atmosphere as a function of time and prunasin concentration while the predator feeds on the bracken fiddleheads, as well as the amount of prunasin that becomes part of its lumen.

$$\mu\text{g HCN evolved} = \left\{\frac{P_1 \times t}{P_2 + t}\right\} \times [\text{Pru}]_t \times \left\{\frac{27}{295}\right\} \times 10 \times W \tag{5}$$

and

$$\mu\text{g HCN (equiv) ingested} = [\text{Pru}]_t \times \left\{\frac{27}{295}\right\} \times 10 \times W \times \left\{100 - \frac{P_1 \times t}{P_2 + t}\right\} \tag{6}$$

where

$[\text{Pru}]_t$ = total prunasin content (mg g dm^{-1}) in the crozier
W = weight of the crozier as dry mass (g)

The 27/295 factor is the ratio of HCN–prunasin molecular weights, and "10" is the resulting conversion factor of mg [Pru] into μg HCN and the 100 to 1 scaling in the plot used (not shown).

Application of these expressions to a chewing insect that, for example, eats 100 mg of fresh crozier head containing 20 mg Pru and 88% moisture, in 10 min, results in 2.01 μg of HCN and by extension 7.89 μg of benzaldehyde being

released in and around the mouth parts of the predator in this time. In this model, we assume that the equimolar amount of benzaldehyde is produced at the same rate as HCN, since both are products of the same reaction. Although the LD_{50} of HCN has been determined for many animal species, it remains to be ascertained whether sufficient HCN diffuses into the air and is capable of affecting the feeding behavior of insect herbivores. Benzaldehyde is repellent against ants (*Myrmica americana*) ($ED_{50} = 375\ \mu g\ ml^{-1}$) in regurgitates of *Malacosoma americanum* larvae when the caterpillars were fed cyanogenic *Prunus* leaves (Peterson et al., 1987). If the fraction of this solution taken up by each individual ant is assumed to be 0.5 μl, ED_{50} would be 1.87 μg of benzaldehyde in Peterson's model. Obviously, this much benzaldehyde would not result from the decomposition of an equivalent amount of crozier tissue (about 0.5 mg) that an individual ant might conceivably nibble from the crozier. Hence, deterrence by the liberated allelochemicals during insects feeding time is likely to be insufficiently effective, at least against *M. americana* unless HCN and benzaldehyde act as synergists, as linamarin and histamine do in *Zygaena* butterflies (Muhtasib and Evans, 1987). No information exists on such synergy in prunasin–HCN–benzaldehyde at this time, nor quantitative data on the alteration of insects feeding behavior and toxicity by these materials acting together is available. Equations (4) and (5) also show that the larger the animal and, therefore, the greater the amount consumed together with feeding rate, the greater the amount of HCN (and benzaldehyde) released (Table 2), so a more effective feeding deterrence may result.

Equations (4) and (5) also reveal that after 10 min of feeding time in our model insect case, less than 10% of the total HCN potential will have been released. The remaining prunasin, which amounts to 91.7% of $[Pru]_t$ will be transferred into the gut, representing a toxic potential of 22.17 μg HCN if it all

TABLE 2. AMOUNTS OF HCN (μg) RELEASED TO THE AIR AND INSIDE THE HERBIVORE GUT AS A FUNCTION OF QUANTITY OF *P. arachnoideum* CROZIERS DEVOURED AND TIME USED IN FEEDING ON THESE

	Amount of crozier eaten (g)					
	0.1		5		100	
Time (min)	Evolved	Eaten	Evolved	Eaten	Evolved	Eaten
1	0.22	23.96	10.79	1,198.2	215.5	23,963.4
5	1.05	23.13	52.27	1,156.7	1,045.0	23,133.7
10	2.01	22.17	100.65	1,108.3	2,013.7	22,166.0
50	7.76	16.42	387.88	821.1	7,757.0	16,421.4
100	12.06	12.12	602.97	606.0	12,059.9	12,119.7

Note. An average content of 20 mg g^{-1}dw of prunasin was assumed. Calculations were performed using Eq. (4) and (5).

decomposes within the next few hours. This quantity, which amounts to 2217 ppm for a 1 g insect, and the accompanying 87.03 μg of benzaldehyde are bound to cause considerable damage unless the rate of rhodanese, β-cyanoalanine, or other enzyme based processing of HCN into innocuous material or the inhibition of prunasin decomposition into HCN is comparable or faster than the rate of prunasin–HCN conversion according to equation (3). At present, these combined digestive rates remain unknown.

Using a different animal model, sheep might be assumed to consume easily 50 g of fiddleheads mixed with pastures of the grassland in 5 min, hence evoking the evolution of 522 μg HCN in this time and ingesting enough prunasin to yield 11.6 mg of HCN in the gut, a potentially hazardous quantity. Adding the benzaldehyde co-product that is formed in an equimolar amount, 2.05 mg will be released into the sheep mouthparts possibly eliciting a sensory aversion reaction to the feed, while 45.5 mg of the aldehyde will be formed in the gut. The higher internal temperature of the herbivore would of course significantly enhance the rate of HCN–benzaldehyde production.

The model figures of Table 2 suggest that the emerging fronds of *P. arachnoideum* possess the potential capacity, in terms of $[\text{Pru}]_t$ and rate of HCN formation at temperatures commonly found in tropical bracken thickets, to embrace the two defense strategies postulated above, causing immediate feeding aversion in some herbivores, and preserving enough latent toxicity in its phytoanticipin form—prunasin—to cause damage inside the attacking organism. However, the balance of both defense routes in the overall HCN-based defense of bracken will depend strongly on the feeding rate, amount ingested by the specific herbivore, and its digestive detoxification reactions, thus opening opportunities to herbivore specialization.

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INFLUENCE OF DIET-RELATED CHEMICAL CUES FROM PREDATORS ON THE HATCHING OF EGG-CARRYING SPIDERS

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Abstract—Previous studies have shown that animals may make adaptive adjustments in response to chemical cues from predators, but hatching responses to diet-related chemical cues from predators have not been previously demonstrated. In the system studied here, the predator is an araneophagic jumping spider (Salticidae), *Portia labiata*, and the prey organism is a subsocial spitting spider, *Scytodes pallida* (Scytodidae). The spitting spider carries its eggs in its chelicerae, and carrying eggs is known to make it more vulnerable to predators. It is also known from an earlier study that the prior diet of the predator alters how dangerous the individual predator is to the spitting spider. In the experiments reported here, incubation time was shorter when volatile cues from the predator were present and longer in control tests when no chemical cues from the predator were present. The previous predator's diet also influenced incubation time: when in the presence of volatile cues from individuals of *P. labiata* that had previously fed on individuals of *S. pallida*, incubation time was shorter than when in the presence of volatile cues from individuals of *P. labiata* that had been feeding instead on house flies.

Key Words—Chemical cues, assessment, predation, anti-predator defence, hatching strategies, spiders, *Scytodes pallida*, *Portia labiata*.

INTRODUCTION

Animals can be envisaged as having ecological switch points at which they may exercise considerable adaptive plasticity. Evidence that chemical cues from predators influence switch-point decisions is now extensive, especially for aquatic species

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(Peckarsky, 1996; Chivers and Smith, 1998; Kats and Dill, 1998; Tollrian and Harvell, 1999; Dicke and Grostal, 2001; Peckarsky et al., 2002). The effects of predator-derived chemical cues include, besides overt behavior such as fleeing from or attacking the predator, adjusting foraging behavior (Cowan and Peckarsky, 1994; Peckarsky and McIntosh, 1998; Abrams, 2000), changing level of general activity (Sih and Wooster, 1994; McIntosh et al., 2003), undertaking morphological transformations during development (Tollrian, 1995; Relyea and Werner, 1999; Peckarsky et al., 2001; Relyea, 2001), adjusting the timing of metamorphosis (Werner, 1986; Skelly and Werner, 1990; Rowe and Ludwig, 1991; Skelly, 1992; DeVito et al., 1998), and adjusting the timing of reproduction (Mangel and Clark, 1988; Crowl and Covich, 1990; Reznick, 1990; Ball and Baker, 1996).

Recent findings suggest that, for some animals, egg hatching is another important life history switch point and that individuals may base hatching on chemical cues from their predators (Sih and Moore, 1993; Moore et al., 1996; Blaustein, 1997; Chivers et al., 2001; Laurila et al., 2002). Embryos may sometimes have sensory and processing capacities more or less comparable to those of the animal after hatching, and embryos sometimes control the timing of their own hatching (Sih and Moore, 1993; Kats and Dill, 1998; Chivers et al., 2001). Alternatively, for species that attend their eggs, the parent may alter the timing of hatching (i.e., "incubation time") when chemical cues from predators are detected (Li, 2002).

Besides detecting cues that simply reveal the presence or absence of predators in the environment, animals may also discern the level of risk from the predator (Keefe, 1992), For example, they may discriminate between fresh and old volatile chemical cues (Venzon et al., 2002), with fresh cues representing greater risk because the probability of a predator still being present in the environment is normally higher when the cues are fresh. In some instances, the predator's sex and how dangerous it is as a predator may be related, and there are instances in which animals rely on chemical cues for discriminating the predator's sex (Lehmann et al., 2004).

Animals may also detect diet-related cues from predators, with especially strong responses being provoked when the predator has recently fed on other individuals of the responder's own species. Besides overt behavioral responses, animals may make life-history adjustments (Crowl and Covich, 1990; Lima and Dill, 1990; Chivers and Smith, 1998; Kats and Dill, 1998; Laurila et al., 1998; Chivers and Mirza, 2001; Venzon et al., 2002). Here, we use a spider predator–prey system to investigate a life-history adjustment that has not been investigated before. We consider whether risk-revealing diet-related cues from predators influence incubation time.

An earlier study suggested that a female of *Scytodes pallida* Doleschall, a subsocial spitting spider, exercises some control over when her eggs hatch. In particular, when egg-carrying females of this species detect chemical cues from *Portia labiata* (Thorell), a web-invading jumping spider that often preys on

S. pallida (Jackson et al., 1998), hatching is speeded up (Li, 2002), but these cues had no evident effect on hatching when eggs were unattended.

When carrying eggs in its chelicerae (Li et al., 1999; Whitfield, 1999), *S. pallida* is more vulnerable to predation by *P. labiata*, and this predator eats the eggs as well as the parent. By spitting, *S. pallida* can defend itself against *P. labiata*. However, before it can spit, it has to release the eggs, introducing a delay and giving *P. labiata* an advantage (Jackson et al., 2002). Compounding the problem, *P. labiata* is predisposed to choose egg-carrying rather than eggless individuals of *S. pallida* (Li and Jackson, 2003). By speeding up hatching, *S. pallida* can free its chelicerae, become ready to spit (Li, 2002), and remove itself from the predator's preferred prey category (Li and Jackson, 2003).

Here, we extend earlier work on spitting spiders by investigating a fine-grain adaptive adjustment in timing of egg hatching. In particular, we investigate, for the first time, whether the predator's recent feeding history influences incubation duration.

METHODS AND MATERIALS

All experiments and all rearing were carried out in a controlled-environment laboratory ($25 \pm 1^{\circ}$C; 60–80% RH; 12L:12D, with lights on at 08:00 hr) using procedures that numerous earlier studies have made standard (Jackson and Hallas, 1986; Li, 2002). Only critical details are provided here.

In each trial, there was a test spider (an egg-carrying adult female of *S. pallida*; body length ca 7 mm) and a source spider (an adult female of *P. labiata*; body length ca 10 mm). For details concerning how egg-carrying *S. pallida* were obtained, see Li (2002). Scytodid eggsacs are only sparsely wrapped in silk, and we could readily discern the status of the eggs through the silk. No individual was used more than once as a source spider or a test spider. In the experiment, the source spider provided a substrate (blotting paper) used as a source of potential chemical cues. For 2 wk before collecting the substrate, each individual of *P. labiata* was fed one prey item every second day (i.e., it fed on a total of 7 house flies or 7 scytodids). Source spiders were assigned at random to one of two groups. Individuals in one group were fed house flies (*Musca domestica* L.) (fly-fed group), and the individuals in the other group were fed eggless scytodid females (scytodid-fed group). The body length of each prey organism (house fly or scytodid) was about 7 mm.

The spiders we used originated from Labrador Park, Singapore. Individuals of *S. pallida* used as *P. labiata*'s prey were collected from the field as needed, but the individuals of *S. pallida* used as test spiders were from cultures (reared from eggs in the laboratory). We used a split sibship design for assigning individuals

from the culture to the experiment, where a "sibship" is defined as the progeny of a particular male and female parent. Eight sibships were used, with the number of test spiders from each sibship being about equal. The maintenance diet of *S. pallida* in the laboratory was a variety of insects. Before the experiment, none of the test spiders had been exposed to *P. labiata*, and none had been in the presence of potential cues from *P. labiata*.

All test spiders were in webs when testing began. This was achieved by placing the scytodid in a cage ($150 \times 100 \times 50$ mm^3) together with a leaf from a camwood tree (*Baphia nitida* Lodd). These waxy leaves have a slightly concave upper surface and are known to be optimal web sites for *S. pallida*.

Substrate was prepared by putting each source spider in a sterile Petri dish (diam 90 mm), with a circular piece of blotting paper covering the inside bottom surface of the dish. The source spider was left in the Petri dish for 24 hr, after which it was returned to its cage. The lid of the Petri dish was replaced with fine mesh (0.1 mm), and the blotting paper was left inside the dish. Testing began about 12 hr later. During testing, the mesh stayed in place, preventing the test spider from contacting the substrate.

One day after oviposition, test spiders were assigned at random to one of three treatment groups, defined by the substrate (20 test spiders in each group). For scytodids in the scytodid-fed group, the substrate was from an individual of *P. labiata* that had fed on scytodids. For scytodids in the fly-fed group, the substrate came from an individual of *P. labiata* that had been feeding on house flies. For scytodids in the control group, there was only clean blotting paper in the Petri dish.

Immediately before testing began (at about 09:00 hr), the test spider was transferred to the experimental cage ($200 \times 200 \times 200$ mm^3). The leaf, with the scytodid on board, was fastened with Blue Tack (Bostik, Australia) to one of the top corners of the cage. One test spider was present in a cage per trial, and two house flies were provided once a week during the trial.

A Petri dish containing the substrate was placed at the center of the inside bottom of the cage. For the duration of each trial, one Petri dish was present in the test spider's cage, but each day each dish was replaced daily with a new one containing freshly prepared substrate of the same type (or new blotting paper for the control). Cages were monitored daily until eggs had either hatched or died. For each test spider, we recorded incubation time, hatching success (the number of eggs that hatched divided by the total number of eggs oviposited), and hatchling size (body length of spiderlings when they hatched). These three variables are referred to collectively as "hatching traits."

Batch size (i.e., the total number of eggs oviposited per eggsac) was determined once all hatchlings had emerged from the eggsac or once 30 d had elapsed after oviposition. This was done by counting the hatchlings and by teasing away the silk and counting any unhatched eggs that were found. For each trial, we

determined hatchling size by calculating the mean body length of five hatchlings chosen at random.

All data met the assumptions for parametric statistics. After showing treatment effects using multivariate analysis of variance (MANOVA), we used univariate analysis of variance (ANOVA) for further analysis. The multivariate F-statistic (Wilks' λ) was used because it does not require compound symmetry. For *post-hoc* comparisons, Tukey honestly significant difference (HSD) tests were used to test for differences between treatment means (Zar, 1996). All statistical tests were run by using SPSS.

RESULTS

Batch size did not differ significantly among treatments (mean $\pm$ SE $=$ 20.8 ± 0.6, 22.5 ± 0.5, and 21.6 ± 0.5 for the control, fly-fed, and *Scytodes*-fed *Portia* treatments, respectively; ANOVA, $F_{2,57} = 2.332$, $P = 0.106$), but there were significant hatching-trait differences across treatments (Figure 1, Table 1). Regardless of diet, incubation time was shorter when substrate from the predator was present than when only clean blotting paper was present (Tukey HSD, fly-fed vs. control, $P < 0.01$; *Scytodes*-fed vs. control, $P < 0.001$). Moreover, when the substrate was from predators fed on scytodids, hatching time was shorter than when the substrate was from predators that had fed on house flies (Tukey HSD, Tukey HSD, $P = 0.034$).

For both diets, hatchling size was smaller in the presence of substrate from the predator than in the presence of clean blotting paper (Tukey HSD, fly-fed vs. control: $P < 0.001$; *Scytodes*-fed vs. control: $P < 0.001$). However, hatchling sizes when in the presence of cues from scytodid-fed and fly-fed predators were not significantly different (Tukey HSD, $P = 0.336$). Hatching success did not differ significantly among treatments (Table 1).

DISCUSSION

Egg-carrying females of *S. pallida* evidently detect and respond adaptively to diet-mediated cues from *P. labiata*, an araneophagic jumping spider and a natural predator. Although previous studies have shown the importance of predator risk on egg hatching traits in a wide range of animals (Sih and Moore, 1993; Moore et al., 1996; Warkentin, 2000; Chivers et al., 2001; Li, 2002), our findings are different because they show that the chemical signature of *P. labiata* is influenced by *P. labiata*'s diet, and that *S. pallida* responds to the *P. labiata*'s diet-derived signature by reducing incubation time. In particular, we showed that volatile chemical cues from individuals of *P. labiata* that had recently fed on *S. pallida* induced shorter incubation times than did volatile chemical cues from individuals

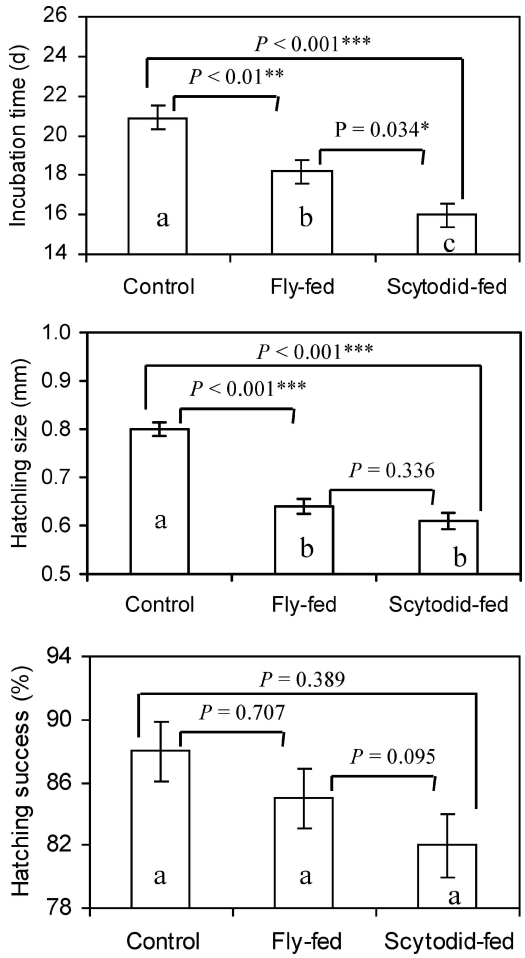

FIG. 1. Mean (±1 SE) incubation time, hatchling size, and hatching success of egg-carrying females of *Scytodes pallida* exposed to different potential volatile chemical cues. Cues from different substrates held in covered Petri dishes. Control: clean blotting paper. Fly-fed: blotting paper that had been in contact with a predator (*Portia labiata*) that had earlier been feeding on house flies. Scytodid-fed: blotting paper that had been in contact with a predator (*P. labiata*) that had earlier been feeding on *S. pallida*. Incubation time: interval between oviposition and hatching. Hatchling size: body length of spiderlings at hatching. Hatching success: number of eggs that hatched divided by total number of eggs oviposited. Different letters within bars indicate significant differences at $P < 0.05$, based on *post-hoc* Tukey's HSD tests.

TABLE 1. MANOVA SHOWING OVERALL EFFECTS ON *Scytodes pallida* OF TREATMENT (PREDATOR DIET) ON EGG HATCHING TRAITS (INCUBATION TIME, HATCHLING SIZE, AND HATCHING SUCCESS) AND ANOVA SHOWING EFFECTS OF EACH TRAIT

	Wilks' λ	*F*	*df*	*P*
MANOVA	0.264	17.379	6,110	<0.001
ANOVA				
Incubation time		15.633	2,57	<0.001
Hatchling size		52.748	2,57	<0.001
Hatching success		2.289	2,57	0.111

of *P. labiata* that had recently fed on house flies. When *S. pallida*'s eggs hatched sooner, they hatched at a smaller size (about 25% smaller) when in the presence of predator cues than in the controls. Additional research is needed for determining whether reduced size at hatching is in some way costly to the *S. pallida* female.

In a variety of animals (Chivers and Smith, 1998; Kats and Dill, 1998; Wisenden, 2000; Chivers and Mirza, 2001), including spiders (Persons et al., 2001), it is known that chemical cues from a predator that has been eating conspecifics elicit stronger anti-predator responses from the prey animals, and that chemical cues from predators that have been eating other types of prey elicit weaker responses. A reasonable interpretation of this literature might be that, after preying on a given species, various predators become more dangerous to other individuals of the prey species on which they just fed (see Persons et al., 2001), but this specific hypothesis is typically left implicit or else accepted as a foregone conclusion. However, this hypothesis was investigated explicitly in a recent study on *Scytodes–Portia* interactions (Jackson and Li, 2004). This study showed that *P. labiata*, after feeding on a single individual of *S. pallida*, forms search images for, and becomes more dangerous to, scytodids.

Scytodes pallida appears to be a prey species in which threat-sensitivity (Helfman, 1989; Bishop and Brown, 1992) is especially pronounced (i.e., it appears to be a species in which plastic response to different levels of threat from predators is pronounced). Evidently, the strength of *S. pallida*'s hatching response to *P. labiata* varies adaptively in relation to how dangerous the individual *P. labiata* becomes as a consequence of this individual's prior diet. This study provides the first experimental support for this conclusion.

There is a need for research designed to clarify the precise origin of, and to derive a biochemical characterization of, the volatile cues by which *P. labiata* influences the hatching of *S. pallida*. More is known about similar systems from aquatic animals (reviews: Chivers and Smith, 1998; Wisenden, 2000; Chivers and Mirza, 2001), and the extensive experimental work on aquatic systems will be a

useful starting point for future work on the chemical-mediation of *Scytodes–Portia* interactions.

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PARALLEL ARMS RACES BETWEEN GARTER SNAKES AND NEWTS INVOLVING TETRODOTOXIN AS THE PHENOTYPIC INTERFACE OF COEVOLUTION

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Abstract—Parallel "arms races" involving the same or similar phenotypic interfaces allow inference about selective forces driving coevolution, as well as the importance of phylogenetic and phenotypic constraints in coevolution. Here, we report the existence of apparent parallel arms races between species pairs of garter snakes and their toxic newt prey that indicate independent evolutionary origins of a key phenotype in the interface. In at least one area of sympatry, the aquatic garter snake, *Thamnophis couchii*, has evolved elevated resistance to the neurotoxin tetrodotoxin (TTX), present in the newt *Taricha torosa*. Previous studies have shown that a distantly related garter snake, *Thamnophis sirtalis*, has coevolved with another newt species that possesses TTX, *Taricha granulosa*. Patterns of within population variation and phenotypic tradeoffs between TTX resistance and sprint speed suggest that the mechanism of resistance is similar in both species of snake, yet phylogenetic evidence indicates the independent origins of elevated resistance to TTX.

Key Words—Coevolution, parallel evolution, resistance, *Taricha*, tetrodotoxin, TTX, *Thamnophis*, toxicity.

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INTRODUCTION

Both the fitness consequences that drive an "arms race" and the traits that evolve as a result depend on the phenotypic interface of coevolution. In other words, the phenotypic traits that mediate the interaction between ecological partners serve as both agents and targets of selection (Brodie and Brodie, 1999b; Brodie and Ridenhour, 2003). In chemically mediated interactions, the phenotypic interface may revolve around a toxin. If the toxin is severe enough to drive an arms race between predator and prey, we would expect to see similar coevolutionary patterns in multiple predator–prey pairs wherein prey possesses this toxin. This scenario, of course, requires that the usual prerequisites for coevolution are met, including the genetic ability of each species to respond to reciprocal selection and the occurrence of ecological interactions that allow the traits to generate selection (Berenbaum and Zangerl, 1992). The existence of such "parallel arms races" would be evidence that the phenotypic interface in question is a driving force behind the patterns of trait covariation observed. Although parallel arms races might be observable as a single prey species with different successful predators in different parts of its range, the strongest case for the evolutionary significance of the phenotypic interface would be independent species pairs of coevolving predators and prey.

Parallel arms races might involve identical traits on both sides of the phenotypic interface or on only one. If two prey species share a common deadly toxin, their respective predators might have responded by evolving the same means of circumventing the toxin, or they might have evolved different mechanisms of exploitation. If both a chemical and mechanism of resistance to the chemical are similar in parallel arms races, this suggests that constraints of some type are involved in determining the evolutionary response to selection in the parallel systems. Some of the best examples of constrained parallel evolution come from the phylogenetically diverse but mechanistically similar adaptations of insects to insecticides (Mallet, 1989; McKenzie and Batterham, 1994). Conversely, other cases of insecticide resistance involve traits as varied as behavioral, enzymatic, and physiological defenses to the same classes of chemicals, indicating that evolutionary responses need not always play out in similar dimensions (Mallet, 1989; Denholm et al., 1999). Analogous possibilities exist for the other side of the interaction as well, such that different prey species might repeatedly evolve similar defenses with respect to a single predator, or evolve unique defenses with respect to a common exploitative trait of its predators. Characterization of the existence and nature of parallel arms races is a first step in understanding the generality of the ecological context related to any phenotypic interface of coevolution.

The phenotypic interface of the interaction between the garter snake *Thamnophis sirtalis* and the newt *Taricha granulosa* is the neurotoxin tetrodotoxin

(TTX). Tetrodotoxin binds to voltage-gated sodium channels in nerves and muscles, thereby blocking action potential propagation. Because TTX has high binding affinity for most sodium channel types in most species (Hille, 1992; Narahashi, 2001), it has broad and extreme toxicity. TTX is found in a wide variety of taxa (Miyazawa and Noguchi, 2001) including all species of newts in the genus *Taricha* (Brodie et al., 1974; Yotsu et al., 1990; Yotsu-Yamashita, 2001). Levels of TTX in *Taricha granulosa* can be extremely high, making the newts lethal prey to almost all potential predators (Brodie, 1968; Hanifin et al., 1999). The common garter snake, *Thamnophis sirtalis*, appears to have entered such an arms race. Some populations of *T. sirtalis* that are sympatric with toxic *T. granulosa* have evolved physiological resistance to TTX, and levels of resistance generally covary with toxicity of newts across a broad geographic range (Brodie et al., 2002). Phylogenetic comparisons suggest that the entire genus *Thamnophis* has slightly elevated resistance to TTX, predisposing this group to engagement at a phenotypic interface involving TTX (Motychak et al., 1999). Despite this predisposition to and apparent evolutionary lability of TTX resistance, *Thamnophis sirtalis* is the only species known to be apparently evolving with toxic newts. The apparent lack of parallel arms races involving TTX and resistant snake predators is paradoxical with predictions of the importance of dangerous prey to predator–prey coevolution (Brodie and Brodie, 1999b).

Following the observations of predation in the wild, we investigated TTX resistance and toxicity in sympatric populations of a second species pair of garter snakes (*T. couchii*) and newts (*T. torosa*) from California. Our results indicate that not only has this species of garter snake evolved elevated resistance to TTX present in local newts, but also that similar patterns of costs to TTX resistance exist, suggesting similar mechanisms of resistance. The phylogenetic relationships of *T. couchii* and *T. sirtalis* (de Queiroz et al., 2002) indicate independent origins of elevated TTX resistance in these two species. The similarity of both predator and prey sides of the phenotypic interface in these apparent parallel arms races indicates not only that TTX is a potent driving force behind coevolution in these taxa, but also that some form of evolutionary or physiological constraint has led to parallel phenotypic evolution in the predatory traits mediating this coevolution.

METHODS AND MATERIALS

Population Samples. Adult female garter snakes (*Thamnophis couchii*) and both juvenile and adult newts (*Taricha torosa*) were collected from Cold Springs Creek, nearby Tyler Creek, and small adjacent ponds, from the Greenhorn Mountains in the Sierra Nevada Range, Tulare County, CA, USA. Subjects were collected (18–20 May and 12–14 June 2001) and will be deposited at the California Academy of Sciences.

Snake Resistance. Tetrodotoxin resistance data were collected on 68 neonate snakes born in the laboratory to six wild-caught females 4 August-5 September, 2001. Females were housed individually in 10 gallon glass aquaria with a thermal gradient (24–30°C) and a 14:10 L:D photoperiod. Females had constant access to water and were fed farm-raised mollies (*Poecillia* sp.) weekly. After parturition, neonates were measured for mass, snout-vent length (SVL), and total length, housed individually in plastic tubs (15 cm diam by 10.5 cm tall), and watered once daily.

Resistance to TTX was scored by using a bioassay of whole organism performance (Brodie and Brodie, 1990; Brodie et al., 2002). At 3–5 d after birth, each individual was raced for 2 m on a 4-m by 0.1-m racetrack with a substrate of indoor/outdoor carpet and equipped with infrared sensors to electronically time sprint speed over 0.5-m intervals. Each neonate was tested twice on one day (3–4 hr apart) to determine "baseline speed." The maximum 0.5-m speed in each trial was taken as a measure of maximum sprint speed.

The following day (20–21 hr after the last speed trial) each neonate was given an intra-peritoneal injection of a known dose [see below) of TTX [crystalline $3\times$ in citric acid–sodium citrate buffer (Sigma) diluted in amphibian ringer solution]. Thirty min after injection snakes were tested on the racetrack to determine "postinjection speed." Forty-eight hr later, snakes were again tested, up to three times total per snake. Control injections of physiological saline have no effect on snake performance (Brodie and Brodie, 1990). "Resistance" was scored as the percentage of an individual's baseline speed crawled after injection (postinjection speed/baseline speed). Individuals that are greatly impaired by TTX crawl only a small proportion of their normal speed, while those unaffected by a dose of TTX crawl 100% of their baseline speed.

A population-level dose response curve was calculated from individual neonate responses to five levels of TTX injections (0.5, 1, 2, 5, and 10 μg) using the linear regression $y' = \alpha + \beta x'$, with the transforms $y' = \ln((1/y) - 1)$, where y is resistance as a percentage of baseline crawl speed, and $x' = \ln(x)$, where x is the dose of TTX in mass-adjusted mouse units ("MAMU") (for further details of analysis, see Ridenhour et al., 2004). From this regression model, we estimated the "50% dose," defined as the amount of TTX required to reduce the average snake to 50% of its baseline speed. Because TTX resistance is related to body size within and among some populations of *Thamnophis sirtalis* (Brodie and Brodie, 1990, 1999a; Ridenhour et al., 2004), and to compare levels of TTX resistance to populations of *Thamnophis sirtalis* (Brodie et al., 2002), we transformed doses using a population-level mass-adjusted measure. A dose in MAMUs was calculated by dividing a given dose of TTX by the mean neonate mass of the population (as measured after the final baseline speed trial), then dividing by the amount of TTX sufficient to kill 1 g of mouse in 10 min (Brown and Mosher, 1963); 1 "mouse unit" = 0.0143 μg of TTX. One MAMU is, therefore, one mouse

unit of TTX per gram of snake. Neonate garter snakes ($N = 56$) were tested at one common dose (0.005 mg TTX) to determine whether families differed with respect to average resistance at this dose; resistance scores were analyzed using a one-way ANOVA in JMP v 5.01 (JMP, 1989–2002).

Phenotypic tradeoffs between locomotor performance and TTX resistance have been detected in populations of *Thamnophis sirtalis* (Brodie and Brodie, 1999a). To investigate the presence of similar tradeoffs in *T. couchii*, we examined the slope of a regression of postinjection speed on baseline speed. If TTX affects all individuals equally, then the slope of the regression will be one, and the effect of TTX is purely additive and reflected in the intercept. If, however, the effect of TTX is related to the speed of an individual, then the slope should differ from one: a slope <1 indicates a tradeoff wherein the fastest individuals have low resistance, while a slope >1 indicates that faster individuals have greater resistance. Regression analyses were performed in JMP v 5.01 (JMP, 1989–2002). Because both variables are measured with error, reduced major axis (RMA) regression was also examined. Results of RMA converge quantitatively with Model I regression as the error ratio exceeds 2, and so only Model I results are reported.

Newt Toxicity. Newts were brought to the laboratory (Utah State University), weighed, SVL measured, and frozen at -80°C within 5 d of field collection. Individual tissue samples from each subject were taken from the dorsal surface between the pelvic and pectoral girdle. This region of skin has a uniform distribution of skin glands, and TTX levels from the dorsum show little within individual variation (Hanifin et al., 2004). We removed a small (5 mm diam) circle of skin with a human skin-biopsy punch (Acu-Punch^{TM}, Acuderm Inc.) for toxin analysis. Only skin and the thin layer of connective tissue between the skin and dorsal muscle was removed.

Toxin was extracted from each skin sample by grinding a single tissue plug (0.19 cm^2) with 800 μl extraction solution (0.1 M aqueous acetic acid). Samples were shaken, heated, and spun following procedures described previously (Hanifin et al., 1999, 2004). The levels of TTX were quantified by fluorometric HPLC following the protocol of (Yasumoto and Michishita, 1985; Hanifin et al., 1999). Data acquisition and chromatographic analysis were performed with System Gold software (version 8.1, Beckman Inc.). Peak area concentration curves were calculated with standards prepared from commercial TTX (Sigma).

We estimated whole newt toxicity by using the relationship of dorsal skin toxicity (from skin punches) to whole animal toxicity described by Hanifin et al. (2004). The relationships between TTX concentration and newt size SVL, and between whole newt toxicity and newt SVL were estimated with regression using JMP v5.01 (JMP, 1989–2002). Graphical comparisons of whole newt toxicity and snake resistance at the population-level were made using these whole newt toxicity measures and a projection of average snake resistance as a function of body size.

To determine how much toxin would reduce the performance of the average snake in a population by a given amount, we used the relationship of injected to oral doses of TTX (Williams et al., 2001) and interpolation of the population average MAMU dose from the population curve (described above or from Brodie et al., 2002 for Benton Co., OR *T. sirtalis*) yielding the equation: mg TTX = [(Mouse Unit × oral/injected dose) × snake mass] × resistance in MAMU. Curves were estimated for 15% (near immobility) and 50% baseline performance for the Cold Springs Creek population of *T. couchii* and sympatric *T. torosa*, and for the Benton Co., OR population of *T. sirtalis* and sympatric *T. granulose*. Data for *T. sirtalis* and *T. granulosa* were taken from Brodie et al. (2002) and Hanifin et al. (1999, 2002).

RESULTS

Snake Resistance. An adult *T. couchii* (50 g, 480 mm SVL-CAS 212868) was observed (J. V. Vindum and C. R. F., personal observation) swallowing a large juvenile *T. torosa* (1.85 g, 41 mm SVL-CAS 212869) in the wild at the Cold Springs Creek locality on 7 June, 2000. When discovered, the newt was visibly covered with secretion and was swallowed head first as far as its forelimbs. Upon collection, the snake disgorged the newt and both animals appeared unharmed for 3 d, after which they were preserved as voucher specimens.

At testing, the mean mass of neonate *Thamnophis couchii* ($N = 68$) was 3.7 ± 0.07 (SE) g, mean SVL was $195 + 1.3$ (SE) mm, and mean total length was 258 ± 1.7 (SE) mm. The average litter size was 11.2 and ranged from 7 to 15.

Neonate *Thamnophis couchii* from this population exhibit high levels of resistance to TTX. The population resistance curve was characterized by the regression: $y' = -6.91 + 1.55x'$. This relationship yielded an estimated 50% dose of 86.5 MAMU (95% CI 70.3–106.3 MAMU) for the population (Figure 1). Significant family level variation in resistance to 0.005 mg of TTX was detected (ANOVA: $F_{4,50} = 11.85$, $P < 0.001$).

Tradeoffs between resistance and locomotor performance were detected at the phenotypic level (Figure 2). The regression of postinjection speed on baseline speed was postinjection speed = 0.106 + 0.185 [baseline speed]. The slope of this regression was less than 1 ($t = -4.30$, df = 1, $P < 0.001$), indicating that slower snakes were relatively more resistant than faster snakes. An insufficient number of families prevented analysis of the genetic tradeoffs.

Newt Toxicity. Tetrodotoxin was detected in each of the newts collected from the Cold Springs Creek locality. Adult newts (>65 mm SVL; $N = 8$) had an average concentration of 0.065 mg TTX/cm^2 dorsal skin (range 0.028–0.133); juvenile newts (<50 mm SVL; $N = 11$) had an average concentration of 0.009 mg TTX/cm^2 dorsal skin (range 0.001–0.026). The concentration of

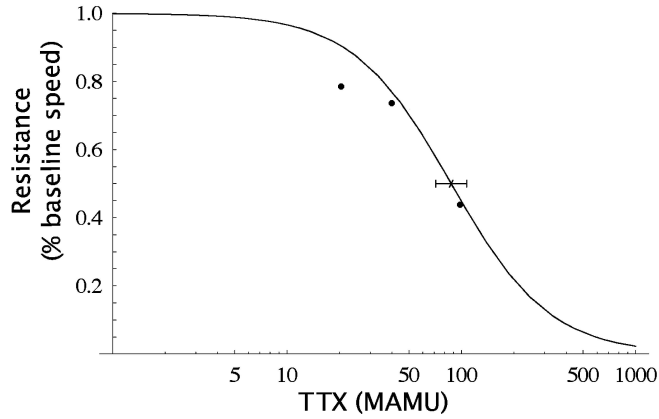

FIG. 1. Tetrodotoxin resistance in *Thamnophis couchii* from Cold Springs Creek, CA. The dose–response curve for neonate *T. couchii* was estimated over a range of TTX doses using curvilinear regression. The 50% dose (with 95% CI) is shown as a bar at 86.5 MAMU of TTX. Symbols indicate average response at each tested dose.

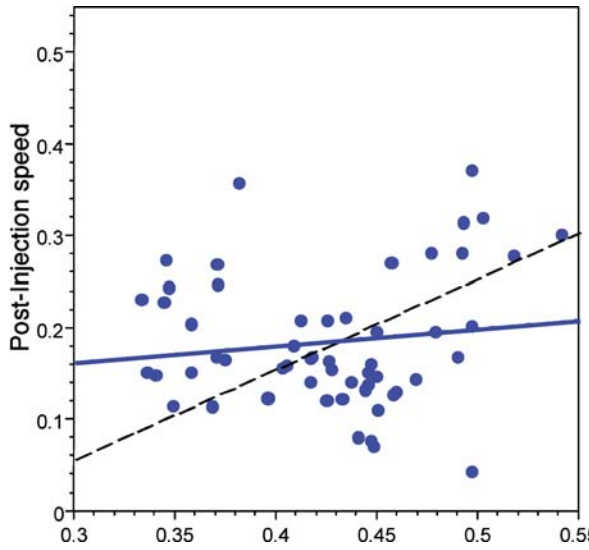

FIG. 2. Costs of tetrodotoxin resistance in *Thamnophis couchii*. Regression of postinjection speed on baseline speed (m/s) reveals a slope (solid line) significantly less than 1 (dashed line), indicating that slower snakes are relatively more resistant than faster snakes. Regression based on data from neonates snakes ($N = 56$) tested at 95.4 MAMU of TTX.

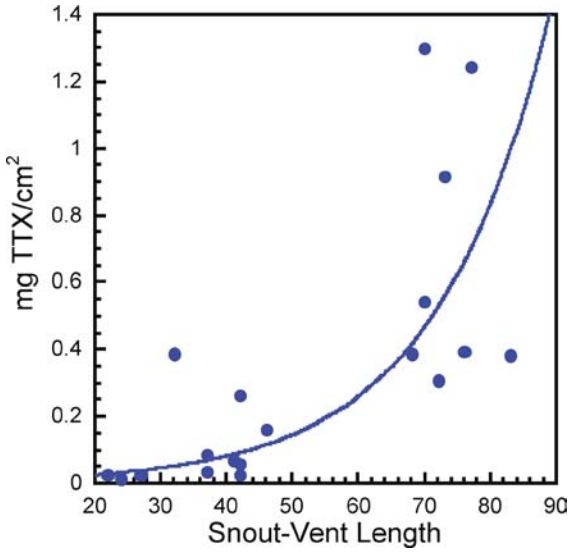

FIG. 3. Tetrodotoxin levels of *Taricha torosa* as a function of SVL (mm). The concentration (by area) of TTX in dorsal skin increases exponentially with SVL. This relationship appears to be driven primarily by differences between juvenile (<50 mm) and adult (>65 mm) newts.

TTX in dorsal skin increased with size of newt, but varied greatly among individuals [Figure 3; $\ln(\text{TTX/cm}^2)$ skin $= -7.596 + 0.642$ [SVL], $F_{1,17} = 54.34$, $P < 0.001$]. Whole animal estimates of TTX per individual indicated the average juvenile newt possessed 0.081 mg TTX in its skin, whereas the average adult newt possessed 2.41 mg of TTX. Whole animal TTX also increased with body size [TTX/individual $= -7.197 + 1.072$ [SVL]; $F_{1,17} = 145.77$, $P < 0.001$]. The effect of size on both skin concentration of TTX and total body TTX appeared to be primarily due to difference between newly metamorphosed and adult newts.

DISCUSSION

Thamnophis couchii from the Cold Springs Creek region of California have evolved high levels of resistance to tetrodotoxin (TTX). Previous studies of *T. couchii* and congeners from other parts of western North America have not detected TTX resistance in this group, even where these snakes are sympatric with newts of the genus *Taricha* (Motychak et al., 1999). As is the case for TTX resistance in another species of garter snake, *T. sirtalis*, among-family variation in level of resistance is apparent. Furthermore, tradeoffs between resistance and

locomotor performance similar to those detected in populations of *T. couchii* are observed in *T. sirtalis* (Brodie and Brodie, 1999a). The presence of such a cost suggests that similar physiological mechanisms might underlie TTX resistance in both species of snakes. Relatively high levels of TTX are present in sympatric *T. torosa*, though the quantity of the toxin varies among individuals and increases dramatically with size after metamorphosis (Figure 3). Field observations of an adult *T. couchii* eating a newt in the wild indicate at least some frequency of ecological interaction between these species.

Compared to known populations of *T. sirtalis*, the Cold Springs Creek population of *T. couchii* has relatively high resistance to TTX; this population would rank in the second highest category with only four populations of *T. sirtalis* exceeding its average 50% level resistance of 86.5 MAMU (Brodie et al., 2002). Interpopulational comparisons of snake resistance and newt toxicity in the *T. sirtalis* and *T. granulosa* interaction suggest fairly close phenotypic matching (Brodie et al., 2002). Projections of relative toxicity and resistance for a range of body sizes (Figure 4) suggest that adult *T. couchii* would be able to ingest most adult newts in the population without experiencing a complete loss of locomotor function. By contrast, adult *T. sirtalis* from the Benton County population in Oregon (one of the more resistant populations of snakes) are expected to frequently encounter newts toxic enough to fully immobilize them (Figure 4). Thus, the Cold Springs population of *T. couchii* appears to be more resistant relative to the toxicity of its sympatric prey than most studied populations of *T. sirtalis*.

Elevated resistance to TTX is a derived character state in the genus *Thamnophis* previously known only in *T. sirtalis*, despite investigations of other species of garter snakes that prey on amphibians and co-occur with *Taricha*. *Thamnophis sirtalis* and *T. couchii* are distantly related within the genus; *T. sirtalis* is a member of the sister group (with ribbon snakes) to all other garter snakes, whereas the aquatic garter snake species group that includes *T. couchii* is relatively derived (de Queiroz et al., 2002). The elevated resistance of Cold Springs Creek *T. couchii* likely represents an independent origin of TTX resistance within the genus, though other alternatives such as introgression of resistance alleles through hybridization currently cannot be ruled out. Species relationships among newts of the genus *Taricha* are less well known (Tan and Wake, 1995), but the presence of TTX in all three members of the genus, combined with the presence of TTX in *Notophthalmus* (the sister genus to *Taricha*) and other salamandrids (e.g., *Cynops* spp.) suggests that some level of TTX toxicity is ancestral in the group (Brodie et al., 1974; Yotsu et al., 1990; Yotsu-Yamashita, 2001). Previously examined populations of *T. torosa* from the Sierra Nevada of California possess TTX at levels well below those observed at the Cold Springs Creek locality (C. Hanifin, unpublished data). Although the source of TTX toxicity in newts is unclear, recent work (Hanifin et al., 2002; Shimizu, 2002; Cardall et al., 2004; Lehman et al., 2004) provides evidence that newts may be more active in the biosynthesis of their

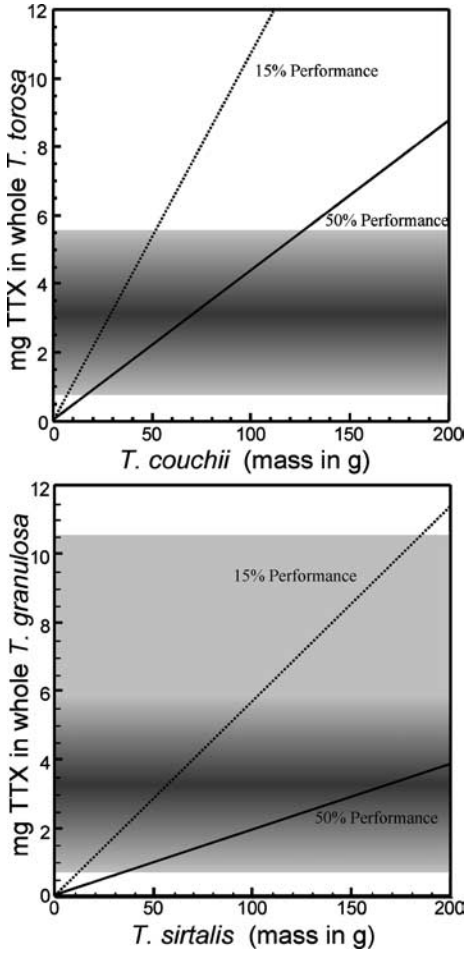

FIG. 4. Comparisons of prey toxicity and predator resistance from two localities. The average resistance for snakes as a function of body size is shown predicting the amount of ingested toxin that would reduce a snake to 15% (near immobility; dotted line) and 50% (solid line) baseline performance. On the vertical axis, the total skin toxicity of adult newts is shown, with the range of observed values shaded and the mean shown as a darker band. For a given size snake, the predicted effect of ingesting a newt of given toxicity can be estimated by finding the intersection of performance lines and newt toxicity. (A) Most adult *T. couchii* from the Cold Springs Creek, CA population (approximately 60–200 g) can ingest the average adult newt without suffering a performance reduction of 50%. (B) Few if any adult *T. sirtalis* (approximately 40–200 gm) from Benton Co., OR could ingest the average adult newt with less than a 50% reduction, and most would be expected to suffer much greater reductions of performance.

TTX than previously thought and that variation in newt toxicity within and among populations of newts may have a genetic basis. Taken together, these ecological and phylogenetic observations suggest that the predator–prey interaction between *T. couchii* and *T. torosa* in the southern Sierra Nevada of California has evolved in parallel to the interaction between *T. sirtalis* and *T. granulosa* in other parts of western North America.

Parallel "arms races" among distinct species pairs of TTX resistant garter snakes and toxic newts reveal both evolutionary lability and phylogenetic bias. The results presented herein show that resistance to TTX has evolved in at least two unrelated species groups in the genus *Thamnophis*, as well as at least twice within one species (*T. sirtalis*; Brodie et al., 2002; Geffeney et al., 2002). This pattern of diversity suggests considerable flexibility for a fundamental neurophysiological feature that is conserved across most vertebrates, the structure of voltage-gated sodium channels in skeletal muscle (Geffeney et al., 2002). Conversely, all known occurrences of elevated resistance to TTX by predators occur in just this single genus of snakes. Such restricted phylogenetic distribution of evolutionary response to dangerous prey is consistent with some form of historical bias, possibly related to the ancestral occurrence of low level resistance to TTX observed in the genus *Thamnophis* (Motychak et al., 1999). Similarly, TTX has been found throughout the genus *Taricha*, as well as in other salamandrids (Mosher et al., 1964; Brodie et al., 1974; Yasumoto et al., 1988), but not in other groups of urodeles. This restricted pattern of TTX distribution is difficult to interpret, however, because the mechanism of production of TTX in these species is still unclear.

The selective importance of deadly toxicity, and TTX in particular, as a feature driving predator–prey arms races is emphasized by the discovery of these parallel coevolutionary systems. The fact that independently derived predator lineages have evolved similar phenotypic responses to prey toxicity further suggests that there are phenotypic constraints to the array of evolutionary counteradaptations available to combat TTX toxicity. Lineage-specific constraints might also contribute to the phenotypic similarity in response, since the predator species in question belong to the same clade. Similar parallel evolution is known from other sorts of biological systems involving deadly toxins, most notably the evolution of insecticide resistance. Many phylogenetically diverse groups of insects have evolved resistance to human-introduced toxins through strikingly similar physiological and genetic mechanisms. In many cases, the parallelism of these adaptations can be attributed to the specific actions of insecticides designed to target fundamental metabolic or neurological pathways. Resistance to such toxins often is achieved by common changes to binding sites or enzymes targeted by the toxins (Mallet, 1989; McKenzie and Batterham, 1994; Ffrench-Constant et al., 2000). Parallelism in these cases is thought to result because there are limited ways to disable the toxins. A similar phenomenon might underlie the parallelism in predator resistance to TTX. Tetrodotoxin is known to bind to sodium

channels in nerves and muscles, thereby blocking action potentials. Resistance to TTX in one species of garter snake (*T. sirtalis*), as well as in other organisms that utilize TTX as a defense (pufferfish, newts), involves changes in the binding affinity of the sodium channel to TTX (Kao and Fuhrman, 1967; Kidokoro et al., 1974; Yotsu-Yamashita et al., 2000; Geffeney et al., 2002). If *T. couchii* shares a similar mechanism as suggested by the observed patterns of variation in and tradeoffs with resistance, then predator–prey arms races involving deadly prey may be under the same sort of metabolic control constraints as insect–insecticide systems.

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INHERITANCE OF RESISTANCE TO MAMMALIAN HERBIVORES AND OF PLANT DEFENSIVE CHEMISTRY IN AN *Eucalyptus* SPECIES

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Abstract—Hybridization in plants provides an opportunity to investigate the patterns of inheritance of hybrid resistance to herbivores, and of the plant mechanisms conferring this resistance such as plant secondary metabolites. We investigated how inter-race differences in resistance *of Eucalyptus globulus* to a generalist mammalian herbivore, *Trichosurus vulpecula*, are inherited in their F_1 hybrids. We assessed browsing damage of 3-year-old trees in a common environment field trial on four hybrid types of known progeny. The progeny were artificial intra-race crosses and reciprocal inter-race F_1 hybrids of two geographically distinct populations (races) of *E. globulus* north-eastern Tasmania and south-eastern Tasmania. Populations of trees from north-eastern Tasmania are relatively susceptible to browsing by *T. vulpecula*, while populations from south-eastern Tasmania are more resistant. We assessed the preferences of these trees in a series of paired feeding trials with captive animals to test the field trial results and also investigated the patterns of inheritance of plant secondary metabolites. Our results demonstrated that the phenotypic expression of resistance of the inter-race F_1 hybrids supported the additive pattern of inheritance, as these hybrids were intermediate in resistance compared to the pure parental hybrids. The expression of plant secondary metabolites in the F_1 hybrids varied among major groups of individual compounds. The most

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common pattern supported was dominance towards one of the parental types. Together, condensed tannins and essential oils appeared to explain the observed patterns of resistance among the four hybrid types. While both chemical groups were inherited in a dominant manner in the inter-race F_1 hybrids, the direction of dominance was opposite. Their combined concentration, however, was inherited in an additive manner, consistent with the phenotypic differences in browsing.

Key Words—*Trichosurus vulpecula*, hybrid, plant secondary metabolites, generalist herbivore, additive inheritance, *Eucalyptus globulus*, hybridization.

INTRODUCTION

Understanding the genetic control of plant resistance to herbivores is crucial in understanding the evolutionary processes behind plant/herbivore interactions (Marquis, 1990; Mauricio and Rausher, 1997), and hybridization offers an ideal opportunity to investigate some of these processes (Bailey et al., 2004). The investigation of the differential resistance of hybrid plants to herbivores, compared to the pure parental plants, has provided insight into the mode of inheritance of resistance in plants. Studies have shown that responses of herbivores to hybrids can vary greatly among plant species and also among different herbivores within one plant species. Various patterns of hybrid resistance have been observed. Hybrid plants may be more susceptible to herbivores than the parental plants (hybrid susceptibility pattern; Floate et al., 1993; Fritz et al., 1998; Dungey and Potts, 2003), more resistant than the parental plant (hybrid resistance pattern; Boecklen and Spellenberg, 1990; Eisenbach, 1996), and exhibit resistance that is intermediate to the parental plants, which indicates additive inheritance of resistance (additive pattern: Aguilar and Boecklen, 1992; Fritz, 2001; Scott et al., 2002). In addition to these patterns, hybrids have also exhibited resistance similar to one of the parental plants (dominance pattern; Hjältén, 1997; Orians and Floyd, 1997; Fritz et al., 1998; Hjältén et al., 2000). If hybrids resemble the susceptible parent, then there is dominance for susceptibility, while if the hybrid resembles the resistant parent, there is dominance for resistance. Finally, hybrids can exhibit resistance that is no different to either of the parental plants (no difference pattern; Fritz et al., 1996, 1998; Orians and Floyd, 1997).

Research exploring the response of herbivores to hybrids has predominantly focused on invertebrates, and this has been highlighted in several reviews on the ecological and evolutionary processes of hybridization in plants (Strauss, 1994; Fritz, 1999; Fritz et al., 1999; Orians, 2000). In comparison, the susceptibility of hybrids to mammalian herbivores has received considerably less attention (but see Vila and D'Antonio, 1998; Dungey and Potts, 2002; Scott et al., 2002; Anderson and Paige, 2003). Additionally, while many studies have focused on the phenotypic patterns of resistance of hybrids to herbivores, relatively few have investigated the

inheritance patterns of the defensive plant characteristics conferring resistance, such as plant secondary metabolites (Orians, 2000 for review; Byrd et al., 1999; Orians et al., 2000; Fritz, 2001; Hallgren et al., 2003).

During the past two decades, aspects of the ecological interactions of *Eucalyptus* species and the mammalian herbivores that feed on them have been investigated (e.g., Landsberg, 1987; Hume and Esson, 1993; Lawler et al., 2000; Close et al., 2003). There is clear inter- and intraspecific variation in browsing damage to some eucalypt species by mammalian herbivores (Lawler and Foley, 1999; Lawler et al., 2000; O'Reilly-Wapstra et al., 2002; Scott et al., 2002; Close et al., 2003). Plant secondary metabolites play an important role as the mechanisms behind this variation in resistance (McArthur and Sanson, 1993; Lawler et al., 2000; Wallis et al., 2002; Marsh et al., 2003). Recently, the role of plant genotype in influencing the resistance of a eucalypt species, *Eucalyptus globulus*, to browsing by a generalist marsupial folivore, the common brushtail possum (*Trichosurus vulpecula* Kerr 1792), was documented (Dungey and Potts, 2002; O'Reilly-Wapstra et al., 2002), and inter-race differences accounted for the majority of the genetically based variation observed by O'Reilly-Wapstra et al. (2002). A group of plant secondary metabolites known as formylated phloroglucinol compounds (FPCs) (Eschler et al., 2000) were found to be the most significant defensive leaf trait conferring resistance in juvenile coppice foliage of *E. globulus* (O'Reilly-Wapstra et al., 2004). The mode of inheritance of plant resistance and plant defensive traits within this system and in other eucalypt/mammalian herbivore systems, however, remains unclear.

Hybrid resistance to herbivores is determined not only by the genetic background of the plant but is also influenced by variation in environmental factors (Fritz, 1999) such as access to nutrients (Orians and Floyd, 1997). To understand the consequences of herbivore feeding on hybrid plants, it is necessary to differentiate the genetic and environmental causes of the hybrid resistance. Many investigations have been conducted in natural plant systems and, in some cases, these studies have not controlled for environmental variation and often do not know the genetic status of the hybrid plants (Aguilar and Boecklen, 1992; Whitham et al., 1994; Gange, 1995). Managed plant systems that grow hybrids of known pedigree in common environment field trials are ideal for investigating the inheritance of resistance in hybrids, without confounding effects of environmental influences. In this study, we aimed to investigate the inheritance of resistance to browsing by *T. vulpecula* and the inheritance of the defensive plant compounds within *E. globulus*. We assessed the browsing damage by *T. vulpecula* on known F_1 progeny of two genetically differentiated geographical races of *E. globulus* from north-eastern Tasmania and south-eastern Tasmania (see Dutkowski and Potts, 1999 for race classifications of *E. globulus)*. The pedigreed progeny used in this present study were grown in a common environment field trial and, therefore, differences in susceptibility to browsing is most likely due to host genetics, not

environmental effects. The progeny were composed of artificial intra-race and reciprocal inter-race F_1 hybrids involving trees from north-eastern Tasmania and south-eastern Tasmania. Populations of trees from north-eastern Tasmania are relatively susceptible to browsing by *T. vulpecula* (O'Reilly-Wapstra et al., 2002) and contain lower concentrations of FPCs (O'Reilly-Wapstra et al., 2004). Conversely, populations from south-eastern Tasmania are relatively more resistant to browsing (O'Reilly-Wapstra et al., 2002) and have higher concentrations of FPCs (O'Reilly-Wapstra et al., in press). Therefore, this genetic material provided an ideal opportunity to investigate three main questions: 1. How are the racial differences in resistance of *E. globulus* to *T. vulpecula* inherited in their F_1 hybrids in a common environment field trial? 2. Is the variation in resistance in the field trial consistent with patterns of intake of *E. globulus* by captive animals? 3. Are there quantitative differences between the hybrids in leaf chemical characteristics, and does this reflect the observed pattern of resistance?

METHODS AND MATERIALS

Field Trial and Plant Material. Browsing *of Eucalyptus globulus* juvenile foliage on 3-year-old trees by *Trichosurus vulpecula* was assessed in a field trial located at Weilangta, in south-east Tasmania, Australia (42° 44′ S 147° 49′ W). The trial design is described in detail in Lopez et al. (2003). In brief, the trees planted in the field trial were progeny from an 8 × 8 diallel crossing (80% complete excluding selfs) design where all possible crosses among eight parents were carried out. The parents were random samples of trees in native strands of *E. globulus* from both north-eastern Tasmania (termed north) and south-eastern Tasmania (termed south) races (following Dutkowski and Potts, 1999). The parental trees were growing more than 10 km apart and were considered unrelated. Four parental trees were from the north and four were from the south. These crosses resulted in four hybrid types; intra-race crosses, north × north (NN) and south × south (SS) and two reciprocal inter-race F_1 hybrids, north × south (NS) and south × north (SN). The female parent is stated as the first letter in the cross, and the male parent is stated as the second letter in the cross. The field trial at Weilangta was planted in 1999, and a duplicate trial was planted at the same time at Geeveston, in south-east Tasmania, Australia (43° 09′ S 146° 51′ W). Trees were planted in a randomized block design with each hybrid represented as a single tree plot. There were 18 field replicates at Weilangta and 17 replicates at Geeveston. The Geeveston field trial was fenced to prevent mammal browsing, while the Weilangta field trial was not. In July (winter) 2002, all trees (648 in total) in the Weilangta field trial were assessed for browsing damage. Each tree was observed for damage by *T. vulpecula* and given a "browsing damage score," which was as follows: 0 = no damage; 1 = minor browsing damage evident in the crown; 2 = major structural branch damage

in the crown (due to *T. vulpecula*), with none to little browsing damage; 3 = major browsing damage in the crown; 4 = major structural branch damage and major browsing damage in the crown.

Feeding Trial. After assessing browsing damage in the Weilangta field trial, we tested the preferences of six captive *Trichosurus vulpecula* (three male and three female, 2.5–4.1 kg body mass) for juvenile foliage from the four hybrids (NN, NS, SN, SS) in a feeding trial. All animals were caught, housed, and maintained following McArthur et al. (2000). Foliage used in the feeding trial was selected from the Geeveston field trial, as it was un-browsed and hence avoided any possible effects of induced chemistry responses of the foliage. Juvenile foliage (cut branches) was selected from as many trees as possible from several replicates to ensure a full representation of each family in each hybrid sample. Cut branches from each hybrid type were combined to make up four bunches of foliage; NN, NS, SN, SS. The foliage for each trial was cut from the field at the beginning of each trial, and foliage was stored in a cool room (5°C) with cut stems in water. Animals were offered foliage as four, of the possible six, paired choice feeding trials, as the four pairs provided adequate comparisons to rank the relative preference of each hybrid type. The pairs were: Trial 1 = SN and SS; Trial 2 = NN and SS; Trial 3 = NS and SN; and Trial 4 = NN and NS. Each trial ran for 3 d and all 6 animals received the same foliage at the same time. For each trial, a random selection of cut stems were presented to each animal in its cage as two bunches (one bunch for each cross race type) in a container of water.

Sufficient foliage was presented to each animal to allow *ad libitum* feeding. On each day (at ~1500 hr) total fresh mass (g) of each bunch was measured before being placed in the container. The next morning (at ~0800 hr), the remains of each bunch were weighed (including any plant fragments found on the cage floor). Intake was estimated as the amount of foliage consumed (weight difference) from each bunch, and all intakes were expressed in terms of grams dry matter per kilogram of body mass of the consumer (gDM/kgBM), based on the fresh to dry weight ratios calculated from the control plants. During the feeding trial, animals were offered a basal diet (see McArthur et al., 2000) at the same time as the experimental foliage. The amount of basal diet offered was 75% of that required to maintain body mass. This ensured that animals would eat the test foliage by choice, not because they were hungry, and ensured that all animals maintained body mass throughout the trial. On each day of the trial, two bunches of each hybrid fed to animals were also prepared as controls. The mean overnight change in mass of the two controls was used to adjust for changes in mass of the experimental plants. On each morning of the trial, one bunch was stripped of the leaves, which were oven dried (35°C for 1 wk and 80°C overnight before weighing) to determine their absolute dry matter (DM). The second bunch was frozen for subsequent chemical analysis.

Chemical Analysis. For each hybrid type, the control plants from the feeding trials were combined, and foliage was randomly subsampled 4 times for each analysis for each variable. We assayed plants for nitrogen, plant cell wall components (neutral detergent fibre, acid detergent fibre, and lignin; $N = 3$ for these constituents), total essential oils and 1,8-cineole, total phenolics, condensed tannins, and six formylated phloroglucinol compounds (FPCs) (sideroxylonal A, sideroxylonal B, sideroxylonal C, macrocarpal A, macrocarpal B, and macrocarpal G; $N = 3$ for these constituents).

Primary Chemistry. Nitrogen was determined following the sulphuric acid and hydrogen peroxide method of Lowther (1980) using air-dried ground foliage, ground in a cyclone grinder through a 1-mm mesh sieve. Digested samples were colorimetrically analyzed for nitrogen (QuikChem method 10-107-06-2E, Lachat Instruments, Wisconsin, USA) on a continuous flow injection analyzer (QuikChem 800, Lachat Instruments). Standard samples of known nitrogen concentration and blank samples were included to validate the efficiency of digestion and elemental analysis. Nitrogen results are expressed as % DM.

Plant cell wall components were analyzed by sequentially fractioning dried ground foliage. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (lignin) were determined following the ANKOM Technology procedures (ANKOM $^{200/220}$Technology Operator's Manual, 1997). Results are expressed as % DM.

Secondary Chemistry. Essential oils (terpenes) were extracted using dichloromethane with heptadecane as an internal standard (100 mg of heptadecane was diluted in 1 l of dichloromethane) (O'Reilly-Wapstra et al., 2004). One g of thawed foliage, cut into 1-cm pieces was soaked in the dichloromethane solvent for 1 hr. Extracts were analyzed by combined gas chromatography-mass spectrometry (GC-MS) on a Varian 3800 Gas Chromatograph coupled to a Varian 1200 triple quadruple mass spectrometer, using a 30 m $\times$ 0.25 mm Varian "Factor Four" VF-5 ms column with a 0.25-micron film. One μl aliquots were injected into a Varian 1177 split/splitless injector at 200°C using a split ratio of 20:1, and a GC column temperature profile of 60–260°C at 12 degrees per min. The electron energy was 70 eV, and the range from m/z 35–350 was scanned on quadruple 1 three times per sec. Total ion currents (TIC) were determined separately for 1,8-cineole, the sum of all oil components (referred to as total oils), and for the heptadecane internal standard. Results for 1,8-cineole and total oils were standardized by dividing by the internal standard. A response factor for cineole relative to the heptadecane internal standard was determined from a pure standard, and the amount of 1,8-cineole was expressed as mg/g DM. The amount of total oils was expressed as equivalents of cineole (mg/g DM).

Total phenolics and condensed tannins were assayed using the modified Prussian blue assay for total phenolics (Graham, 1992) and the acid butanol assay for condensed tannins (Porter et al., 1986). Foliage for these assays was prepared

and extracted following the method outlined in Hagerman (1995). In brief, 0.6 g of air dried, ground leaf was weighed into glass tubes, and 5 ml of 70% acetone were added. Samples were sonicated at 4°C for 30 min and centrifuged for 10 min at 2800 g/min. The supernatant was poured off and saved at 4°C. This process was repeated $\times 3$ for each sample. The supernatants were combined for each sample. The concentration of the total phenolics (mg/g DM) was determined in relation to a gallic acid standard (SIGMA G-7384), and the concentration of condensed tannins (mg/g DM) was determined in relation to a purified sorghum tannin standard.

Formylated phloroglucinol compounds were extracted following the method outlined in Wallis et al. (2003). Specific representative FPCs were determined by HPLC using a Waters Alliance 2690 HPLC with a Waters 996 UV/Vis diode array detector. The chromatogram at 280 nm was generated for peak area measurements from diode array data acquired between 230 and 400 nm. The column was a 250 $\times$ 4.6 mm Wakosil C18RS 3micron (SGE), using a flow rate of 1 ml/min. A tertiary gradient system was used; solvent A was 0.1% trifluoroacetic acid in acetonitrile, solvent B was Milli-Q water, and solvent C was "hexanes" (85% *n*-hexane, Mallinckrodt). The initial mix was 93:7 A and B, then a linear gradient to 100% A between 13 and 15 min was used. At 20 min, the solvent mix was immediately changed to 95:5 A and C, and this was held until 27 min, when it was immediately changed to 90:10 A and C to help remove late eluting FPCs. At 38 min, it was ramped back to starting conditions through 100% A and equilibrated for 7 min before the next sample. Peaks were identified by retention times and UV spectra in comparison to authentic standards, and representative samples were also analyzed by combined negative ion electrospray HPLC-MS to confirm that the molecular weights and tandem MS data of the peaks selected from the UV trace were also consistent with the standards (Eyles et al., 2003). Under these conditions, retention times were: sideroxylonal A 10.53 min, sideroxylonal B 15.32 min, sideroxylonal C 11.11 min, macrocarpal A 8.26 min, macrocarpal B 9.86 min and macrocarpal G 25.00 min. Results are expressed as mg/g DM for sideroxylonal A and C and macrocarpal A and B. Results for sideroxylonal B are expressed as mg/g DM equivalents of sideroxylonal A and results for macrocarpal G are expressed as mg/g DM equivalents of macrocarpal A.

Statistical Analysis. Results from the field trial were analyzed by first fitting a mixed model to the individual tree browsing data to simply determine if there was any significant difference in browsing score across the four hybrids and to test for replicate effects in the field trial design. Residuals were checked for normality and homoscedasticity with the general linear model procedure (PROC GLM) in SAS (SAS Institute Inc., SAS version 8), and the data were log transformed. Log transformed browse score was the dependent variable and the fixed effect was the hybrid type. The random effects were replicate and replicate by hybrid type interaction. The model was fitted with a PROC MIXED procedure in SAS

(SAS Institute Inc., SAS version 8). *A posteriori* multiple pair-wise comparisons of significant effects (least squares means) were made using the Tukey-Kramer adjustment. We performed *a priori* contrasts (contrast statement, SAS Institute Inc., SAS version 8) to test among predicted patterns of browsing damage on the parental and F_1 hybrids (Fritz et al., 1996; Hjältén et al., 2000). Figure 1 illustrates the contrasts performed and the possible expected outcomes.

Results from the feeding trial were analyzed as four separate trials: Trial 1 = SN and SS; Trial 2 = NN and SS; Trial 3 = NS and SN; and Trial 4 = NN and NS. The PROC UNIVARIATE procedure in SAS was performed for each trial to determine if the difference in intake between the two pairs was significantly different from zero (SAS Institute Inc., SAS version 8).

Results for each chemical constituent were first analyzed by fitting a one factor general linear model (PROC GLM) for each chemical variable with hybrid type (SAS Institute Inc., SAS version 8). Residuals were checked for normality and

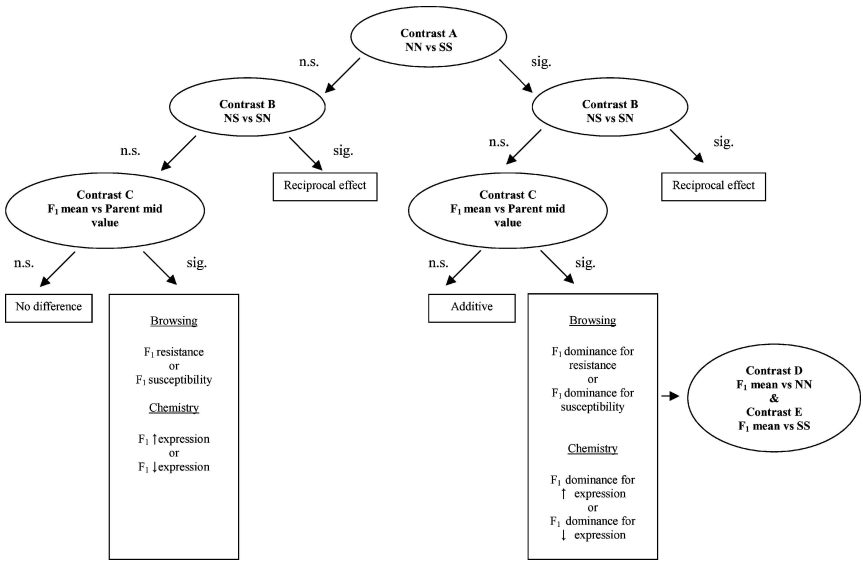

FIG. 1. Description of the contrast procedures and the potential outcomes of inheritance patterns of the browsing resistance to *T. vulpecula*, as assessed in the field trial, and of the concentration of chemical constituents of *E. globulus* foliage from the captive feeding trial. *NN* the hybrid from the northern Tasmania × northern Tasmania cross; *SS* the hybrid from the southern Tasmania × southern Tasmania cross; *NS* the hybrid from the northern Tasmania × southern Tasmania cross; *SN* the hybrid from the southern Tasmania × northern Tasmania cross. The first letter in the cross represents the maternal parent, *sig* a significant contrast; *ns* a non significant contrast.

homoscedasticity. Data for total oils and 1,8-cineole were log transformed, and data for macrocarpal A were inverse transformed (1/macrocarpal A). Following this, if there were significant effects, we performed *a priori* contrasts (PROC MIXED, SAS Institute Inc., 1990) to test among predicted patterns of concentration of chemical constituents on the parental and F_1 hybrids (Figure 1). If an effect was not significant following the general linear model analysis, no contrasts were performed.

RESULTS

Browsing damage by *T. vulpecula* on individual trees at the Weilangta field trial ranged in browsing damage score from 0 (no damage) to 4 (major structural branch damage and major browsing damage in the crown). There were no significant replicate or replicate * hybrid effects ($P > 0.05$). There was a highly significant difference in damage score among the four F_1 hybrid types ($F_{3,51} = 37.90$, $P < 0.001$, Figure 2). The intra-race NN cross was browsed more than the other three hybrid types, while the SS cross was browsed less than the other three hybrid types. There was no significant difference between the inter-race reciprocal F_1 hybrids (NS and SN), and these were intermediate in amount of browsing damage compared to the parental intra-race crosses, supporting the hypothesis of additive inheritance on the log transformed scale (Figure 2).

Table 1 illustrates foliage intake by *T. vulpecula* of each hybrid pair and results of the univariate statistical analysis for each feeding trial. In trial 1, there was no significant difference in intake between the two hybrids SS and SN. In trial 2, foliage from the NN cross was significantly more preferred by *T. vulpecula* than foliage from the SS cross. In trial 3, there was no significant difference in

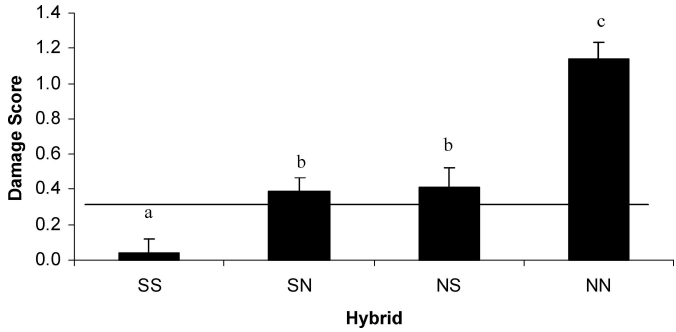

FIG. 2. Browsing damage by *T. vulpecula* for each hybrid type in the field trial. Values are least squares means with SE bars. Letters that differ indicate significant differences ($\alpha = 0.05$ after Tukey-Kramer adjustment for multiple comparisons). The line represents the back transformed mid parent value.

TABLE 1. MEAN INTAKE (GDM/KGBM) BY *T. Vulpecula* OF FOLIAGE FOR EACH HYBRID TYPE IN EACH CAPTIVE FEEDING TRIAL AND RESULTS FOR THE UNIVARIATE ANALYSIS OF EACH TRIAL

Trial	Hybrid pair	Intake (±SE)	*T*-value	*P*-value
1	SS	6.2 (0.59)	1.28	0.256
	SN	5.1 (1.20)		
2	NN	7.7 (1.51)	−3.81	0.012
	SS	4.6 (1.03)		
3	NS	7.7 (2.01)	−0.78	0.471
	SN	5.7 (1.36)		
4	NN	10.1 (1.66)	−2.27	0.073
	NS	6.7 (1.40)		

intake of foliage between the NS and the SN hybrids, while in trial 4 there was no significant difference in intake of the NN and NS hybrids, although the NN cross tended to be more preferred.

Several chemical constituents varied significantly ($P < 0.05$) among the four hybrid types (Tables 2 and 3). There was a significant difference in the nitrogen content between the four hybrid types. The NN hybrid cross had higher nitrogen content than the SS cross. A significant difference between the two F_1 hybrids indicates a reciprocal cross effect for nitrogen concentration (Table 3), with each F_1 hybrid tending to be closer to their maternal parent indicating a maternal reciprocal effect. However, the range in nitrogen concentration from 1.3 to 1.4%, may not be biologically significant. Results for acid detergent fiber show significantly higher acid detergent fiber content for the SS cross compared to the NN cross (Tables 2 and 3) and F_1 dominance for decreased expression (Table 3). There was significant variation in total oil and 1,8-cineole concentration among hybrid types (Table 3). Contrast A shows the two intra-race hybrid crosses differed in total oils and 1,8-cineole; SS had higher concentrations of oil than NN (Tables 2 and 3). There was no significant difference in concentration of total oils and 1,8-cineole between the two F_1 hybrids (contrast B), however, there was a difference between the mean F_1 hybrid concentration and the mid-parent value (contrast C). Significance at contrast D indicates F_1 dominance for increased expression of oil production. There was a significant difference in concentration of condensed tannins among the four hybrid types (Table 3). The SS intra-race cross had more condensed tannins than the NN cross (Tables 2 and 3) and there was F_1 dominance for reduced expression (Table 3). There was no significant difference between the four hybrid types in concentration of sideroxylonal A, however, there were differences in all other FPCs (Table 3) despite the concentrations of these compounds being low (Table 2). There was F_1 dominance for decreased expression of sideroxylonal B, and F_1 dominance for increased expression of sideroxylonal C, although these

TABLE 2. CONCENTRATION (LEAST SQUARES MEANS (±SE)) OF PRIMARY AND SECONDARY CHEMICAL CONSTITUENTS OF FOLIAGE OF THE FOUR HYBRID TYPES OFFERED TO *T. Vulpecula* IN THE CAPTIVE FEEDING TRIALS

Constituent	Units	Hybrid			
		SS	SN	NS	NN
Nitrogen	(% DM)	1.2(0.03)	1.3(0.03)	1.4(0.03)	1.4(0.03)
NDF	(% DM)	38.6(0.98)	36.4(0.98)	36.3(0.98)	37.3(0.98)
ADF	(% DM)	25.1(0.54)	23.0(0.54)	21.3(0.54)	22.5(0.54)
Lignin	(%DM)	9.3(0.48)	8.5(0.48)	10.4(0.48)	9.3(0.48)
Total oil	(mg/g DM cineole equivalents)	16.0(1.04)	16.1(1.04)	16.0(1.04)	11.0(1.04)
1,8-cineole	(mg/g DM)	8.0(0.55)	7.4(0.55)	8.5(0.55)	5.6(0.55)
Total phenolics	(mg/g DM gallic acid equivalents)	99.3(4.78)	84.2(4.78)	76.5(4.78)	85.4(4.78)
Condensed tannins	(mg/g DM sorghum quivalents)	8.3(0.57)	2.3(0.57)	2.4(0.57)	3.5(0.57)
Sideroxylonal A	(mg/g DM)	0.13(0.004)	0.12(0.004)	0.13(0.004)	0.13(0.004)
Sideroxylonal B	(mg/g DM sideroxylonal A equivalents)	0.10(0.002)	0.09(0.002)	0.10(0.002)	0.11(0.002)
Sideroxylonal C	(mg/g DM)	0.07(0.001)	0.08(0.001)	0.07(0.001)	0.06(0.001)
Macrocarpal A	(mg/g DM)	0.48(0.010)	0.71(0.010)	0.49(0.010)	0.45(0.010)
Macrocarpal B	(mg/g DM)	0.35(0.007)	0.52(0.007)	0.36(0.007)	0.33(0.007)
Macrocarpal G	(mg/g DM macrocarpal A equivalents)	0.76(0.029)	1.31(0.029)	0.78(0.029)	0.66(0.029)

Note. NDF, neutral detergent fiber, ADF, acid detergent fiber.

patterns should be viewed with caution due to the low concentrations. A reciprocal effect was evident for all macrocarpal concentrations with the SN F_1 hybrid having higher concentrations of macrocarpals than all other hybrid types. There are no significant differences in concentration of total phenolics, neutral detergent fiber, and lignin among the four hybrid types (Table 3).

DISCUSSION

In this study, we had an *a priori* expectation that the two parental hybrids would exhibit differential susceptibility to browsing by *T. vulpecula* (O'Reilly-Wapstra et al., 2002). The patterns of browsing by *T. vulpecula* on *E. globulus*, in previous common environment field trials planted across different geographic areas of Tasmania, have consistently shown that populations of *E. globulus* from

TABLE 3. *P*-VALUES FOR THE DIFFERENCE IN BROWSING DAMAGE ACROSS THE FOUR HYBRID TYPES IN THE FIELD TRIAL, THE DIFFERENCE IN CONCENTRATION OF EACH CHEMICAL CONSTITUENT ACROSS THE FOUR HYBRID TYPES (GENERAL LINEAR MODEL ANALYSIS) AND THE CONTRAST COMPARISONS

Variable	GLM	Contrast Comparison					Hypothesis supported
		A	B	C	D	E	
Browsing damage in field trial	0.0001[a]	<0.001	0.439	0.2809	[c]	[c]	Additive[b]
Nitrogen (% DM)	0.0004	0.002	<0.001	[c]	[c]	[c]	Reciprocal effect
NDF (% DM)	0.3632	[c]	[c]	[c]	[c]	[c]	No difference
ADF (% DM)	0.0069	0.009	0.061	0.016	0.649	0.002	F_1 dominance for ↓ expression
Lignin (%DM)	0.1270	[c]	[c]	[c]	[c]	[c]	No difference
Total Oils	0.0037	0.002	0.950	0.016	0.001	0.980	F_1 dominance for ↓ expression[b]
1,8-cineole	0.0072	0.004	0.171	0.042	0.002	0.870	F_1 dominance for ↓ expression[b]
Total phenolics	0.0546	[c]	[c]	[c]	[c]	[c]	No difference
Condensed tannins	0.0002	<0.001	0.861	<0.001	0.219	<0.001	F_1 dominance for↓ expression
Sideroxylonal A	0.0729	[c]	[c]	[c]	[c]	[c]	No difference
Sideroxylonal B	0.0064	0.016	0.072	0.007	0.001	0.266	F_1 dominance for ↓ expression
Sideroxylonal C	0.0001	0.000	0.128	<0.001	<0.001	0.752	F_1 dominance for ↓ expression
Macrocarpal A	0.0001	0.092	0.000	[c]	[c]	[c]	Reciprocal effect
Macrocarpal B	0.0001	0.111	0.000	[c]	[c]	[c]	Reciprocal effect
Macrocarpal G	0.0001	0.036	0.000	[c]	[c]	[c]	Reciprocal effect[d]

Note. Contrast comparison A is between the two intra-race hybrid crosses (parental crosses), Comparison B between the two F_1 hybrids, comparison C between the mean of the F_1 hybrids and the mid-parent value, contrast D between the mean of the F_1 hybrids and NN (the susceptible parent), and contrast E between the mean of the F_1 hybrids and SS (the resistant parent). The final column refers to the hypothesis or inheritance pattern that is supported by the hybrid results. NDF, neutral detergent fiber, ADF, acid detergent fiber.

[a] Indicates mixed model analysis.

[b] Indicates log transformed data.

[c] Indicates either that no contrasts were performed as there were no significant differences between hybrids in the GLM analysis, or that further contrast analysis between hybrids was redundant.

[d] Indicates inverse transformed data.

north-eastern Tasmania are more susceptible than populations from south-east Tasmania (Volker and Orme, 1988; O'Reilly-Wapstra et al., 2002). This was supported in both the field trial and captive feeding trial results in this present study. The pure parental hybrid from the north-eastern Tasmania race (NN) was more susceptible to *T. vulpecula* than the parental hybrid from the south-eastern Tasmania race (SS). The pattern of hybrid resistance, however, was unknown. The phenotypic expression of resistance of the inter-race F_1 hybrids supports the additive pattern (Fritz, 1999) of hybrid resistance. On the log scale, the F_1 hybrid was intermediate in resistance, compared to the parental hybrids. This pattern of browsing was consistent over two separate years of field data (Potts, unpublished data). Numerous studies in plant hybrid/insect systems have demonstrated that the patterns of hybrid resistance are highly variable (e.g., Aguilar and Boecklen, 1992; Eisenbach, 1996; Hjältén, 1997; Fritz et al., 1998). In comparison, relatively few studies have examined the resistance of hybrids to mammalian herbivores (but see Vila and D'Antonio, 1998; Dungey and Potts, 2002; Scott et al., 2002; Anderson and Paige, 2003). However, the patterns of hybrid resistance to mammalian herbivores appear just as varied as those patterns expressed to insect herbivores. Increased hybrid resistance (Vila and D'Antonio, 1998), additive inheritance of resistance (Scott et al., 2002), dominance toward susceptibility (Dungey and Potts, 2002), and no difference in hybrid resistance compared to the parental plants (Anderson and Paige, 2003) have all been demonstrated.

Confirmation of the field trial results (the differential resistance of NN and SS) in the feeding trial indicate that the difference in resistance was most likely due to foliage characteristics, such as plant secondary metabolites, as opposed to other characteristics such as tree form. Based on our previous studies (O'Reilly-Wapstra et al., 2004) and research in similar systems (Lawler et al., 2000; Wallis et al., 2002), we expected the formylated phloroglucinol compounds (FPCs) to follow the pattern of hybrid resistance observed in this study and to provide the mechanism of resistance in these plants. On the contrary, there was no relationship between FPC concentration and relative resistance of the four hybrid types. The most likely reason for this is that, despite some significant differences in concentration of FPC compounds in these leaves, the actual concentrations were very low and, therefore, perhaps biologically ineffective in acting as deterrents to *T. vulpecula* in this particular foliage.

In comparison to the 3-year-old juvenile foliage used in this study, our previous and ongoing research with *E. globulus* and preferences by *T. vulpecula* has utilized foliage from coppice re-growth (O'Reilly-Wapstra et al., 2002; O'Reilly-Wapstra et al., 2004) and young seedlings (O'Reilly-Wapstra, unpublished data). Concentrations of FPCs in these two types of foliage was higher than that measured in the present study; sideroxylonal A concentration was 0.9–8.5 mg/g DM and 0.2–1.8 mg/g DM for coppice and seedling foliage, respectively. It is possible that the production of higher levels of FPCs in *E. globulus* coincides with the

period when the plant is most vulnerable to mammalian herbivores. *Eucalyptus* seedlings are particularly susceptible to browsing by mammals (Gilbert, 1961; Bulinski and McArthur, 1999). We suppose that coppice re-growth is also susceptible to mammal browsers, as has been demonstrated with insect herbivores feeding on *Eucalyptus* (Abbott et al., 1993; Steinbauer et al., 1998), and with *T. vulpecula* (O'Reilly-Wapstra et al., 2002). While the concentration of FPCs in the seedling foliage (O'Reilly-Wapstra, unpublished data) were still quite low, the differences in concentration among coppice, seedling, and 3-year-old juvenile foliage was consistent with this idea. Additionally, although *T. vulpecula* is an arboreal herbivore and is capable of consuming foliage at all successive *E. globulus* life stages, 3-year-old *E. globulus* trees (approximately 4 m in height) may be better capable of recovering from foliage loss. Consequently, defensive mechanisms to mammalian herbivory (FPC production) may not be necessary at this stage, and energy may be allocated to traits such as growth and reproduction. In this species, ontogenetic development of the tree results in heteroblastic leaf change from the seedling leaf phase to the juvenile phase at around 18 wk (James and Bell, 2001). Timing of juvenile to adult foliage phase change is variable (Jordan et al., 2000) but can occur at around 1.5–3 yr (James and Bell, 2001). Many studies have demonstrated developmental changes in leaf resistance to herbivores (e.g., Kearsley and Whitham, 1989; Fritz, 2001), including *E. globulus* resistance to insect herbivory (Steinbauer, 2002; Lawrence et al., 2003). Production of defensive chemistry to mammalian herbivory may, therefore, alter at these phase changes and become relatively unnecessary in older trees. However, further investigation of the chemical profile and timing of expression of plant secondary metabolites in *E. globulus* foliage at different life stages is necessary to elucidate this.

There were clear differences in resistance among the four hybrid types in this study, particularly among the two parental hybrids. We suggest that the combined effect of condensed tannin and essential oil concentrations conferred this difference. Condensed tannins and essential oils were significantly higher in the SS than the NN parent. These two groups of secondary metabolites decrease herbivory by mammalian herbivores in many systems (Harborne, 1991). Tannins decrease intake of *E. rossii* and *E. consideniana* foliage by *T. vulpecula* (Marsh et al., 2003). Tannins can decrease herbivory by mammals by binding with dietary proteins (Hagerman and Butler, 1991). The maximal flow rate of salivary proline-rich proteins (available to bind with dietary tannins) in *T. vulpecula* is relatively slow compared to other marsupial herbivores (McArthur et al., 1995). This may indicate a decreased ability to cope with ingested dietary tannins. The negative effects of increasing 1,8-cineole levels in artificial diets on intake by *T. vulpecula* has also been highlighted (Wiggins et al., 2003). While the levels of 1,8-cineole required to constrain intake in that study were much higher than in *E. globulus* in the present study, clear preferences at lower concentrations were still detected in the "choice" feeding trials in the latter study. We suggest, therefore, that

T. vulpecula respond to foliage chemical characteristics in choice feeding trials, even when levels do not physiologically constrain intake.

We also suggest that the combined effect of tannins and essential oils in *E. globulus* foliage explains the inheritance of resistance demonstrated in the inter-race F_1 hybrids. The SS parent had significantly higher levels of both compounds compared to the NN parent. While the F_1 hybrids deviate from the mid-parent value in each of these compounds, the direction of dominance was opposite. Condensed tannin levels in the hybrids showed dominance for decreased expression (similar to the NN parent), while essential oils showed dominance for increased expression (similar to the SS parent). Consequently, the combined effects of these two groups of compounds (condensed tannins + essential oil) show an additive pattern of expression: SS hybrid had the highest, NN hybrid had the lowest, and the two inter-race hybrids fell intermediate. This pattern is consistent with the observed patterns of additive inheritance of resistance.

In a review of the effects of hybridization on plant secondary chemistry, Orians (2000) found that hybrids typically express concentrations of compounds at intermediate levels (additive pattern) or at levels similar to one of the parents (dominance pattern). The majority of compounds in this study exhibited the dominance pattern of expression but, interestingly, the direction of dominance varied. As discussed above, the inter-race hybrids showed dominance for decreased expression of condensed tannins, while dominance for increased expression of essential oils was also evident. This could arise, for example, if a dominant gene(s) for the reduced expression of condensed tannins (from the susceptible NN parental hybrid) and a dominant gene(s) for the increased expression of essential oils (from the resistant SS parental hybrid) are expressed in these hybrids. Few studies have examined the pattern of inheritance of plant secondary metabolites in eucalypts, but two have reported that the inheritance pattern of essential oils in inter-specific hybrids varies. The additive pattern (Dungey et al., 2000; Farah et al., 2002) and the dominance and no difference pattern (Dungey et al., 2000) have all been demonstrated. The variable pattern of results from Dungey et al. (2000) indicates that different compounds, even from the same group (terpenes) can exhibit different inheritance patterns, as also demonstrated with the FPCs in this study.

In summary, we have demonstrated that the inter-race differences in the phenotypic expression of resistance to *T. vulpecula* in *E. globulus* are inherited in an additive manner. Variation in resistance of the foliage appeared to be due to the combined effects of condensed tannins and essential oils. These compounds were both in high concentrations in the SS cross and significantly lower in the NN cross. At present, there is no evidence to suggest that past or current densities of *T. vulpecula*, and consequently browsing pressures, differ between the north-east and south-east populations of *E. globulus*. Consequently, explanations as to why these populations differ in resistance and chemical profiles remain to be elucidated.

While oils and condensed tannins were inherited in a dominant manner in the inter-race F_1 hybrids, the direction of dominance was opposite, resulting in their combined concentration being inherited in an additive manner. This is consistent with the phenotypic differences in browsing. Five different patterns of hybrid inheritance of plant chemistry were demonstrated in this study. This highlights that patterns of inheritance of resistance and plant secondary metabolites vary not only among plant species, as demonstrated in numerous systems, but also vary considerably within a plant species and within closely related groups of plant compounds.

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STIR BAR SORPTIVE EXTRACTION: A NEW QUANTITATIVE AND COMPREHENSIVE SAMPLING TECHNIQUE FOR DETERMINATION OF CHEMICAL SIGNAL PROFILES FROM BIOLOGICAL MEDIA

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Abstract—Various applications of a novel sampling procedure in chemical ecology are outlined. The stir bar extraction method features the analytical reproducibility needed in recording the analytical profiles of volatile and semivolatile components of biological mixtures. This methodology has been demonstrated here through the examples of small volume urine samples, glandular tissue volatiles, and the air blown through animal cages. Its analytical merits are compared with those of the previously established purge-and-trap (dynamic headspace) technique.

Key Words—Pheromones, chemical signals, hamster urine, methods, mouse urine, rat preputial gland, rat odor, volatiles, stir bar sorptive extraction, gas chromatography–mass spectrometry.

INTRODUCTION

Mammalian chemical communication is at least partially mediated by volatile and semivolatile compounds (mostly small, relatively hydrophobic molecules), which are often encountered in urine and glandular secretions (Novotny et al., 1990; Novotny, 2003). Some of these molecules process pheromonal activities, influencing both the social behavior and reproductive function in the recipient animals. At least some of these biological activities are presumably mediated through the vomeronasal organ, in which these molecules exhibit selective activities at their

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target neurons (Moss et al., 1997; Leinders-Zufall et al., 2000; Boschat et al., 2002).

While searching for putative pheromones in biological materials, it is often advantageous to compare substance profiles under different genetic, endocrinological, or behavioral conditions. Such comparisons are now typically performed through the use of capillary gas chromatography–mass spectrometry (GC–MS), with a prior use of a sample extraction procedure. While capillary GC–MS has now been developed as a highly reproducible analytical tool, variations still exist in the sample preparation procedures. Quantitative analyses of the chemosignaling compounds in animal urine and other biological specimens are challenging due to a complexity of many biological matrices, frequently low concentrations of bioactive compounds, and a wide variation in their physical and chemical properties. Frequently, one sample isolation approach may be optimal for a class of compounds, but it may be discriminating toward other substance types. Typical approaches for isolating low-molecular weight compounds of a volatile and semivolatile nature in the analysis of aqueous biological samples are liquid–liquid extraction (Rasmussen et al., 1990; Burger et al., 1996), dynamic headspace extraction (purge-and-trap) (Novotny et al., 1974a,b; Schwende et al., 1984; Ma et al., 1999), and a solid-phase microextraction (SPME) using coated silica fibers (Zhang and Pawliszyn, 1993).

Solvent extraction is seemingly beneficial for extraction of moderately volatile compounds at relatively high concentrations. However, when solvent evaporation is needed for concentrating the analytes, loss of the more volatile compounds becomes unavoidable. Solventless trapping of volatiles on the surface of adsorption materials (e.g., porous polymers, such as Tenax GC—Novotny et al., 1974a,b) has long provided a means of effective preconcentration of urinary volatiles in pheromone studies in our laboratory (Novotny et al., 1980; Schwende et al., 1984; Ma et al., 1999). While this "purge-and-trap" (dynamic headspace) approach has been satisfactory in semiquantitative studies to observe large differences in pheromone excretion, it will be less satisfactory in the cases where subtle quantitative differences in profile constituents (Schwende et al., 1984, 1986) may account for recognition of a biological message. Typically, through this approach, multiple sampling is necessary to gain more reliability in quantification of volatiles in biological matrices (Jemiolo et al., 1994). Generally, adsorption-trapping procedures are beneficial in handling highly volatile, nonpolar compounds.

There are several disadvantages of the dynamic headspace, adsorption-based systems in a routine, quantitative profiling of biological samples. Even small variations in the preparation of trapping precolumns (amounts of the sorption material and its batch-to-batch variations) can lead to lower reproducibility. In addition, strong adsorption and nonquantitative desorption of certain solutes can hamper quantification, even when an appropriate internal standard is utilized to compensate for physical and chemical properties. A typical setup for the purge-and-trap

or the dynamic headspace system also requires specialized glassware and additional care to periodically clean the system's components with relatively large surface areas. Consequently, sample throughput using this methodology becomes a limiting step for the analysis of a large number of samples that are increasingly required in biological studies.

Current developments in analytical sampling seem to favor *absorption* rather than *adsorption* phenomena. Specifically, the organic polymers can be chemically attached to a mechanical device (a "sample holder") such as a silica fiber (Zhang and Pawliszyn, 1993), or a polymer-coated magnetic stir bar (Baltussen et al., 1999, 2002) to allow sample sorption and subsequent thermal desorption in a heated port of a gas chromatograph. Using SPME fibers, quantitative analyses have been documented for environmental pollutants and aroma components (Pawliszyn, 1997; Holt, 2001; Pino et al., 2002). Both aqueous (Luo et al., 1998) and headspace versions (Fustinoni et al., 1999; Dehnhard et al., 2001) of SPME have been utilized for recovering volatiles. However, the SPME fibers used typically in these extractions have relatively low extraction efficiency due to the low volume of a sorption polymer. This limits the use of such techniques in the applications targeting analytes at low concentrations. Further developmental steps to improve extraction efficiency of the SPME techniques have been to use larger surface to volume ratio of the extraction polymer. Recently, a thin-film microextraction approach was demonstrated as beneficial for the analysis of polyaromatic hydrocarbons (PAHs) in lake water (Bruheim et al., 2003).

The recently developed stir bar absorptive extraction techniques appear advantageous to deal with very dilute media and low-level samples. It has been demonstrated that a wide range of volatile and semivolatile substances (from both aqueous and gaseous media) can be retained on a polymer-coated magnetic bar (Twister^{TM}) (Baltussen et al., 1999, 2002; Tienpont et al., 2000). The volume of the polydimethylsiloxane (PDMS) coating on a stir bar can be precisely controlled, increasing the reliability of extraction. A typical polymer phase volume in the stir bar sorptive extraction (SBSE), which is 24–100 μl, well exceeds that used with the SPME technique (typically, 0.5 μl). Through the fast stirring action by a magnetic rod in the aqueous medium, the equilibrium state on the PDMS layer is ensured during the sorption, so that the analyte concentration stays constant and reproducible, leading to good analytical precision. A review dealing with the technical differences between the adsorptive and absorptive sampling has recently been published (Baltussen et al., 2002). The PDMS sorption-based analyses have been described in environmental studies (Baltussen et al., 1998; Peñalver et al., 2003), essential oils (Kreck et al., 2002), food flavor investigations (Tredoux et al., 2000; Bicchi et al., 2002), and analysis of volatile phenols in wines (Díez et al., 2004). Utilization of similar techniques for human biological samples has been reported for determination of polychlorinated biphenyls in human sperm (Benijts

et al., 2001) as well as terpenes, steroids, and drug residues in urine (Tienpont et al., 2002, 2003).

Following the success of SBSE sampling techniques in other areas, we wished to adapt this methodology to studies in chemical ecology. Specifically, we became interested in developing this methodology for routine and precise profiling analyses in mammalian communication projects. As an example of aqueous media, we demonstrate here a highly precise determination of urinary volatiles from hamsters (*Phodopus cambpelli*) and house mouse (*Mus domesticus*). The applicability of the SBSE technique was further tested with the rat preputial tissue and the air sample blown through the rat cages. Performance of the SBSE methodology was compared with that of a traditionally used dynamic headspace method. We further discuss its advantages in a parallel sample preparation and increased sample throughput.

EXPERIMENTAL

Sample Collection. Hamster urine originated from the colony of *Phodopus cambpelli* (Animal Physiology, Institute of Zoology, University of Tübingen, Germany) and mouse urine (Indiana University) samples were collected in metabolic cages and kept frozen at −20°C until analysis. Rat preputial glands originated from Long-Evans rats (Department of Psychology, Center of Alcohol Studies, Rutgers University, NJ) and were frozen at −20°C until analysis. Air quality in the rat cage was measured periodically according to the scheme shown in Figure 1 (Star Enterprises, Inc., Bloomington, IN, USA; a design for NASA-supported studies). In the case of air sampling, the analyses were performed immediately after sample collection. Unless otherwise stated in the text, each representative application was chosen through a single chromatographic profile to demonstrate different analytical assets of our procedure.

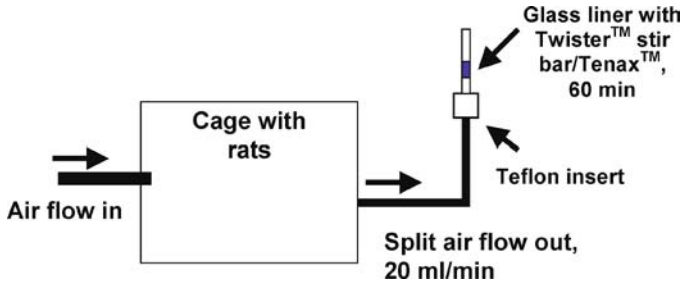

FIG. 1. Schematic of the air sampling system for a rat cage using Twister™ or Tenax™ for trapping volatiles.

Urine Sample Extraction by SBSE. All glassware used was washed with distilled water and acetone, and dried at 80°C. Volatile and semivolatile compounds were typically extracted from 1.0 ml of undiluted or water-diluted urine by sorptive extraction with a Twister™ PDMS polymer coated stir bar (10 mm × 0.5 mm film thickness, 24 μl PDMS volume, Gerstel GmbH, Mülheim an der Ruhr, Germany). A stir bar was placed in the 20 ml capped vial containing urine and stirred for 60 min. Stirring speed was 800 + rpm on the Variomag Multipoint HP 15 stirplate (H + P Labortechnic, Oberschleissheim, Germany). After extraction, stirbars were rinsed with a small volume of distilled water, dried gently on paper tissue, and placed in the glass injector liner for mass-spectrometric (MS) identification, or in the TDSA autosampler tube for the gas-chromatographic (GC) quantitative analysis. Standard compounds were obtained from Aldrich Chemical Company (Milwaukee, WI) or synthesized in our laboratory.

Urine Sample Extraction by Tenax™ Sorbent by Purge-and-Trap Analysis. Urine samples (1.0 ml) were placed in the glass vessel supplied with a helium flow (70 ml/min) purging the urine sample and collecting the sample headspace, as shown in Figure 2. The vessel was placed in the water bath at 40°C to improve transportation of volatiles. Helium flow carrying volatiles passed through an ice-water chilled section to minimize excess water before the volatiles were trapped on 8 mg of Tenax™ sorbent (TA, 35/65 mesh, Alltech, Deerfield, IL).

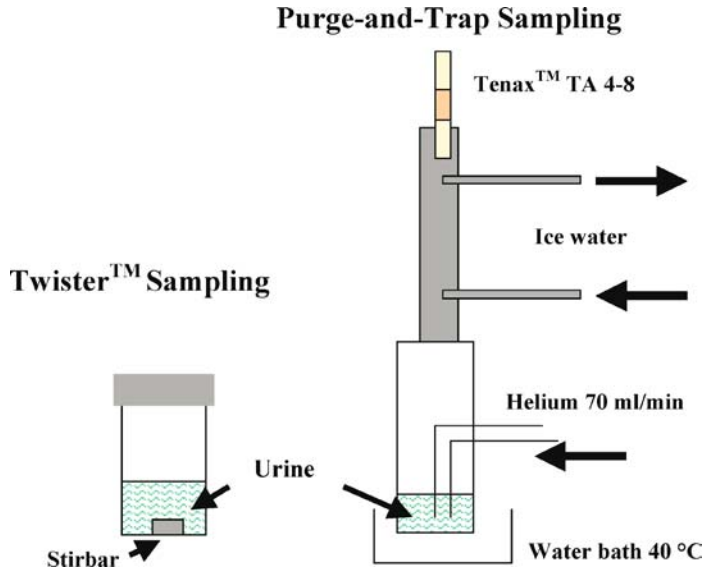

FIG. 2. Aqueous Twister™ and purge-and-trap Tenax™ sampling device for trapping volatiles from urine.

Rat Preputial Gland Extraction by SBSE. Whole gland was frozen with the aid of a small amount of liquid nitrogen and homogenized. About 500 mg of the homogenate were weighed into a 20-ml capped extraction vial. Stir bar extractions (Twister™) were performed with 20 ml of water or 5–10% (v/v) acetonitrile–water for 60 min.

Air Sampling. Rat cages were built as closed units with an air inlet and outlet. Air was pumped through the cage with a constant flow rate. Collection tubes were installed in the air outlet of the cage. Air samples were collected from the rat cages for 60 min with the air flow adjusted to 20 ml/min flowing through the collection tube equipped with Tenax™ or a Twister™ stir bar (as shown in Figure 1).

Gas Chromatography. GC equipment for the quantitative analysis consisted of an Agilent GC Model 6890 with an Atomic Emission Detector (AED) Model G2350A (Agilent Technologies, Inc., Wilmington, DE) and a Thermal Desorption Autosampler (TDSA, Gerstel GmbH, Mülheim an der Ruhr, Germany). The separation capillary was DB-5 (30 m $\times$ 0.25 mm, i.d., 0.25 μm film thickness, from J&W Scientific, Folsom, CA). Samples were thermally desorbed in a TDSA automated system, followed by injection into the column with a cooled injection system CIS-4. TDSA operated in the splitless mode. Temperature program for desorption was 20°C (0.5 min), then 60°C/min to 280°C (10 min). Temperature of the transfer line was set at 280°C. CIS was cooled with liquid nitrogen to −60°C. After desorption and cryotrapping, CIS was heated at 12°C/sec to 280°C with the hold time of 10 min. Temperature program during the GC analysis was 40°C for 5 min, then increasing to 200°C at the rate of 2°C/min. The final temperature was held for 10 min. Carrier gas head pressure was 14 psi (flow rate, 1.2 ml/min). The GC unit was operated in the constant flow mode. The emission lines for carbon (193 nm), sulfur (181 nm), and nitrogen (174 nm) were monitored in the atomic plasma emission detection unit.

Capillary Gas Chromatography–Mass Spectrometry. Finnigan MAT Magnum ion trap GC-MS system was used for the compound identification (Finnigan MAT, San Jose, CA). The system was provided with a DB-5 capillary column (30 m $\times$ 0.25 mm, i.d., 0.25 μm film thickness, J&W Scientific, Folsom, CA). Helium carrier gas head pressure was 12 psi. At the beginning of the column, a loop of uncoated deactivated silica tubing (30 cm $\times$ 0.25 mm, i.d.) was attached using a universal Press-Tight Connector (Restek Corporation, Bellefonte, PA) as described earlier (Ma et al., 1999). The loop was cooled in liquid nitrogen, while the Twister™stirbar was held in the injector liner for 15 min at 250°C for the thermal desorption of the analytes. Subsequently, the desorbed compounds were cryotrapped into the liquid nitrogen cooled loop. After removing liquid nitrogen cooling, the GC temperature was held at 40°C for 5 min and increased to 200°C at the rate of 2°C/min. The final temperature was held for 10 min. The manifold and transfer line temperatures were 220°C and 300°C, respectively. The ion trap

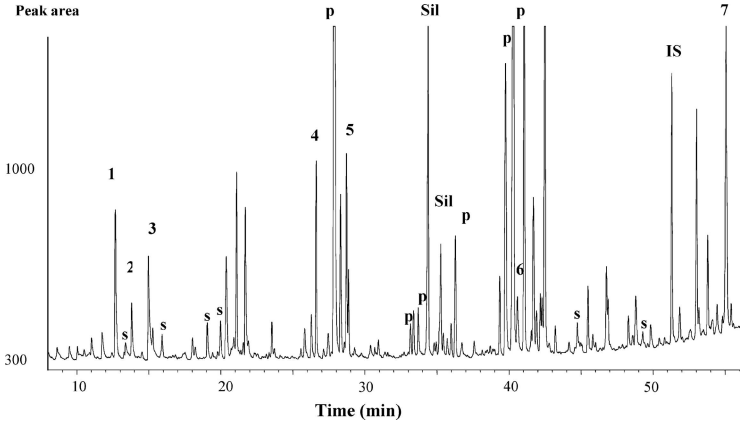

FIG. 3. Chromatogram of the SBSE-extracted and thermally desorbed compounds from male hamster urine (GC-AED, carbon emission, 193 nm). The numbers (1–7) indicate quantitatively measured compounds as seen in Table 1, "s" = sulfur compounds verified on sulfur emission (181 nm); "p" = partially identified pyrazines by GC-MS; Sil = siloxanes from a stir bar; IS = an internal standard, 7-tridecanone. Analytical conditions: as described in the text.

was operated in the positive ionization mode. Spectra were scanned from 40 to 350 amu (1 scan/sec).

RESULTS AND DISCUSSION

Urinary Profiles. GC profiles of the SBSE extracted compounds were compared using the GC-MS instrument, while the peak identities were established based on retention indices, mass spectra, and comparisons with known standard compounds. As an example, a urinary volatile compound GC profile from a pooled male *Phodopus campbelli* hamster urine sample is shown in Figure 3. The numbers indicate a selected compound identity (see Table 1). Male hamster urine characteristically contained a number of partially identified alkylated pyrazines, indicated as "p." Their strong endocrine dependency (data not shown) suggested that alkylated pyrazines are putative chemosignals for this hamster species (Soini et al., unpublished). Additional work is underway for a complete characterization of the individual pyrazine compounds. The peaks marked as "s" were verified as sulfur compounds using a GC-AED (data not shown). While accurately measurable, their levels were too low for unequivocal mass-spectral characterization.

Profiles of the pooled *Phodopus campbelli* hamster urine were compared between the SBSE method (Twister^{TM}) and the purge-and-trap method using

TABLE 1. REPRODUCIBILITY OF SBSE EXTRACTION FOR SELECTED COMPOUNDS IN A MALE HAMSTER (*Phodopus campbelli*) POOLED URINE SAMPLE MEASURED BY GC-AED (CARBON LINE 193 nm).

Peak number	Compound	log K_{ow}^{a}	Peak area (average, $N = 4$)	SD	RSD (%)	Theoretical recovery (%)	Quantity detected (ng)
1	4-Heptanone	1.73	3,399	116	3.41	12	60
2	2-Heptanone	1.73	1,221	28	2.30	12	21
3	2,5-Dimethylpyrazine	1.03	3,652	86	2.35	3	62
4	4-Nonanone	2.71	3,492	25	0.72	57	12
5	2-Nonanol	3.22	3,774	111	2.95	80	11
6	Geraniol	3.47	1,682	81	4.80	88	69
7	Vitamin K_3	2.21	9,522	526	5.52	30	15

[a] EPISuite™ v. 3.10 from 2000 U.S. Environmental Protection Agency, K_{ow} is a partition coefficient between water and octanol.

Tenax™ as an adsorbent. GC-MS profiles in Figure 4 show that SBSE method extraction efficiency was lower for the very volatile compounds (typically, 5–10%), but an increased extraction efficiency was observed for less volatile compounds like alkylated pyrazines (typically 50–100%). The ability to adsorb and desorb efficiently small nonpolar volatile compounds with Tenax-like adsorbents is based on a fast equilibrium following their adsorption on the surface through the weak van der Waals interactions. Subsequently, thermal desorption is efficient due to such weak interactions, which was shown with the slightly higher levels of the early eluting volatile compounds. Less volatile compounds (semivolatiles) generally reach the adsorption surface in lesser quantities and, furthermore, become difficult to remove thermally due to stronger adsorptive interactions on the surface (Baltussen et al., 2002). This is illustrated in Figure 4 as the almost nonexisting semivolatile compound region on the TIC of hamster urine after using purge-and-trap with Tenax™ sampling. On the other hand, Twister™ efficiently released semivolatile compounds during their thermal desorption. This feature adds substantial information on the previously unknown range of the putative chemical signaling compounds or the less volatile (semivolatile) compounds (later parts of a chromatogram).

Reproducibility and Quantitative Analysis. Partition into the PDMS phase can be estimated through water–octanol partition coefficients (EpiSuite™, 2000 U.S. Environmental Protection Agency). Using the phase ratios of 24 μl PDMS/10 ml water, the theoretical recoveries can subsequently be calculated. Relative standard deviations of the peak areas for seven compounds in male hamster urine ranged from 1 to 5% ($N = 4$), as shown in Table 1. This case is representative of the repeatability of other chromatographic profiles obtained from mammalian samples (Novotny et al., unpublished; Soini et al., unpublished). Theoretical solute

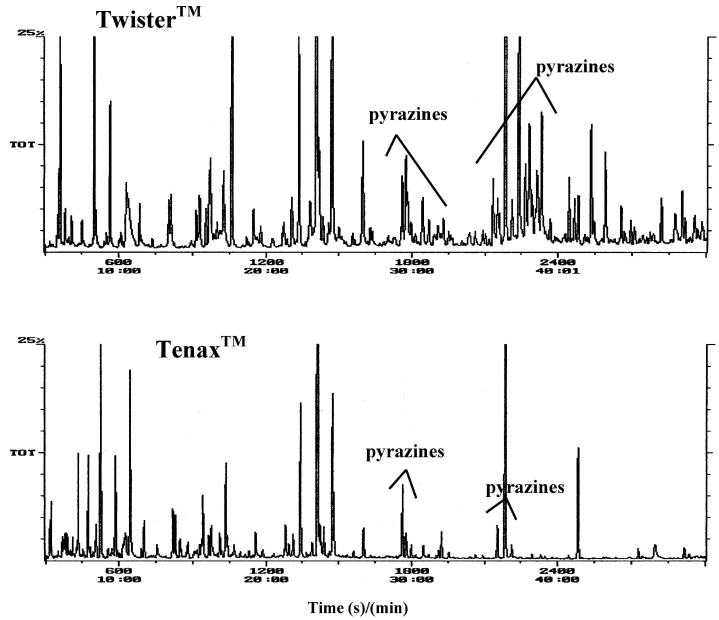

FIG. 4. Comparison of GC-MS total ion chromatogram (TIC) volatile profiles from hamster urine using Twister™ and Tenax™ methods. GC-MS conditions as described in the "Capillary Gas Chromatography-Mass Spectrometry" section.

extraction efficiencies into the PDMS phase varied between 3% and 88%. The more hydrophobic the compound, the higher is the extraction efficiency into the moderately hydrophobic PDMS polymer phase that can be expected. Hydrophobic compounds (e.g., geraniol) with high theoretical recoveries did not show improved extraction reproducibility compared with more hydrophilic compounds (e.g., 2,5-dimethylpyrazine). Overall, quantitative reproducibility of the SBSE extraction of volatiles from hamster urine was relatively constant over a respectable range of hydrophilic and hydrophobic compounds. The linearity of extraction was demonstrated by extracting benzaldehyde from aqueous solutions within the concentration range of 0.25 to 4.0 μg/ml for 60 min. The Coefficient of correlation (R^2, $N = 4$) was >0.99 over the measured range. Furthermore, stir bar extraction has proven to be well suited for the analysis of low levels of benzaldehyde found typically in biological samples. On the contrary, Tenax™ produces benzaldehyde as a degradation product from the polymer and thus would not be suitable for the low-level benzaldehyde analysis. There are often known and suspected artifacts of the interactions between solutes and the adsorbent, in addition to thermal degradation products of this polymer. The stir bar approach is relatively milder in this regard.

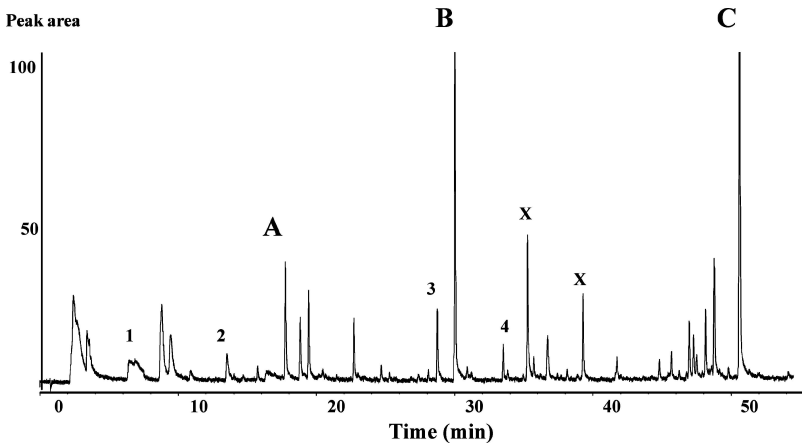

FIG. 5. Chromatogram of the SBSE-extracted and thermally desorbed compounds from male mouse urine (GC-AED, sulfur emission, 181 nm). 1 = dimethyldisulfide; 2 = bis(methylthio)methane; 3 = 2-sec-butyl-4,5-dihydrothiazole; 4 = benzothiazole; A, B, C unknown sulfur compounds; X = sulfur compounds from blank. Chromatographic separation conditions: as described in the text.

Effect of Dilution of Mouse Urine Matrix Proteins. Mouse urine contains relatively high concentrations (1–5 mg/ml) of major urinary proteins, MUPs (Finlayson et al., 1965). Several mouse pheromones are known to bind strongly inside the protein hydrophobic cavity (Zidek et al., 1999). We investigated whether a dilution of mouse urine and MUPs with water would change extractability of the mouse urinary constituents. A chromatogram of the extracted sulfur compounds from a pooled male mouse urine after 50:50 dilution with water is shown in Figure 5. Dilutions of 500 μl, 250 μl, and 100 μl of urine to 1 ml total volume with water decreased almost linearly the peak areas of selected sulfur compounds (Figure 6). Overall, the level of protein did not markedly affect extractability of mouse urine compounds. Detectability of about 0.2 ng of the sulfur urinary components, dimethyldisulfide and bis(methylthio)methane, was achieved with the SBSE method using 100 μl of mouse urine. Thus, the experiment, showed that relatively small biological sample volumes can be utilized reliably with the SBSE technique.

Suitability of SBSE for Tissue Samples. Tissue samples contain higher levels of hydrophobic matrix components, such as lipids and hydrophobic proteins, than urine. Hydrophobic matrix can compete in the sorption processes with the PDMS phase, lowering extraction efficiencies. In our processing of such samples, water proved to be an appropriate solvent for the extraction of volatiles from

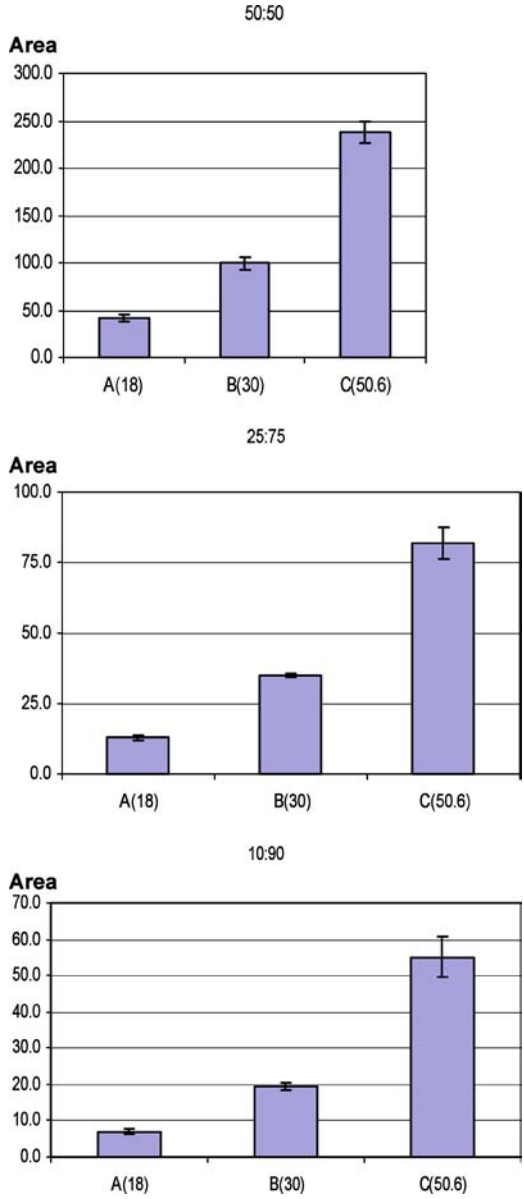

FIG. 6. Effect of dilution for stir bar extraction of selected sulfur compounds A, B, and C in mouse (*Mus domesticus*) urine. Standard deviation bars (SD, $N = 4$) indicate variation among repeated peak area measurements.

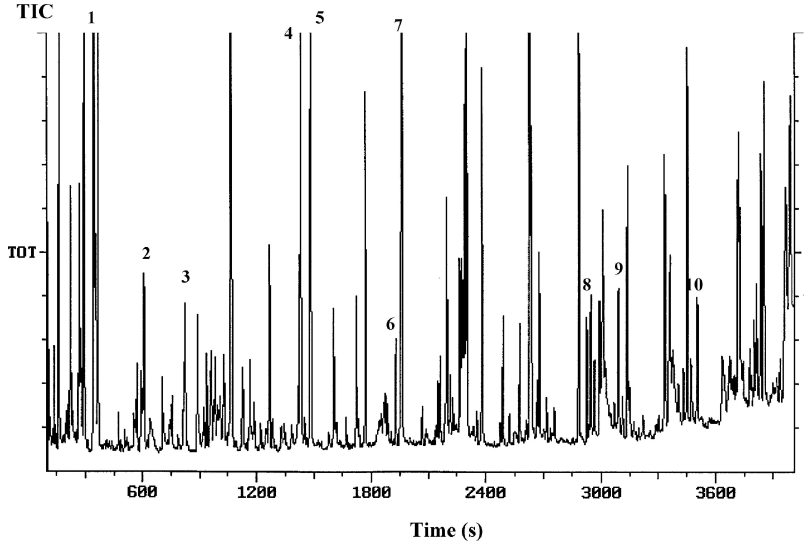

FIG. 7. A typical total ion chromatograph (TIC) obtained from the rat preputial gland by GC-MS after Twister™ extraction. Identified compounds: 1 = hexanal, 2 = heptanal, 3 = benzaldehyde, 4 = 2-nonanone, 5 = nonanal, 6 = decanal, 7 = benzothiazole, 8 = geranylacetone, 9 = α-farnesene, 10 = tetradecane. Analytical conditions: as described in the text.

rat preputial glands. A typical TIC (a total ion chromatogram) by GC-MS is shown in Figure 7. This extraction provided a wide range of tissue volatiles for quantification. We noticed that extraction efficiencies could be increased by using acetonitrile as an organic modifier in the extraction medium. However, acetonitrile (5–10%, v/v) increased also the levels of co-extracted matrix compounds, which were evidenced by the elevated baseline on a total ion chromatogram (data not shown). Work continues to optimize the extraction medium for the different tissue matrices.

SBSE in Sampling Volatiles from Air. Comparison of the extracted compound profiles by Twister™ SBSE and dynamic headspace with Tenax™ on rat odors is shown in Figure 8. During the dynamic headspace sampling of the relatively small air volume (1.2 l), stir bar extraction produced a wider range of compounds on thermal desorption than Tenax™. Difficulties in desorption of the higher molecular weight compounds on Tenax™ sampling were similar to those demonstrated with mouse urine. While no other porous polymers were investigated in this study, it is conceivable that specific methodologies may need to be developed for different applications in this area.

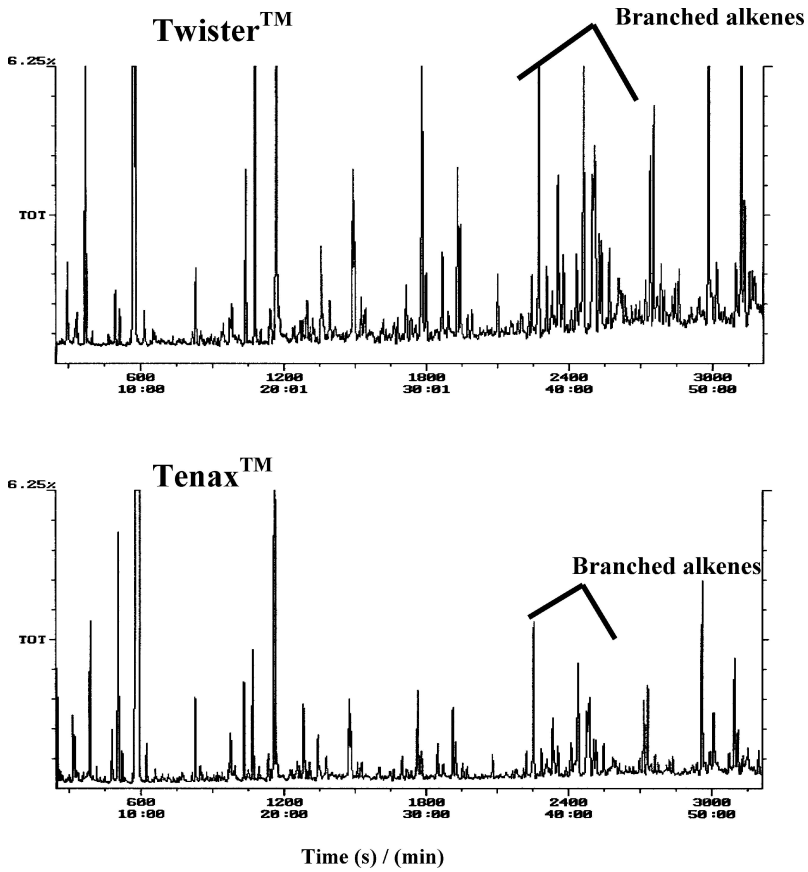

FIG. 8. GC-MS total ion chromatogram (TIC) profiles of volatiles/semivolatiles collected from the rat cage using Twister™ and Tenax™ sampling methods. Analytical conditions: as described in the text.

CONCLUSIONS

In summary, the novel sorptive extraction methodology with a PDMS coated stir bar proved to be highly reproducible and linear in aqueous sampling of volatiles and semivolatiles from mammalian urine and tissue samples. This technique allows the use of relatively small sample volumes (100 μl), as such samples could be diluted with water without significant component losses. In addition, the method was suitable for the extraction of small, organic, animal odor-related compounds in air samples in the dynamic sampling mode. Equilibrium properties of the PDMS material facilitated multiple sample extractions simultaneously and significantly

increased the analysis throughput. Efficient extraction and thermal desorption properties of the PDMS phase also produced previously unseen semivolatile compound patterns in the sample profiles, as demonstrated with the mouse and hamster urine samples. The matrix effects seen due to the urinary proteins (mouse urine) and tissue components (e.g., in glands) need more investigation to assess the quantitative attributes of the method in the presence of higher levels of lipids and macromolecular compounds.

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OPTIMIZATION OF PHEROMONE LURE AND TRAP CHARACTERISTICS FOR CURRANT CLEARWING, *Synanthedon tipuliformis*

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Abstract—Currant clearwing *Synanthedon tipuliformis* (Sesiidae) has been a pioneering and successful target of mating disruption in New Zealand, with virtually universal black currant industry adoption since c. 1990. Recent unexplained control failures using mating disruption lead to questions about pheromone efficacy. In this study, we have investigated the possible reasons for reduced control from mating disruption, and report improvements in trap catch based on pheromone loading and trap color. No differences were found in electrophysiological responses to pheromone components from two New Zealand populations. Male moth catches in traps baited with synthetic lures were disrupted in the presence of mating disruption dispensers (>99.99%) indicating no apparent barrier to efficacy from the pheromone formulation. Field behavioral observations confirmed this result. Male attraction to yellow delta traps was equivalent to green delta traps, but was greater than to red, black, blue, or white traps. Solid yellow delta traps were more attractive than black traps with yellow stripes, the latter designed to mimic the color pattern of the insect. Solid yellow funnel traps were less attractive than a composite of green, yellow, and white funnel traps. Trap catch increased as a function of pheromone loading and trap color. In another experiment conducted in Tasmania, there was no difference in catch with single component [(*E*,*Z*)-2,13-octadecadienyl acetate] or two component lures [97% (*E*,*Z*)-2,13-octadecadienyl acetate:3% (*E*,*Z*)-3,13-octadecadienyl acetate], refuting the suggestion of a different pheromone strain there.

Key Words—Pheromone, mating disruption, currant clearwing, *Synanthedon tipuliformis*, delta trap, funnel trap, color, behavior.

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INTRODUCTION

Currant clearwing *Synanthedon tipuliformis* (Clerck, 1759) (Sesiidae) is a worldwide pest of black currants (*Ribes nigrum*). It is difficult to control using insecticides because the cryptic larvae inhabit stems of black currants (Scott and Harrison, 1979), an important crop in New Zealand worth NZ$9m in 2002/03. The clearwing pest group includes congenerics with known pheromones, such as the apple clearwing *S. myopiformis* (Borkhausen), and peachtree borer *S. exitiosa* (Say) (Tumlinson et al., 1974; El-Sayed, 2004). The identification of the pheromone of *S. tipuliformis* (Priesner et al., 1986; Szöcs et al., 1990) led to the rapid development of mating disruption in New Zealand black currants (Thomas and Burnip, 1991), using polyethylene tubing dispensers.

The small size of the New Zealand industry, as well as the quick operational success of mating disruption across the entire industry, reduced interest in further research on the mechanisms and biology underlying disruption of this day-flying species. During the 1990s, growers progressively lowered application rates to as few as 200 dispensers/ha and appeared to maintain control, despite the original recommendations of 500 dispensers/ha (Thomas and Burnip, 1991). More recently, control failures have occurred in New Zealand, raising the question of whether the tactic was failing due to changes in the formulation, in grower practices (leading to a gradual build-up in the pest), or in the insect itself (e.g., through population adaptation). The mechanism of a possible change in the insect population was hypothesized to be sexual selection favoring individuals that were less affected by the synthetic blend, possibly through selection for additional components.

It was also suggested that Australian and New Zealand populations might be different (Szöcs et al., 1990), because there appeared to be differences between the optimal pheromone blends from New Zealand and Tasmanian trapping experiments, suggesting regional strains of currant clearwing, but the differences were never fully explored. The New Zealand insects responded best to 97% (*E*,*Z*)-2,13-octadecadienyl acetate [(*E*,*Z*)-2,13-18:Ac]:3% (*E*,*Z*)-3,13-octadecadienyl acetate [(*E*,*Z*)-3,13-18:Ac] compared to the Tasmanian population that responded optimally to 100% (*E*,*Z*)-2,13-18:Ac. However, the conclusion of a strain difference in Tasmania was taken from a single experiment with a 2-fold difference based on relatively low catches. Tests in Canada indicated a preference for the two component blend, similar to the European strain (Szöcs et al., 1998). Furthermore, catches in 11 countries across Europe showed little intraspecific variation, but catches in traps baited with (*E*,*Z*)-2,13-18:Ac alone were only 25% as high as catches in traps with the two component blend (Szöcs et al., 1991). James et al. (2001) also found the 97:3 ratio of the two component blend to be present from pheromone gland analyses of a Washington State, USA, population.

The goals of this study were to examine possible reasons for the change in efficacy of mating disruption, and to investigate whether trapping systems could

be improved to provide growers with better quality monitoring information. We first tested whether the ratio of the major and minor components of the black currant clearwing pheromone lures might influence male moth attraction in the presence of disruption (Minks and Cardé, 1988). Pheromone release rate is a key requirement for success of disruption (e.g., Suckling et al., 1999) and was also assessed. Both funnel traps and delta traps were used, depending on the aims of individual experiments.

A second series of experiments tested the effects of visual cues. Stüber and Dickler (1986) reported that male apple clearwing moths (*S. myopiformis*) exhibited upwind orientation to the characteristic red band on the female abdomen, highlighting the importance of visual stimuli in this species. Here, we investigated the color response of *S. tipuliformis* in greater detail with delta traps, because they are more readily manipulated, and available in more colors than funnel traps.

METHODS AND MATERIALS

Orientation Disruption (Experiment 1). This experiment compared catches in delta traps baited with a range of lures including caged virgin female moths, synthetic lures at doses of 0, 200 μg, 1, 3, and 10 mg [97% (*E*,*Z*)-2,13-18:Ac:3% (*E*,*Z*)-3,13-18:Ac (purity >95%)] in the presence or absence of mating disruption with 700 dispensers/ha (Shin Etsu Chemical Co., Tokyo, loaded with 97% (*E*,*Z*)-2,13-18:Ac:3% (*E*,*Z*)-3,13-18:Ac (purity >85%), placed in the field on November 11, 2002. Two separate properties in Canterbury, New Zealand were used for the experiment (Edenfield and Mullaghmore, 0.5 km apart), due to the need to separate blocks and avoid pheromone drift. Mullaghmore had consistently been treated with pheromone dispensers, whereas Edenfield had used mating disruption only intermittently (not in 2002/03 or 2003/04). The block used at Mullaghmore was planted in 1998 and was 0.75 ha in size. The Edenfield block was planted in 1994 and was 1 ha. The cultivar in both properties was cv. "Ben Rua." Six red delta traps (for each treatment above) were spaced within the crop at ≥20 m between traps, on metal poles at ca. 1 m height in the crop rows (total of 18 traps per property). Caged virgin female moths were of field origin as larvae and were 2-d old at the time of testing. A fine mist of water was applied to the inside of the cage enclosures each day between 08:00 and 09:00. Delta traps were operated from November 25 to December 12, 2002, randomly allocated to a position in the row and cycled to a new position daily at the time of insect counts. New sets of female clearwing moths were put out every 3–4 d.

Insect population levels in the treated and untreated blocks were assessed by analysis of canes on April 16, 2003. Destructive sampling of the ensuing larval population inside canes involved harvesting 60 shoots chosen at random (based on a pre-sample pilot study), measuring their length, and counting the number of larvae per shoot, to establish the number of larvae/m.

Orientation Disruption (Experiment 2). The same plots described above were maintained as treated or untreated for a second season. Three composite colored plastic funnel traps (green lid, yellow funnel, and white base) were operated in each block, with either the single component [(*E*,*Z*)-2,13-18:Ac)] or two component [97% (*E*,*Z*)-2,13-18:Ac:3% (*E*,*Z*)-3,13-18:Ac] lures (1 mg doses). Traps were maintained from November 10, 2003 to January 23, 2004, checked weekly, and the trap positions were rotated at each count.

Behavioral observations of male and female moths were made on three occasions in each treatment. One row was randomly selected in the treated and non-treated blocks and visual observations started between 13:00 and 14:00 and lasted for 20 min. Behavioral observations were made in consecutive time periods on warm (20–26°C) sunny days in each treatment (December 11, 12, and 15, 2003). Total incidence of male and female moths, flight activity of males, calling by females, and mating were recorded for each period. Wind speed and temperature were recorded at Lincoln University (5 km from sites).

Dispenser Release Rate (Experiment 3). Ten dispensers were put out in the field on November 11, 2002 at Edenfield, and measured weekly for pheromone content for 19 wk. Dispensers were shaken so that the fluid was at one end of the dispenser, and the rate of pheromone release from dispensers was estimated from the decrease of the length (mm) of the pheromone column inside the dispenser (Suckling and Shaw, 1992). A data logger (HortPlus temperature micrologger, Cambridge, NZ) recorded air temperatures hourly.

Electrophysiological Responses (Experiment 4). Coupled gas chromatography-electroantennogram recordings (GC-EADs) were made using excised male antennae from two New Zealand populations (Canterbury and Nelson) of currant clearwing. Responses of males to the mating disruption dispenser contents ($N = 5$ antennae), and to pheromone gland extracts of Canterbury ($N = 5$ antennae) and Nelson females ($N = 5$ antennae) were analyzed. An excised male moth antenna was positioned between two glass electrodes containing BE Ringer's solution with 10% polyvinylpyrrolidone (mol. wt. 360,000). Each glass electrode held a length of 1 mm silver wire that electrically connected the preparation to the recording unit preamplifier. The EAD exit port temperature was maintained at 200°C and the antennal preparation was placed in a filtered and humidified 400 ml/min airstream. Pheromone glands were excised from actively calling 1–2-d-old females held under ambient temperature and natural light in batches of five between 16:30 and 17:00 hr. All samples were analyzed using a Varian 3800 gas chromatograph (Varian, Walnut Creek, CA, USA), equipped with both HP-Wax and HP-5 capillary columns (Agilent, Palo Alto, CA, USA) of 30 m × 0.25 mm i.d. × 0.5 μm film thickness with a 1:1 split outlet, coupled to an INR-02 Recording Unit (Syntech Research and Equipment, Hilversum, The Netherlands). The retention times of the elicited antennal response peaks were compared with the retention times of standards (isomeric purity >98% (*E*,*Z*)-2,13-18:Ac and

(*E*,*Z*)-3,13-18:Ac; Pherobank, Wageningen, The Netherlands). Gas chromatographic conditions were as follows: splitless injections of either a 1 μl aliquot of a 0.1 mg/ml solution of disruption dispenser contents in hexane, or a 4 μl aliquot of gland extract, vented after 0.6 min to a 50:1 split and then to a 10:1 split after 1 min; injector 220°C; detector 300°C; nitrogen carrier gas at 1 ml/min, temperature program, 80°C for 1 min, 20°C/min to 240°C and held for 15 min. Chromatograms were converted to ASCII and averaged for presentation.

Trap Color Response (Experiment 5). This experiment at the untreated Edenfield site compared catches of males in delta traps of different colors, baited with 1 mg pheromone lures (ratio of 1,000:30 of the major and minor components). Traps were white, black, yellow (no lure), yellow, blue, green, and red [spectral reflectances are reported in Clare et al. (2000)], with three replicates of each deployed daily (and positions rotated) from December 19 to 24, 2002. Traps were placed at 20-m spacings, and the sticky inserts were changed after 25 moths were caught.

Influence of Yellow Banding on Catch (Experiment 6). The visual importance of the two color bands on the black body of the female moth to male behavior was tested at the untreated Edenfield site by comparing catches in delta traps of either solid yellow, white, and black colors, or black with 1–4 yellow stripes ($N = 3$ replicates, baited and operated from December 16 to 18, 2002 as in Experiment 5).

Effect of Color and Blend on Catch in Tasmania (Experiment 7). Delta traps ($N = 4$ replicates) were deployed in an untreated black currant block at a commercial planting near Hobart (Tasmania, Australia) with either the single component (100% (*E*,*Z*)-2,13-18:Ac) or the two component blend [97% (*E*,*Z*)-2,13-18:Ac:3% (*E*,*Z*)-3,13-18:Ac], using lures loaded with 100 or 1,000 μg, in both white or yellow delta traps at the lower dose, or yellow alone at the higher dose. Unbaited control traps of each color were also included. Traps were maintained from November 20, 2003 to January 16, 2004, and checked weekly.

Yellow and Green Funnel Traps (Experiment 8). This experiment compared green and yellow funnel traps [baited with synthetic pheromone (97:3) at 3 mg dose], after preliminary results in Experiment 1 indicated good attraction to this dose. Yellow funnel traps had a green lid, yellow top, and white base. Dichlorovos strips were used inside the funnel traps to kill the insects that were caught. Traps ($N = 9$ replicates) were placed at 20-m spacings in an untreated plot at the Edenfield site, and counts were made and traps rotated in position weekly from December 24, 2002 to January 22, 2003.

Effect of Color on Funnel Trap Catch (Experiment 9). Three different colors of funnel traps ($N = 3$ replicates) were deployed from December 1 to 16, 2003 in an untreated plot at the Edenfield site, using 1 mg lures (two component blend). Traps were checked weekly and re-positioned as above. Traps had three components with lid, funnel and base: (a) yellow, yellow, yellow, (b) green, yellow, green, or (c) green, yellow, white.

Statistical Analysis. In experiment 1, a two-way analysis of variance was conducted on log-transformed counts of trap catch (SAS Institute Inc., 1998), although untransformed means are presented for all experiments. Percentage disruption was calculated from the fraction of untransformed catch in treated over control plots. Except for experiment 8, significantly different treatment means were identified using Fisher's Protected Least Significant Difference Test (SAS Institute Inc., 1998) after analysis of variance on log-transformed counts, to stabilize the variance. In experiment 8, the significance of treatment effect was tested using an unpaired t test (SAS Institute Inc., 1998), after counts were found to be normally distributed ($P = 0.54$ and $P = 0.84$ for the two treatments). In the behavioral observation experiment, differences between treatments were identified using a χ^2 2×2 test of independence (SAS Institute Inc., 1998).

RESULTS

Orientation Disruption (Experiment 1). The orientation of male currant clearwing to traps baited with synthetic pheromone lures was successfully disrupted by dispensers releasing the same ratio of the two components (Figure 1), with over 1,623 moths caught in both treatments. The effects of pheromone treatment and lure loading were both significant, as was the interaction (two-way analysis of variance; treatment: $P < 0.001$, $F_{1,16} = 672$; lure: $P < 0.001$,

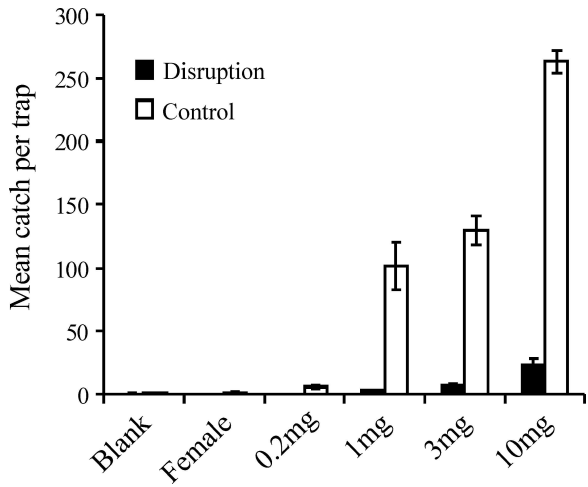

FIG. 1. Mean catch (SEM) of *S. tipuliformis* to synthetic pheromone lures at different doses deployed in delta traps placed in black currant blocks, with and without mating disruption.

$F_{3,16} = 215$; interaction: $P < 0.003$, $F_{3,16} = 6.99$). The significant interaction indicated that a reduction in percentage disruption occurred with increasing lure loading (100%, 97%, 94%, and 91% disruption with doses of 0.2, 1, 3, and 10 mg lures, respectively). There was no difference in catch in traps baited with caged virgin female moths and the unbaited traps, indicating that the female moths were unable to provide a measure of relative attraction of synthetic lures. The lack of attraction to virgin females may reflect insect handling or disruption of normal calling behavior in the cages. Male moths were periodically observed mating with female moths in the treated areas, indicating that mating was not completely prevented. Thomas and Burnip (1991) had reported low or no mating of sentinel females in treated plots, although 3–20% of captured feral females within the treatment were mated. A high population level was apparent in the present study, and cane analysis showed that differences in cane infestation were present between treatments at the end of the season [control: 1.16 ± 0.11 larvae/m of cane, treated: 0.70 ± 0.11 larvae/m of cane (mean $\pm$ SEM); $t = 2.21$, $P < 0.03$, df $= 98$). The lack of plot scale replication here prevents further interpretation of the impact of disruption on larval populations, although the starting population in the disrupted block for Experiment 2 would have been only 63% of the population in the untreated block in the second year.

The 200-μg lure was not effective in catching males compared to higher doses, and attracted no moths in the mating disruption plot. Thomas and Burnip (1991) reported complete trap shut-down of both 100 and 800 μg lures in several (but not all) trials. The experiments here extended this range to higher doses than similar tests with other species in this genus (e.g., Roccini et al., 2003).

Orientation Disruption (Experiment 2). In the second season, catches in the composite colored funnel traps in the untreated plot were high, but almost complete disruption of trap catches (>99%) was observed in the presence of mating disruption dispensers (Table 1), with a total of 2,929 moths caught in the trial. There was a significant effect of disruption ($F_{1,10} = 413.2$, $P < 0.001$), but no difference in trap catches in disrupted blocks with the addition of the second component in the 97:3 ratio. This result was unexpected because of the poor catches reported earlier from New Zealand to the single component (Szöcs et al.,

TABLE 1. CATCH (MEAN $\pm$ SE) OF *S. tipuliformis* IN COMPOSITE FUNNEL TRAPS TO SINGLE AND TWO COMPONENT LURES IN UNTREATED AND DISRUPTED BLACK CURRANT CROPS IN CANTERBURY, NEW ZEALAND

	100% (*E*,*Z*)-2,13-18:Ac	97% (*E*,*Z*)-2,13-18:Ac:3% (*E*,*Z*)-3,13-18:Ac
Disrupted	3.3 ± 1.5	2.3 ± 1.3
Control	481.0 ± 26.3	489.7 ± 50.9
% Disrupted	99.3%	99.5%

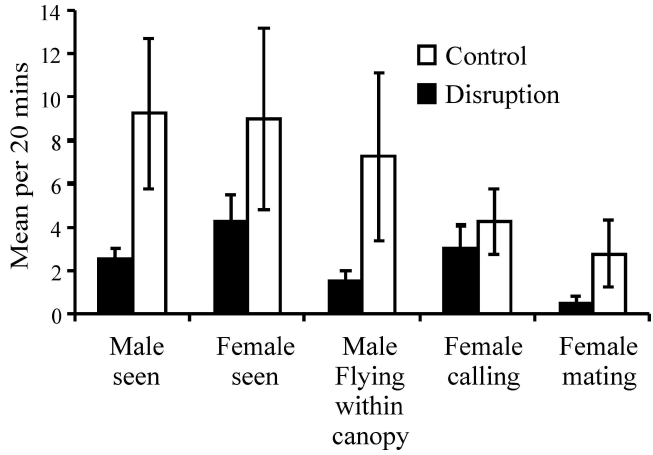

FIG. 2. Behavioral observations of male and female *S. tipuliformis* in black currant crops untreated or treated with mating disruption (same plots as for Table 1).

1990), although Szöcs et al. (1991) reported results close to this from one location in eastern Europe, of 11 locations trapped. Catches here were much larger than those reported from Europe.

Behavioral observations indicated (Figure 2) that male and female density may have been lower in the treated plots (27% and 47% of the control population of males and females, respectively). Although there was no significant difference between treatments, this trend is in the same direction as the estimate from cane sampling (the population in the treated area was 62% of the control population). Males were also observed flying less frequently in the treated crop ($P < 0.05$, $X^2 = 13.51$). In contrast, females were observed calling at relatively similar rates in both treatments (Figure 2), but mating occurred at lower rates in disrupted plots ($P < 0.05$, $X^2 = 11.88$).

Dispenser Release Rate (Experiment 3). The dispensers followed a zero order release, releasing a mean of 2.2% of the total amount of pheromone in the dispenser per week ($R^2 = 0.99$), at an average temperature over the period of ca. 14°C. The 50% life expectancy of the dispensers in the field was 110 d. One of the ten dispensers released pheromone at a rate of 6.3% of the total amount of pheromone per week, but it is unknown whether the sample accurately represents the proportion of malfunctioning dispensers. Dispenser content was confirmed by GC as 97% (*E*,*Z*)-2,13-18:Ac:3% (*E*,*Z*)-3,13-18:Ac (see Experiment 4).

Electrophysiological Responses (Experiment 4). Coupled GC-EAD analysis indicated antennal responses to both (*E*,*Z*)-2,13-18:Ac and (*E*,*Z*)-3,13-18:Ac in the commercial disruptant blend (Figure 3), and confirmed the presence of

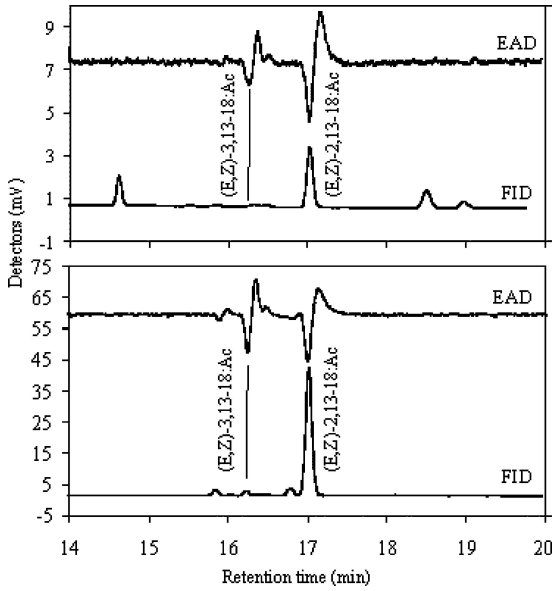

FIG. 3. Coupled gas chromatograph (bottom traces) and electroantennogram (top traces) analysis of the contents of (top) a polyethylene tubing dispenser (97:3 ratio, mean of 5 antennae), and (bottom) a pheromone gland extract (97:3 ratio, mean of 5 antennae).

these components in pheromone gland extracts (as reported by Szöcs et al., 1990; James et al., 2001) of females from Nelson (Figure 3) and Canterbury (not shown) populations of currant clearwing.

Trap Color Response (Experiment 5). Moth catch in delta traps was dependent on trap color ($F_{6,14} = 27.28$, $P < 0.001$). There was no difference between green and yellow delta traps, the two most preferred colors (Figure 4). These green and yellow traps have relatively similar spectral reflectances (Clare et al., 2000), which may account for the lack of difference in catches. Black and blue traps were ineffective, as were unbaited yellow traps. Red delta traps were also not very effective, but had been used earlier in Experiment 1. This suggests that even higher trap catches might have been obtained in mating disruption blocks if yellow traps with high pheromone doses had been used. A total of 473 insects were caught in the experiment.

Influence of Yellow Banding on Trap Catches (Experiment 6). Solid yellow was again favored over white (6-fold) or black traps (180-fold), including black traps with yellow bands designed to simulate the insect coloring ($F_{6,14} = 18.46$, $P < 0.001$) (Figure 5). There were no differences among catches in black traps with 1–4 yellow stripes. A total of 225 insects were caught in the experiment.

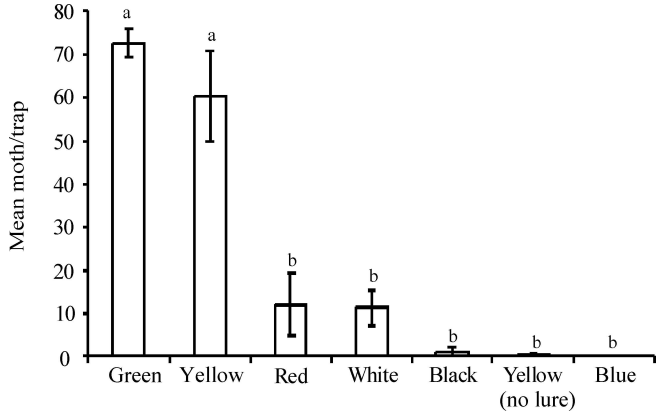

FIG. 4. Mean catches (SEM) of *S. tipuliformis* in a range of colored delta traps baited with pheromone or no lure. Different lower case letters indicate significant differences between treatments ($P < 0.05$).

Effect of Color and Blend on Catch in Tasmania (Experiment 7). A total of 3,016 moths were caught in commercial Tasmanian black currant plantings, with no catch in white or yellow blank traps (Figure 6). A one-way ANOVA on all treatments indicated a significant improvement of the combination of yellow traps

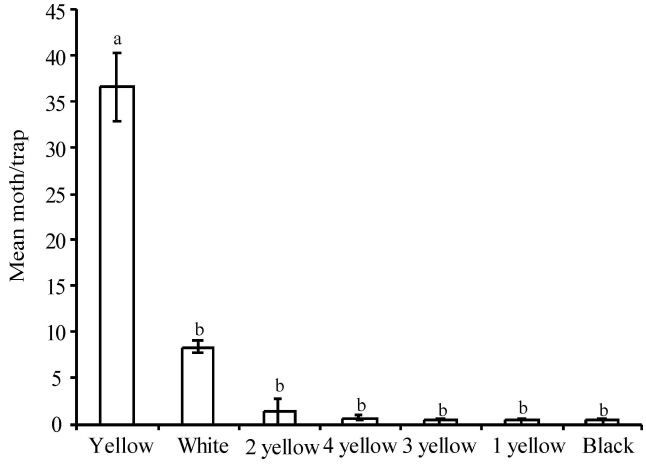

FIG. 5. Mean catches (SEM) of *S. tipuliformis* in pheromone-baited delta traps colored in solid colors, or in black with 1–4 yellow stripes. Different lower case letters indicate significant differences between treatments ($P < 0.05$).

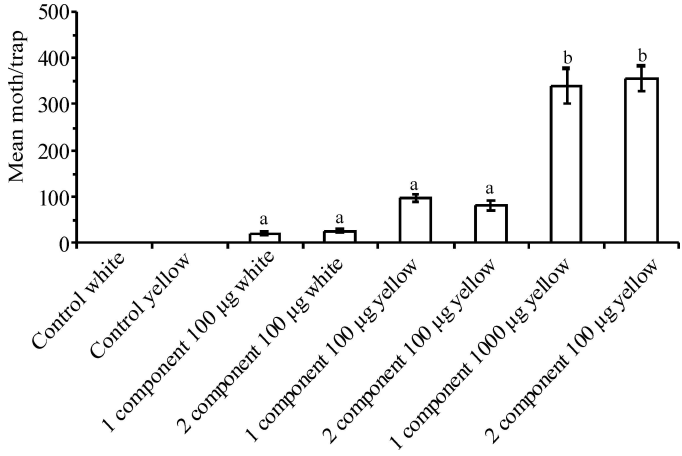

FIG. 6. Mean catches (SEM) of *S. tipuliformis* to pheromone-baited delta traps colored white or yellow, with single or two component blends at two doses. Different lower case letters indicate significant differences between treatments ($P < 0.05$).

baited with the high dose over the other treatments ($F_{7,24} = 19.58$, $P < 0.001$). There was no evidence of a blend effect, with single or two component lures, after one-way ANOVA of all baited treatments ($F_{1,22} = 0.05$, $P = 0.83$).

Yellow and Green Funnel Traps (Experiment 8). Composite yellow and white funnel traps caught more currant clearwing males (mean ± SEM: 238.9 ± 26.0) than green funnel traps (46.2 ± 9.2) ($t = 7.03$, $P < 0.001$, df = 9). This result suggests that larger catches could have been made in Experiments 5 and 6, if funnel traps had been used instead of delta traps.

Effect of Color on Funnel Trap Catch (Experiment 9). There were differences in catch between funnel traps of the three composite color patterns ($F_{2,6} = 18.34$, $P = 0.003$) (Figure 7), with the greatest catch in composite yellow and white funnel traps.

DISCUSSION

Disruption of male moth catches in pheromone-baited traps was successfully achieved in the block treated with polyethylene tubing pheromone dispensers, reducing the likelihood that large-scale changes in the response of the insect population to the pheromone had occurred. Such changes due to co-adaptation or "resistance" to the tactic of mating disruption have been hypothesized (Cardé and Minks, 1995), but there was no evidence for either in our study. At the start of this study, we hypothesized that resistance might be occurring due to selection for a

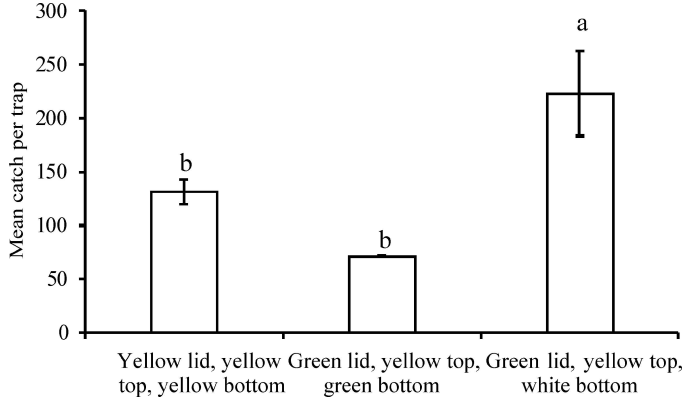

FIG. 7. Mean (±SEM) catches of *S. tipuliformis* in plastic funnel traps with different colored components.

shift in blend by calling females, possibly influenced by the presence of a mixture of the purported Tasmanian and New Zealand pheromone strains (Szöcs et al., 1990). Furthermore, the analysis of gland extracts found the presence of the two electrophysiologically active components, but did not find evidence of additional components that might provide the basis for mate location in the presence of two component mating disruption (as adopted across the majority of the habitat of this monophagous species since 1990).

There was no evidence of an intrinsic failure of mating disruption in controlling *S. tipuliformis* in black currant crops. It therefore seems more likely that the explanation for failure of mating disruption was the gradual increase in pest populations over time, possibly because of dispensers being deployed at densities below the threshold required for efficacy, and the industry shift away from any insecticides in the crop. A reduction in insecticide use after the introduction of mating disruption may have exacerbated pest populations during the 1990s. Because mating disruption works best at low population levels, it may be necessary to either return to higher dispenser rates or to use alternative tactics to regain control in areas with high pest populations.

Equivalent catches with the single component and two component lures in both New Zealand and Tasmanian populations (with data sets comprising high catches) challenge the earlier reports of the importance of the second component, even though this can be seen in the gland extract (Figure 3). The earlier reported preference in Tasmania for the single component over the two component blend (Szöcs et al., 1990) was not supported by the trapping in Tasmania reported here, and there was no evidence for differences between Australian and New Zealand populations in this regard.

Experiments with different trap colors and patterns showed that traps of solid yellow color were most attractive. This also highlights the importance of visual and olfactory cues in mate location in this day-flying species, although it is unclear why this species should be attracted to patches of yellow color. While there were hints of differences in catch with different multiples of yellow stripes, more work would be needed to provide a definitive understanding of the visual acuity of this insect. The combination of a high dose lure in combination with yellow traps improved catches, and this improvement suggests that the possibility of mass trapping of males may warrant examination for currant clearwing control. These results on visual cues also suggest that mating disruption using yellow dispensers could provide an improvement in control over clear dispensers, by combining false trail following to the odor plume with visual cues for mate location, and this approach may warrant further examination. Funnel traps were successful at catching large numbers of *S. tipuliformis*, and did not suffer from trap saturation under high pest densities (as noted for *S. myopifomis* by Voerman and van Deventer, 1984). Roccini et al. (2003) found no difference in catch of the Douglas fir pitch moth, *S. novaroensis* (Hy. Edwards) between sticky traps and funnel traps, but this may have been due to low catches in that study. The composite colored funnel traps with a yellow funnel were more effective than fully yellow traps, further suggesting a high degree of visual discrimination by this insect.

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6-ALKYLSALICYLIC ACIDS AND 6-ALKYLRESORCYLIC ACIDS FROM ANTS IN THE GENUS *Crematogaster* FROM BRUNEI

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Abstract—The defensive chemistry of two species of ants from Brunei in the genus *Crematogaster* (*Physocrema* group) has been investigated. Ants in this group release a white secretion from hypertrophied metapleural glands on their thorax when they are disturbed. Previously, one species in this group has been shown to produce alkylphenols and alkylresorcinols. In the present investigation, similar compounds along with salicylic acids and resorcylic acids that are anacardic acid and olivetolic acid homologs, respectively, are described from two species. The structures of these compounds were suggested by their spectroscopic data and confirmed by direct comparison with synthetic samples. Some of these compounds occur in lichens and have well documented physiological activities.

Key Words—*Crematogaster* spp., Hymenoptera, Formicidae, phenols, resorcinols, resorcylic acids, salicylic acids, chemotaxonomy, metaplural glands.

INTRODUCTION

The exocrine chemistry of ants in the genus *Crematogaster* has been investigated in many species. Typically, the alarm pheromones in these ants are volatile, acyclic alcohols and ketones produced in the mandibular glands (Crewe et al., 1969,

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1970; Schlunegger and Leuthold, 1972; Leuthold and Schlunegger, 1973; Brand and Pretorius, 1986; Scheffrahn and Rust, 1989). On the other hand, the Dufour's gland of this genus is the source of numerous long-chain, cross-conjugated dienone derivatives and furanocembrane diterpenes that function as defensive compounds (Daloze et al., 1987, 1998; Leclercq et al., 1997, 2000a,b; Pasteels et al., 1989) along with, in at least one case, some novel long chain trihydroxycyclohexanes (Laurent et al., 2003). Additionally, *Crematogaster difformis* (= "*deformis*") in the *Physocrema* group, collected at Gombak Valley Field Study Center of the University of Malaya, produces alkylphenols, alkylresorcinols, and mellien in the hypertrophied metapleural glands on its thorax (Attygalle et al., 1989).

In its more typical form, the metapleural gland occurs across major groups of ants, though it has been lost secondarily in some arboreal taxa and males of some species (Hölldobler and Wilson, 1990). Distinguishing the Formicidae from bees and wasps, which impregnate brood cells with antimicrobial compounds, this gland has likely played a key role in maintaining the cleanliness of the nest environment, enabling ants to colonize moist, microbe-ridden nest sites (Hölldobler and Wilson, 1990). Prior to studies of *C. difformis* products, secretions of this gland were known only for highly derived leafcutter ants (*Atta*), in which the gland produces antibiotic phenylacetic acid (Maschwitz et al., 1970). However, antifungal activity has been demonstrated for unidentified metaplural gland products of other ants (Beattie et al., 1986).

Here, we report a detailed analysis of the extracts of *C. difformis* F. Smith, *sensu lat*. and *C. inflata* F. Smith collected in Brunei Darussalam, showing that the major aromatic compounds present in these ants are salicylic acids and resorcylic acids, respectively, the apparent biological precursors of the alkylphenols and resorcinols. Analysis of the former species has revealed the presence of some of the previously reported phenols and isocoumarins along with larger quantities of 6-alkylsalicylic acids, while *C. inflata* contains 5-alkylresorcinols and larger quantities of the corresponding 6-alkylresorcylic acids.

METHODS AND MATERIALS

Field Studies. Collections of *C. difformis sensu lat*.(June 2002) and *C. inflata* (October 2003) in methanol were made at the Kuala Belalong Field Studies Center, run by the University of Brunei Darussalam, and located in the Batu Apoi Forest Reserve, Temburong District, Brunei Darussalam (4°32′N, 115°10′E). Specimens collected in ethanol from the same colonies were compared with type material, and vouchers have been deposited in the entomological collections of both the Brunei Museums and the Natural History Museum of Los Angeles County. *C. difformis sensu lat*. nested in the low arboreal zone and foraged arboreally, and *C. inflata* nested high in an isolated canopy tree and mass foraged in the leaf litter.

Chemical Analysis. Gas chromatography-mass spectrometry (GC-MS) was carried out in the EI mode using a Shimadzu QP-5000 GC-MS equipped with a RTX-5, 30 m × 0.25-mm i.d. column. The instrument was programmed from 60°C to 250°C at 10°/min. Vapor phase FT-IR spectra were obtained using an Hewlett-Packard model 5965B detector interfaced with an H-P 5890 gas chromatograph fitted with a 30 m × 0.25 mm RTX-5 Amine column. High-resolution mass spectrometry was performed on a JEOL SX102 instrument in the positive ion fast atom bombardment mode using a direct probe. Nuclear magnetic resonance (NMR) spectrometry was carried out in $CDCl_3$ solutions using a Varian Mercury 400 NMR spectrometer. The phenols and isocoumarins were suggested from their mass spectra (NIST/EPA/NIH, 1999) and retention times, and were confirmed by comparison with commercial or synthetic authentic samples.

Initial GC-MS analysis of the methanol extract of *C. difformis sensu lat.* revealed the presence of 3-*n*-propylphenol (**1a**), 3-*n*-pentylphenol (**1b**), and 3-*n*-heptylphenol (**1c**) in ca. a 1:1:1 ratio comprising ∼80% of the volatiles detected, along with small amounts of hydroxyisocoumarin (**2**) and mellein (**3**), 3% and 2%, respectively (Table 1). The mass spectra of the remaining poorly resolved components had a base peak at $m/z = 134$, suggesting that they might be methyl salicylates. The solvent was removed from a small amount of the extract *in vacuo*,

TABLE 1. AROMATIC COMPOUNDS DETECTED IN *Crematogaster* SPECIES

	% Composition	
	Before methylation	After methylation[a]
C. difformis sensu lat.		
3-*n*-Propylphenol (**1a**)	26.7	4.5
3-*n*-Pentylphenol (**1b**)	26.7	<1
3-*n*-Heptylphenol (**1c**)	26.7	<1
Hydroxyisocoumarin (**2**)	3	—
Mellein (**3**)	2	—
6-*n*-Propylsalicylic acid (**4a**)	—	41
6-*n*-Pentylsalicylic acid (**4b**)	—	34
6-*n*-Heptylsalicylic acid (**4c**)	—	19
C. inflata		
5-*n*-Pentylresorcinol (**5a**)	42.0	4.7
5-*n*-Heptylresorcinol (**5b**)	57.7	9.4
5-*n*-Nonylresorcinol (**5c**)	<1	<1
6-*n*-Pentylresorcylic acid (**6a**)	—	28.4
6-*n*-Heptylresorcylic acid (**6a**)	—	56
6-*n*-Nonylresorcylic acid (**6a**)	—	<1

—Not detected.

[a]Samples were treated for 24 hr with excess CH_3I/K_2CO_3, and compounds were detected as their methyl esters and ethers. In this way, **4a–4c** were detected as **8a–8c**, and **6a–6c** were detected as **10a–10c**.

replaced with an equal volume of acetone, and treated with anhydrous K_2CO_3 and a few drops of CH_3I. After 24 hr, GC-MS analysis of the mixture showed the presence of equivalent amounts of three new homologous components, methyl 2-methoxy-6-propylbenzoate (**8a**), methyl 2-methoxy-6-pentylbenzoate (**8b**), and methyl 2-methoxy-6-heptylbenzoate (**8c**) (Table 1). The gas chromatographic retention times and mass spectra of **8a–8c** were identical to those of synthetic samples. The remaining volatile components were the three peaks whose mass spectra corresponded to the methyl ethers of the alkylphenols (**1a–1c**) MS *m/z* (rel.%) 121 (100).

The only aromatic compounds detected in the initial GC-MS analysis of the methanol extract of *C. inflata* were 5-*n*-pentylresorcinol (**5a**), and 5-*n*-heptylresorcinol (**5b**) (Attygalle et al., 1989; NIST/EPA/NIH, 1999), and a trace of 5-*n*-nonylresorcinol (**5c**) MS *m/z* (rel.%) 236 (M^+, 4), 138 (15), 137 (13), 136 (14), 124 (100), 123 (25), 107 (13), and 91 (17) (Table 1). The solvent was removed from a small amount of the extract *in vacuo*, replaced with an equal volume of acetone, and treated with anhydrous K_2CO_3 and a few drops of CH_3I. After 24 hr, GC-MS analysis of the mixture showed the presence of three new homologous components, methyl 2,4-dimethoxy-6-pentylbenzoate (**10a**), methyl 2,4-dimethoxy-6-heptylbenzoate (**10b**), and a very small amount of the two carbon homolog, methyl 2,4-dimethoxy-6-nonylbenzoate (**10c**) MS *m/z* (rel.%) 322 (M^+, 4), 291 (8), 223 (3), 210 (100), 191 (25), 179 (12), 152 (20), 151 (30), 135 (5), 121 (4), 109 (10), and 91 (7) (Table 1). The gas chromatographic retention times and mass spectra of **10a** and **10b** were identical to those of synthetic samples.

3-Heptylphenol (**1c**). A solution containing 0.49 g of 3-hydroxybenzaldehyde (4 mmol) in 5 ml of THF was added slowly to a THF solution of *n*-hexylmagnesium bromide made from 2.64 g (4 equiv.) of *n*-hexylbromide. The resulting mixture was heated to reflux for 1 hr, cooled, diluted with ether, and carefully acidified with 10% HCl. The layers were separated, and the aqueous layer was washed two times with 50 ml of ether. The combined ether layers were washed once with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo* to give 0.9 g of a waxy solid. This solid was taken up in 50 ml of ethanol and shaken under 3 atm. of hydrogen in the presence of 300 mg of 10% Pd/C until the uptake of hydrogen ceased. After filtration and removal of the solvent, kugelrohr distillation provided 0.8 g of **1c** that was >95% pure by gas chromatographic analysis. ^{1}H NMR (400 MHZ, $CDCl_3$) δ: 0.84 (t, $J = 7.6$ Hz, 3H), 1.24 (m, 8H), 1.53 (q, $J = 7.6$ Hz, 2H), 2.48 (t, $J = 7.6$ Hz, 2H), 5.9 (br s, 1H), 6.61 (d, $J = 7.6$ Hz, 1H), 6.63 (s, 1H), 6.70 (d, $J = 7.6$ Hz, 1H), 7.07 (t, $J = 7.6$ Hz, 1H). ^{13}C NMR (100 MHZ, $CDCl_3$) δ: 14.39, 22.97, 29.48, 29.59, 31.61, 32.10, 36.12, 112.85, 115.68, 121.21, 129.65, 145.25, 155.62. MS *m/z* (rel.%) 192 (7, M^+), 149 (2), 121 (9), 108 (100), 107 (35), 77 (16), 43 (18), 41 (19). HRMS: Calculated for $C_{13}H_{20}O$ (M^+), 192.1514; observed 192.1506.

Methyl 2-Methoxy-6-Propylbenzoate (**8a**). A solution containing 0.47 ml of diisopropylamine in 7 ml of THF was cooled to −78°C and treated with 2.1 ml of 1.6 M *n*-butyllithium in hexanes. The mixture was warmed to 0°C for 15 min then cooled to −78°C, and a solution of 300 mg (1.3 mmol) of methyl 2-methoxy-6-methylbenzoate (**7**) in 2 ml of THF was added dropwise. After 10 min, a solution containing 0.2 ml of ethyl iodide and 0.5 ml of DMPU in 2 ml of THF was added, and the mixture was allowed to warm to room temperature. The mixture was poured into excess ether, extracted with 5% HCl, and water, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo* to give 0.4 g of **6a** that was >90% pure by gas chromatographic analysis. ^{1}H NMR (400 MHZ, $CDCl_3$) δ: 0.92 (t, $J = 7.6$ Hz, 3H), 1.60 (sextet, $J = 7.6$ Hz, 2H), 2.51 (t, $J = 7.6$ Hz, 2H), 3.81 (s, 3H), 3.909 (s, 3H), 6.76 (d, $J = 7.6$ Hz, 1H), 6.81 (s, 1H), 7.26 (t, $J = 7.6$ Hz, 1H). ^{13}C NMR (100 MHZ, $CDCl_3$) δ: 14.25, 24.48, 35.72, 52.41, 56.08, 108.59, 121.75, 123.71, 130.45, 141.31, 156.42, 168.24. MS *m/z* (rel.%) 208 (23), 177 (37), 161 (100), 147 (31), 105 (20), 91 (33), 77 (28), 51 (25). HRMS: Calculated for $C_{12}H_{16}O_3$ (M^+), 208.1099; observed 208.1107.

Methyl 2-Methoxy-6-Pentylbenzoate (**8b**). The procedure described for **8a** with butyl iodide produced 0.3 g of **8b** that was >90% pure by gas chromatographic analysis. ^{1}H NMR (400 MHZ, $CDCl_3$) δ: 0.86 (t, $J = 7.6$ Hz, 3H), 1.30 (m, 4H), 1.57 (quintet, $J = 7.6$ Hz, 2H), 2.53 (t, $J = 7.6$ Hz, 2H), 3.79 (s, 3H), 3.89 (s, 3H), 6.74 (d, $J = 8$ Hz, 1H), 6.81 (d, $J = 8$ Hz, 1H), 7.25 (t, $J = 8$ Hz, 1H). ^{13}C NMR (100 MHZ, $CDCl_3$) δ: 14.17, 22.64, 31.04, 31.87, 33.64, 52.30, 56.05, 108.85, 121.71, 123.71, 130.45, 141.57, 156.47, 169.15. MS *m/z* (rel.%) 236 (14), 205 (18), 179 (12), 161 (100), 148 (13), 121 (16), 105 (8), 91 (15), 77 (13), 65 (6), 41 (12). HRMS: Calculated for $C_{14}H_{20}O_3$(M^+), 236.1412; observed 236.1418.

Methyl 2-Methoxy-6-Heptylbenzoate (**8c**). The procedure described for **8a** with hexyl iodide produced 0.4 g of **8c** that was >90% pure by gas chromatographic analysis. ^{1}H NMR (400 MHZ, $CDCl_3$) δ: 0.87 (t, $J = 7.6$ Hz, 3H), 1.28 (m, 8H), 1.57 (br q, 2H), 2.53 (t, $J = 7.6$ Hz, 2H), 3.80 (s, 3H), 3.89 (s, 3H), 6.74 (*d*, $J =$ 8 Hz, 1H), 6.81 (d, $J = 8$ Hz, 1H), 7.25 (t, $J = 8$ Hz, 1H). ^{13}C NMR (100 MHZ, $CDCl_3$) δ: 14.30, 22.86, 29.28, 29.67, 31.37, 31.97, 33.70, 52.30, 56.06, 108.57, 121.71, 123.70, 130.44, 141.59, 156.47, 169.16. HRMS: Calculated for $C_{16}H_{24}O_3$(M^+), 264.1725; observed 264.1725.

Methyl 2,4-Dimethoxy-6-Pentylbenzoate (**10a**). By using the procedure described for the preparation of **6a–6c**, 0.42 g of methyl 2,4-dimethoxy-6-methylbenzoate (**9**) was alkylated with butyl iodide. Radial chromatography of a sample gave 23 mg of **10a** that was >90% pure by gas chromatographic analysis. ^{1}H NMR (400 MHZ, $CDCl_3$) δ: 0.81 (t, $J = 6.8$ Hz, 3H), 1.2 (m, 4H), 1.5 (m, 2H), 2.47 (t, $J = 8$ Hz, 2H), 3.73 (s, 3H), 3.74 (s, 3H), 3.81 (s, 3H), 6.243 (d, $J = 2.4$ Hz, 1H), 6.252 (d, $J = 2.4$ Hz, 1H). ^{13}C NMR (100 MHZ, $CDCl_3$) δ: 14.23, 22.68, 31.11, 31.91, 34.14, 52.31, 55.58, 56.09, 96.28, 105.96, 116.41, 143.32, 158.15, 161.57, 169.15. MS *m/z* (rel.%) 266 (21), 235 (30), 211 (10), 210

(100), 209 (18), 191 (37), 179 (12), 152 (10), 151 (45), 135 (11), 121 (7), 91 (17), 77 (22), and 41 (32).

Methyl 2,4-Dimethoxy-6-Heptylbenzoate (**10b**). By using the procedure described for the preparation of **8a**, 0.24 g of methyl 2,4-dimethoxy-6-methylbenzoate (**9**) was alkylated with hexyl iodide. Radial chromatography of a sample gave 86 mg of **10b** that was >90% pure by gas chromatographic analysis. ^{1}H NMR (400 MHZ, $CDCl_3$) δ: 0.80 (t, $J = 6.8$ Hz, 3H), 1.2 (m, 8H), 1.49 (m, 2H), 2.46 (t, $J =$ 8 Hz, 2H), 3.72 (s, 3H), 3.74 (s, 3H), 3.80 (s, 3H), 6.239 (d, $J = 2.4$ Hz, 1H), 6.258 (d, $J = 2.4$ Hz, 1H). ^{13}C NMR (100 MHZ, $CDCl_3$) δ: 14.34, 22.88, 29.32, 29.72, 31.45, 32.00, 34.19, 52.30, 55.57, 56.08, 96.28, 105.95, 116.40, 143.33, 158.16, 161.47, 169.16. MS *m/z* (rel.%) 294 (15), 263 (18), 223 (5), 211 (10), 210 (100), 209 (12), 191 (25), 179 (5), 152 (10), 151 (35), 135 (7), 91 (12), 77 (10), 55 (10), 43 (20), and 41 (30).

The ^{13}C NMR spectra, mass spectra and gas chromatographic retention times of **10a** and **10b** were identical to those of samples prepared by the exhaustive methylation of authentic samples of **6a** and **6b**, respectively.

RESULTS AND DISCUSSION

The initial analysis of the methanol extracts from *C. difformis sensu lat.* revealed that over 80% of the detectable volatiles were a 1:1:1 mixture of 3-propylphenol (**1a**), 3-pentylphenol (**1b**), and 3-heptylphenol (**1c**) accompanied by small amounts of 3,4-dihydro-8-hydroxyisocoumarin (**2**) and its 3-methyl homolog, mellien (**3**) (Figure 1). Except for **1c**, these compounds are well-known

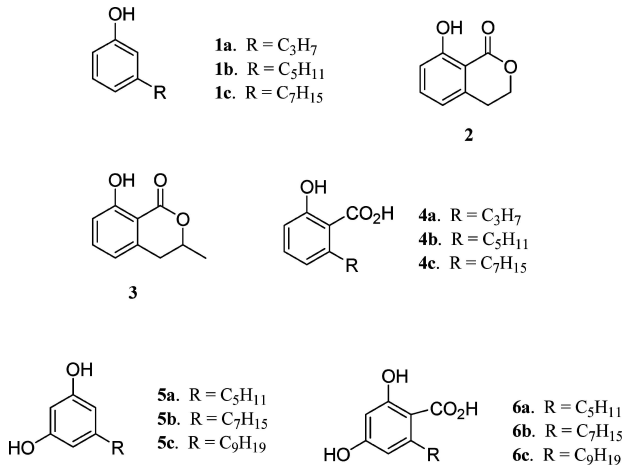
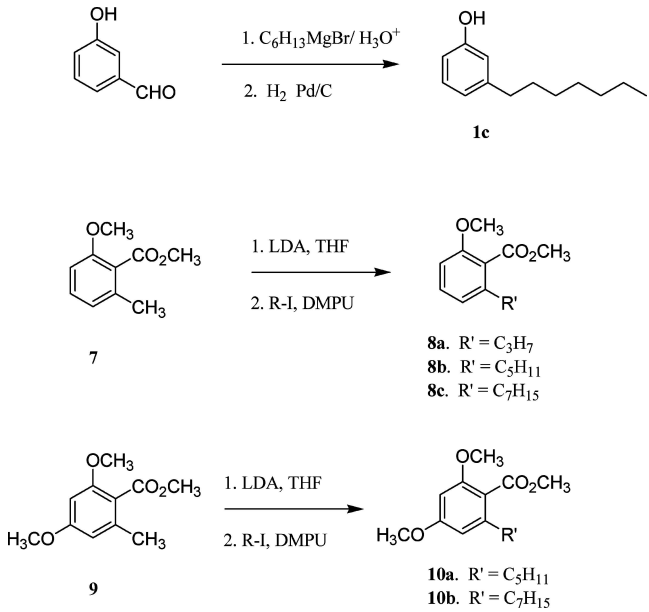


FIG. 1. Compounds found in *Crematogaster* cf. *difformis* and *C. inflata*.

from insect sources and were identified from their published mass spectra (Lloyd et al., 1978; Attygalle et al., 1989). In the case of **1c**, identification was made by direct comparison with synthetic material.

In this analysis, some of the poorly resolved minor components had mass spectra with a base peak at $m/z = 134$ (100), suggesting the presence of hydroxybenzoic acids that had been esterified by the solvent. Exhaustive methylation with CH_3I/K_2CO_3 showed that the major components of the extract were the 6-alkylsalicylates **4a–4c**, which had been converted to the methyl 6-alkyl-2-methoxybenzoates **8a–8c** (Table 1). The structures of these esters were confirmed by direct comparison with synthetic compounds prepared by lateral lithiation and subsequent alkylation (Tyman and Visani, 1997) of methyl 6-methyl-2-methoxybenzoate (**7**) (Hauser and Pogany, 1980; Hauser and Ellenberger, 1987) with the appropriate alkyl iodide (Scheme 1). It was found that by using a solution of the alkyl iodide in THF containing an equal volume of DMPU instead of HMPA, this reaction provided a crude product that was nearly 90% pure.

Since the methoxyphenol derivatives of **1a–1c** were only a small percentage of the mixture after exhaustive methylation, and the dihydroisocoumarins **2** and **3** amount to only 1–2% of the aromatic volatiles present, these results indicate that



SCHEME 1. Syntheses of *m*-heptylphenol (**1c**), methyl 2-alkyl-6-methoxybenzoates (**6a–6c**), and methyl 2-alkyl-4,6-dimethoxybenzoates (**10a–10b**).

the major components of the extracts of *C. difformis sensu lat.* appear to be the 6-alkylsalicylic acids **4a–4c** (Table 1). Obviously, the underivatized 6-alkylsalicylic acids **4a–4c** are not amenable to gas chromatography under normal conditions.

In a similar manner, the initial analysis of the methanol extracts from *C. inflata* revealed only the presence of 5-alkylresorcinols **5a–5c**, along with the usual fatty acid methyl esters resulting from the methanol that the ants were collected in. Exhaustive methylation with CH_3I/K_2CO_3 showed that the apparent major components of the extract were the 2-alkyl-resorcylic acids **6a–6c**, which had been converted to the methyl 6-alkyl-2,4-dimethoxybenzoates **10a–10c** by this treatment (Table 1). The structures of esters **10a** and **10b** were confirmed by direct comparison with synthetic compounds prepared by lateral lithiation and subsequent alkylation (Tyman and Visani, 1997) of methyl 6-methyl-2,4-dimethoxybenzoate (**9**) (Wedekind and Fleischer, 1923; Gaucher and Shepherd, 1971) with the appropriate alkyl iodide (Scheme 1). The structure of the minor component **10c** was inferred from its mass spectrum. The relative amounts of the resorcinols (**5a–5c**) to the resorcylic acids (**6a–6c**) as their completely methylated derivatives are shown in Table 1.

The 6-alkylsalicylic acids **4a–4c** are lower homologs of the "anacardic" acids, extensively studied components of cashew nut oil (*Anacardium* spp.) and *Ginkgo biloba* (Tyman, 1979). These compounds and some of their analogs have antibacterial, antifungal, and antifeedant properties (Matsumoto and Sei, 1987; Muroi and Kubo, 1993; Kubo et al., 1993; Begum et al., 2002). Additionally, they are known to impart mite resistance to geraniums (Walters et al., 1988). It should be noted that the antibacterial activity of these compounds increases with increasing alkyl chain length (Muroi and Kubo, 1993). The antibiotic functions of these compounds are consistent with the current hypothesis for the functional significance of the metaplural gland (see "Introduction").

The resorcylic acids **6a** and **6b** are well known components of lichen depsides (Elix, 1974; Elix et al., 1994; Djura and Sargent, 1976). The repellency of the alkyl resorcinols such as **5a** has been described (Attygalle et al., 1989) so the resorcylic acids may only be present as their precursors, or they may also serve as antimicrobials (Fujikawa et al., 1953).

Although the ecology of *C. difformis sensu lat.* remains poorly studied, the species has been placed in the subgenus *Physocrema*, together with *C. inflata*, which also has hypertrophied metapleural glands [Forel, 1912, as *C.* (sic) *deformis*]. In the latter species, the propodeum, which contains the glands, is colored bright yellow and may function as aposematic coloration. Under attack by highly predacious weaver ants (*Oecophylla smaragdina*), *C. inflata* workers release a white secretion from their metapleural glands upon direct contact with the propodeum (Hölldobler and Wilson, 1990). It is likely that the metapleural glands of *C. difformis sensu lat.* function similarly. The integument of this ant is weak, and light pressure on the anterior thorax or head serves to evert the metapleural

glands, even in dead individuals. This, along with the observed behavior of the related *C. inflata*, and the noxious nature of the "anacardic" acids and alkyl phenols suggests a defensive role in interspecific combat with ants and other predators.

The chemistry before methylation of both *C. difformis sensu lat.* and *C. inflata* shows some similarity to that described for the Malaysian *Crematogaster* species (Attygalle et al., 1989), although no exhaustive methylation was carried out in that case. The phenols **1a–1b** and the isocoumarins **2** and **3** were detected in the Bruneian *C. difformis* and the Malayan species, while only the 5-pentylresorcinol **5a** is common to *C. inflata* and the Malayan species. On the other hand, no heptyl phenols or resorcinols were detected in the Malayan species. The specific name *C. difformis* has routinely been incorrectly rendered as "*C. deformis*" by various authors. It seems probable that our *C. difformis sensu lat.* from Brunei is a different species from the *"C. deformis"* from Malaya. In that case, a series of resorcinols, not observed in the Bruneian *C. difformis*, was detected along with alkylphenols, although neither 3-heptylphenol (**1c**) nor 6-alkylsalicylic acids were detected (Attgalle et al., 1989). Unfortunately, voucher specimens of the Malaysian samples were either never retained or were lost in subsequent years (U. Maschwitz, personal communication to RRS). There is thus no way to compare "*C. deformis*" with our *C. difformis*.

Crematogaster (*Physocrema*) is a well circumscribed assemblage within the genus, characterized by the hypertrophied metapleural glands, and limited to southeastern Asia. At present, there are nominally eight species plus an additional five infraspecific forms. These taxa are, on the whole, known with confidence only from original material; differentiation based solely on the available descriptions is nearly impossible. The present confusion reinforces the need for the deposition of voucher specimens in museum collections. Analyses of metapleural gland secretions may be useful in separating sibling species and investigating phylogenetic relationships among members of this group. However, they can only support the grouping of these taxa under *Physocrema* if similar compounds are determined to be unique to the group.

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VOLATILE EXUDATES FROM THE ORIBATID MITE, *Platynothrus peltifer*

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Abstract—Gas chromatographic-mass spectrometric analysis of whole body extracts of *Platynothrus peltifer*, a desmonomatan oribatid mite that belongs to the family Camisiidae, exhibited a basic profile of seven compounds, comprising the monoterpenes neral, geranial, and nerylformate; the aromatics 3-hydroxybenzene-1,2-dicarbaldehyde (= γ-acaridial) and 2-formyl-3-hydroxybenzyl formate (= rhizoglyphinyl formate), and two unsaturated C_{17}-hydrocarbons, 6,9-heptadecadiene and 8-heptadecene. Neryl formate, γ-acaridial, and rhizoglyphinyl formate were the main components and amounted to 80% of the extracts. With the exception of γ-acaridial (relative abundance varied considerably among samples), this chemical profile was consistently present in extracts of *P. peltifer* from nine different localities in SE-Austria. In addition, two further components, 2,3-dihydroxy benzaldehyde and 7-hydroxyphthalide, both probably of non-oil gland origin, infrequently were detected in the extracts. The aromatic compound rhizoglyphinyl formate, also known from Astigmata, was found for the first time in extracts of Oribatida, whereas all other compounds have already been reported from other oribatid species. The hydrocarbons are generally considered to represent plesiomorphic characters of mite oil gland secretions, whereas the monoterpenes and γ-acaridial form a part of the so-called "astigmatid compounds" that are thought to be characteristic for middle-derivative Mixonomata and all more highly derived oribatid groups (including Astigmata).

Key Words—Astigmatid compounds, oil glands, opisthonotal glands, Oribatida, *Platynothrus*, rhizoglyphinyl formate.

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INTRODUCTION

Oil glands (opisthonotal glands) represent the largest exocrine system in both oribatid and astigmatid mites, and constitute paired, large-reservoir glands that open to the notogastral surface via a single pore latero-dorsally on either side of the hysterosoma (Howard et al., 1988; Raspotnig et al., 2003). Apart from their biological significance as pheromonal, defensive, and protective organs (Kuwahara, 1991; Shimano et al., 2002; Raspotnig et al., 2003), these glands have proven useful as paradigms for oribatid and astigmatid phylogeny. With regard to current classification models, the presence of oil glands divides the Oribatida into basal nonglandulate groups (Palaeosomata and Enarthronota) and into the monophyletic group of glandulate oribatids, which includes the four more derived cohorts of Oribatida (Parhyposomata, Mixonomata, Desmonomata, and Brachypylida) and the Astigmata (Norton, 1998). Astigmatid mites, sharing the occurrence of oil glands with glandulate Oribatida, may have evolved from glandulate oribatid ancestors and thus in a strict sense may represent just a further oribatid group (Norton, 1998).

Beyond the phylogenetic implications of the occurrence or absence of oil glands, the study of the oil gland chemistry among glandulate groups has introduced a valuable and novel data set for further phylogenetic analyses. Chemical profiles of oil gland secretions have been shown to be species-specific (e.g., Sakata and Norton, 2003) and have been instrumental in the study of the evolutive origin of Astigmata, most probably from a mixonomatan-desomonatan lineage, confirming morphological evidence (Norton, 1998; Raspotnig et al., 2001; Sakata and Norton, 2001). From the multitude of components hitherto detected, Sakata and Norton (2001) have evaluated a characteristic set of five oil gland secretion components, so-called astigmatid compounds, which are widespread in Astigmata and also found in the middle-derivative oribatid species hitherto studied (Sakata et al., 1995, 2003; Raspotnig et al., 2001; Sakata and Norton, 2003). On the other hand, in early-derivative glandulate Oribatida such as Parhyposomata and basal Mixonomata, astigmatid compounds are conspicuously lacking. Thus, this set of compounds—including the monoterpenes neral, geranial, and neryl formate and the aromatics 2-hydroxy-6-methylbenzaldehyde (= 2,6-HMBD) and 3-hydroxybenzene-1,2-dicarbaldehyde (= γ-acaridial)—most likely has evolved first in middle-derivative Mixonomata, and would be expected to characterize the more derived part of Mixonomata and all groups above.

A prerequisite for such a hypothesis is a sufficient matrix of chemical data. However, the study of oribatid oil glands is fragmentary, not allowing generalizing statements on the chemistry of oribatid cohorts or even families. Whereas 67 substances from 52 species of Astigmata have been identified (Kuwahara, 1991; Sakata et al., 2003), chemical data are only available for two species of Parhyposomata, three species of Mixonomata (Raspotnig et al., 2001; Sakata and Norton,

2001), and five species of Desmonomata, four of them belonging to one family, the Trhypochthoniidae (Sakata et al., 1995, 2003; Shimano et al., 2002; Sakata and Norton, 2003).

As a first example of chemical characters from oil gland secretions of a further desmonomatan family, we have studied the oil gland chemistry of *Platynothrus peltifer*, a parthenogenetic and soil-dwelling representative of Camisiidae.

METHODS AND MATERIALS

Specimen Collection and Sample Preparation. Specimens of *P. peltifer* (C. L. Koch, 1839) (Oribatida: Camisiidae) were collected from the litter and fermentation layer of mixed forests, repeatedly from nine different localities in Austria (see Table 1). Altogether, 16 collections were carried out over a 2-yr period (from early spring 2002 to late autumn 2003), giving 33 different samples of adult *P. peltifer* (in all comprising 367 specimens) and 5 samples of juveniles (each sample containing a mixture of nymphal stages, i.e., proto-, deuto-, and tritonymphs, in all comprising 19 individuals). Oil gland secretions were extracted from whole mite bodies: due to the minute amounts of oil gland secretions and the small size of *P. peltifer*, pooled extracts were prepared by transferring a number of freshly collected specimens (usually 4–20) into solvent (100 μl hexane or ethyl acetate) and subsequent extraction for 30 min.

TABLE 1. SPECIMEN COLLECTION AND SAMPLE PREPARATION

Locality	Sample number (number of individuals)	Stage	Solvent
I. Ferlach, Osce (Carinthia)	1 (19), 2 (20)	Adults	Ethyl acetate
	3 (3), 4 (5), 5 (7), 6 (9)	Adults	Hexane
	7 (2)	Juveniles	Hexane
II. Ferlach, Moatsche (Carinthia)	8 (10), 9 (14)	Adults	Hexane
III. Ferlach, Rauth (Carinthia)	10 (7), 11 (5)	Adults	Hexane
IV. Deutschlandsberg (Styria)	12 (23), 13 (30)	Adults	Ethyl acetate
V. Heiligengeistklamm (Styria)	14 (18), 15 (15), 16 (11), 17 (7), 18 (10), 19 (10), 20 (10), 21 (11), 22 (11)	Adults	Hexane
	23 (6)	Juveniles	Hexane
VI. Tamischbachgraben (Styria)	24 (4)	Adults	Hexane
VII. Göltschach (Carinthia)	25 (4)	Adults	Hexane
VIII. Vasoldsberg (Styria)	26 (10), 27 (7), 28 (5), 29 (6), 30 (6), 31 (7), 32 (7), 33 (5), 34(44)	Adults	Hexane
	35 (6), 36 (2), 37 (3)	Juveniles	Hexane
IX. Graz, Plabutsch (Styria)	38 (7)	Adults	Hexane

Chemical Analysis. After extraction, the extracts were separated from mite bodies and stored at −20°C until analysis. Finally, an aliquot of crude mite extracts, in most cases between 1 and 3 μl, was analyzed by GC-MS, using a Fisons MD 800 GC-MS system (Thermo-Quest, Vienna, Austria). The GC column (ZB-5 30 m × 0.25 mm × 0.25 μm; Phenomenex via HPLC SERVICE, Vienna, Austria) was directly connected to the ion source of the MS. We kept the splitless Grob injector at 260°C and used helium as carrier gas at a constant flow rate (1.5 ml/min). The ion source (EI-source) was kept at 200°C, the transfer line at 310°C. We used the following temperature program: 50°C for 1 min, then 10°C/min to 200°C, then 15°C/min to 300°C and hold at 300°C for 5 min. DMDS (dimethyl disulfide) derivatives, to determine the positions of double bonds in unsaturated hydrocarbons, were prepared according to Vincenti et al. (1987).

Reference Compounds. Nerol, geraniol, and citral were purchased from Aldrich (Vienna, Austria). Isomerically pure neral and geranial were prepared by diluting the corresponding alcohols in acetone and stirring the solution with activated MnO_2 at room temperature and under nitrogen until the oxidation was complete. The reaction products were purified by silica gel chromatography using acetone: hexane (9:1) as eluent. Purity of compounds was checked by GC-MS (purity >99%). Neryl formate was prepared from nerol according to Kuwahara and Sakuma (1982). Rhizoglyphinyl formate (= 2-formyl-3-hydroxybenzyl formate) was synthesized according to Sato et al. (1993). As a reference for γ-acaridial (= 3-hydroxybenzene-1,2-dicarbaldehyde), we used a North American *Perlohmannia* species from West Virginia with γ-acaridial being an already characterized constituent of its oil gland secretion (Sakata and Norton, 2001).

Scanning Electron Microscopy. For scanning electron microscopy (SEM), specimens were fixed in glutaraldehyde, dehydrated, and mounted on small dishes prior to sputtering with gold. SEM was carried out on a Philips XL30 ESEM at the Institute of Plant Physiology, Karl-Franzens-University of Graz, Austria.

RESULTS

Oil Glands in P. peltifer. Oil glands in *P. peltifer* are well developed and open through one slit-like pore at either of the distal latero-dorsal margins of the notogaster (Figure 1). Oil glands turn brownish when specimens are stored in 70% alcohol or in Faure's liquid and then are visible through the cuticle.

Evaluation of Extract Profiles. From all 38 samples investigated, a total of nine different components could be separated by gas chromatography. Only seven of the detected components were present in all samples investigated, forming a characteristic and consistent basic extract profile (peaks A–G in Figure 2). Qualitatively, no differences between extracts of adults and juveniles (nymphs) were detected.

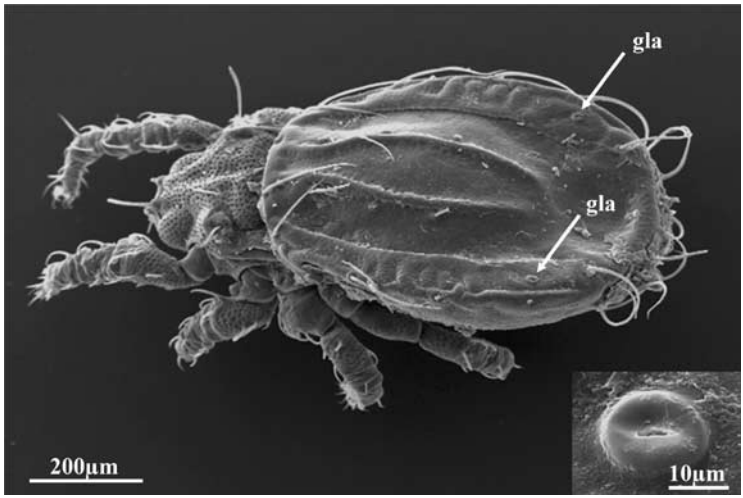

FIG. 1. Scanning electron micrograph of an adult individual of *Platynothrus peltifer*, dorsal view. White arrows point to oil gland openings (gla). Insert: Magnification of right oil gland pore.

Identification of Compounds. Gas chromatographic and mass spectral data of compounds A–G are summarized in Table 2. Peaks A and B exhibited a similar fragmentation pattern that only differed in the intensity of certain fragment ions. Both components appeared to be acyclic monoterpenes with their most prominent fragments at *m*/*z* 69 (from allylic cleavage) and *m*/*z* 41, and molecular ions of weak intensity at *m*/*z* 152. Peaks A and B were identified as neral and geranial, respectively, and their identities were confirmed by comparisons with authentic standards. Peak C also exhibited a monoterpenoid fragmentation pattern but did not show a molecular ion (ion of highest mass at *m*/*z* 136). The compound was identified as neryl formate by comparison with a standard. Peak D appeared to be an aromatic di-aldehyde, showing a prominent molecular ion at *m*/*z* 150 and repeated loss of CO and CHO, respectively, leading to the fragments at *m*/*z* 122 and *m*/*z* 121 (the base peak), and the fragments at *m*/*z* 93 and 92. The isotopic peak at *m*/*z* 151 had an intensity of 8.74% of the molecular ion, indicating the presence of eight carbon atoms, and supporting a molecular formula of $C_8H_6O_3$. The mass spectrum of the compound matched the spectrum of 3-hydroxybenzene-1,2-dicarbaldehyde (γ-acaridial) as recently reported (Sakata and Kuwahara, 2001), and a comparison of the retention time to authentic γ-acaridial from extracts of *Perlohmannia sp.* (Sakata and Norton, 2001) confirmed its identity. Peak E showed an aromatic fragmentation pattern with pronounced high and low aromatic series and the characteristic aromatic ion at *m*/*z* 77. The diagnostic fragments at *m*/*z* 151 and at *m*/*z* 134 (base peak) indicated the loss of an aldehyde group and the neutral loss of

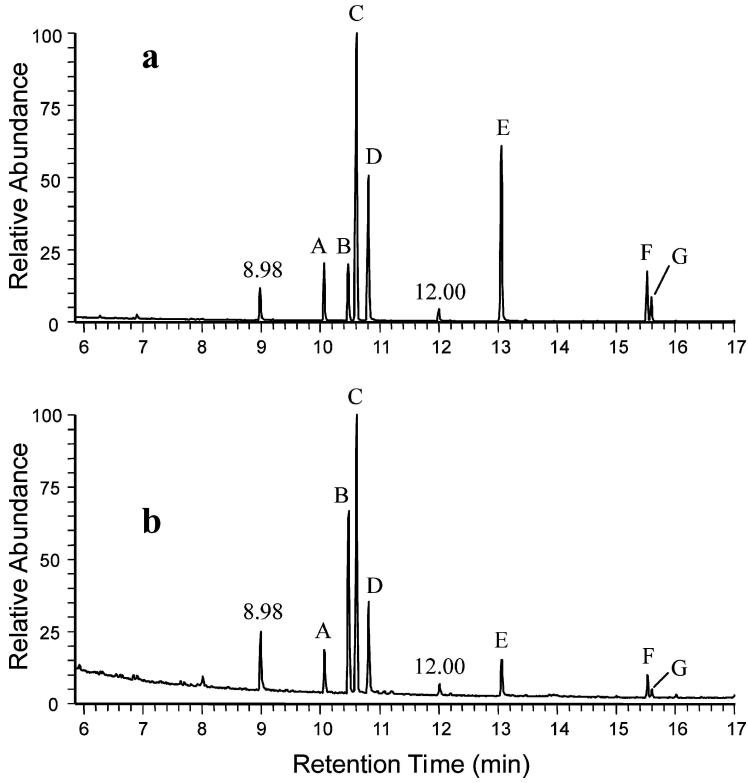

FIG. 2. Gas chromatogram of extract components in adults (a) and nymphs (b) of *Platynothrus peltifer*. Basic extract profile consists of compounds A–G: (A) neral; (B) geranial; (C) neryl formate; (D) γ-acaridial; (E) rhizoglyphinyl formate; (F) 6,9-heptadecadiene; and (G) 8-heptadecene. Infrequently occurring compounds are 2,3-dihydroxy-benzaldehyde at rt = 8.98 min and 7-hydroxyphthalide at rt = 12.00 min.

formic acid, respectively, from the molecular ion (at *m*/*z* 180). The isotopic peaks at *m*/*z* 181 ($M^+ + 1$ peak) and *m*/*z* 182 ($M^+ + 2$ peak) had relative intensities of 9.3% and 1.5% of the molecular ion, respectively, and suggested the presence of nine carbon and at least four oxygen atoms, and thus a molecular formula of $C_9H_8O_4$. The compound was finally identified as rhizoglyphinyl formate (= 2-formyl-3-hydroxybenzyl formate) by synthesis of an authentic standard. Peaks F and G had mass spectra typical of unsaturated hydrocarbons with molecular ions at *m*/*z* 238 and *m*/*z* 236, being consistent with a heptadecene and a heptadecadiene, respectively.

Determination of Double Bond Positions. The DMDS adduct of the heptadecadiene showed a molecular ion at *m*/*z* 362 (52% relative intensity), indicating

TABLE 2. GAS CHROMATOGRAPHIC AND MASS SPECTRAL DATA OF BASIC EXTRACT PATTERN

Peak No.	Retention time (min)	EI-fragmentation (relative intensity)	Identified as
A	10.06	152 (1), 137 (7), 134 (10), 123 (5), 119 (17), 109 (25), 95 (19), 94 (28), 84 (24), 81 (20), 69 (99), 67 (27), 59 (13), 57 (19), 55 (14), 53 (21), 41 (100)	Neral
B	10.47	152 (4), 137 (9), 134 (5), 123 (7), 119 (8), 109 (12), 94 (19), 91 (17), 84 (24), 81 (11), 69 (100), 67 (15), 57 (10), 55 (8), 53 (13), 43 (12), 41 (77)	Geranial
C	10.60	182 (M^+; trace), 137 (2.1), 136 (20), 121 (10), 107 (4), 80 (12), 79 (19), 77 (17), 69 (100), 53 (17), 41 (63)	Neryl formate
D	10.80	152 ($M^+ + 2$ peak; 0.69), 151 ($M^+ + 1$ peak; 4.2), 150 (M^+; 48), 122 (49), 121 (100), 93 (42), 65 (36), 63 (14), 57 (37), 43 (24), 41 (31)	γ-Acaridial
E	13.05	182 ($M^+ + 2$ peak; 0.3), 181 ($M^+ + 1$ peak; 1.86), 180 (M^+; 20), 151 (33), 135 (18), 134 (100), 106 (32), 105 (30), 79 (8), 78 (37), 77 (51), 65 (13), 63 (11), 51 (19)	Rhizoglyphinyl formate
F	15.51	236 (13), 152 (2), 138 (8), 124 (10), 110 (12), 109 (16), 96 (33), 95 (43), 82 (43), 81 (60), 79 (25), 77 (10), 69 (21), 68 (34), 67 (100), 57 (9), 55 (47), 43 (21), 41 (42)	Heptadecadiene
G	15.58	238 (8), 139 (3), 125 (15), 111 (36), 97 (72), 83 (83), 71 (35), 69 (100), 57 (80), 55 (91), 43 (77), 41 (81)	Heptadecene

the formation of a cyclic thioether with the loss of MeSMe (m/z 236 plus $2 \times m/z$ 94 minus m/z 62 = m/z 362). Diagnostic ions, originating from α-cleavage, comprised two acyclic fragments (formed by α-cleavage from the two MeS-bearing α-atoms, at m/z 131 [67%] and m/z 159 [68%]) and two ions containing the cyclic moiety (formed by α-cleavage at both sides of the cyclic thioether function at m/z 231 [41%] and m/z 203 [44%]). From ions containing the cyclic moiety, neutral loss of MeSH was observed (loss of 48 amu), leading to the most intense fragments in the spectrum at m/z 155 (base peak) and at m/z 183 (97%), respectively. These mass spectrometric data are consistent with a 6,9-heptadecadiene only. The DMDS adduct of the heptadecene

showed a molecular ion at *m*/*z* 332 (*m*/*z* 238 + *m*/*z* 94) and diagnostic ions from α-cleavage at *m*/*z* 159 and *m*/*z* 173, respectively, corresponding to 8-heptadecene.

Additional Components. In 21 (of the 38) samples, a minor component with short retention time (rt = 8.98 min) and the following mass spectrum could be detected: *m*/*z* 138 (M^+, 100), 137 (71), 120 (21), 109 (15), 92 (35), 81 (34), 79 (8), 64 (14), 63 (19), 55 (18), 53 (24), 52 (23), 51 (18). The stable and intense molecular ion along with hydride abstraction suggested the presence of an aromatic aldehyde. Diagnostic fragments showed the loss of water (*m*/*z* 120), loss of an aldehyde group (*m*/*z* 109), and loss of water and decarbonylation (*m*/*z* 92) from the molecular ion. The compound was tentatively identified as a dihydroxy-benzaldehyde, confirmed by comparison of retention time and mass spectral data with that of authentic 2,3-dihydroxybenzaldehyde.

A further component with a mass spectrum similar to γ-acaridial appeared in small amounts at rt = 12.00 min in 19 (of the 38) samples [*m*/*z* 150 (37), 149 (7), 122 (7), 121 (100), 93 (15), 65 (19), 63 (11), 51 (15)]. It mainly differed from the spectrum of γ-acaridial in the weak relative intensity of *m*/*z* 122 and was identified as 7-hydroxyphthalide by comparison of retention times with those of the authentic substance.

Quantitative Analysis. A quantitative analysis of the extracted compounds was done for 30 hexane extracts of adults (in all containing 275 individuals) and the 4 hexane extracts of nymphs (in all containing 17 individuals) by determining the abundances of single components in relation to the amount of the whole 7-compound profile (given in % peak area in the following). The basic pattern of the extracts occurred in constant proportions, irrespective of the locality of sampling or population. In all samples of adult *P. peltifer* investigated, neryl formate was the main component (36.3 $\pm$ 6.1%), followed by rhizoglyphinyl formate (25.2 $\pm$ 7.3%) and γ-acaridial (19.8 $\pm$ 8.9%), all three components together comprising more than 75% of the extracted material. Only the abundance of γ-acaridial showed high variations between samples, ranging from "not detected" (in one sample of locality VII: Göltschach) up to about 1/3 (36%) relative abundance in another sample. Usually, and independent of localities, it was the third-most abundant compound in the extracts. Mean values of the remaining compounds were as follows: neral 4.5 $\pm$ 2.2%, geranial 6.1 $\pm$ 3.0%, 6,9-heptadecadiene 5.2 $\pm$ 3.5%, and 8-heptadecene 3.0 $\pm$ 2.2%. In samples that had been collected in autumn 2003, the relative abundances of neral, geranial, and the hydrocarbons had decreased considerably and consistently were found to be below mean values.

In nymphal extracts, neryl formate (40.1 $\pm$ 3.7%), γ-acaridial (24.7 $\pm$ 10.5%) and geranial (22.2 $\pm$ 4.0%) were the main components. The mean relative abundances for the remaining components were neral 5.3 $\pm$ 2.7%, rhizoglyphinyl formate 5.4 $\pm$ 3.9%, 6,9-heptadecadiene 1.6 $\pm$ 1.2%, and 8-heptadecene 0.7 $\pm$ 0.5%.

DISCUSSION

Origin of Extract Components. All seven compounds that form the basic pattern of whole body extracts of *P. peltifer* most likely belong to the oil gland secretion of this species, because oil glands are the only exocrine glands in *P. peltifer* that are large enough to produce the detected amounts of secretion. Second, all seven components from extracts are already known from oil gland secretions of Astigmata (Kuwahara, 1991) and, with the exception of rhizoglyphinyl formate, also from other Oribatida (Sakata et al., 1995, 2003; Raspotnig et al., 2001; Sakata and Norton, 2001, 2003). Third, in whole body extracts of oribatid mites that do not possess oil glands—such as early-derivative species from Enarthronota—no sign of these components, which typically are found in oil gland-bearing groups, were detected (Sakata and Norton, 2001). Fourth, a definitive proof of the oil gland origin of some of these compounds has been carried out in the large mixonomatan *Collohmannia gigantea*. Whole-body extracts of individuals with different filling status of oil gland reservoirs were compared, resulting in a proportionate decline of oil gland compounds in the case of exhaustion of oil gland reservoirs (Raspotnig et al., 2001). Moreover, in *C. gigantea*, histochemical proofs have shown the presence of aldehydes (a predominant class of oil gland components) in oil gland reservoirs only, but not in other regions of the body. Comparably, a direct line of evidence for the oil gland origin of astigmatid compounds is provided by a recent study by Sakata and Norton (2003) who extracted the same set of components from whole mite bodies of *Archegozetes longisetosus* and from dissected glands of exuvial cuticles, respectively.

As in *C. gigantea*, oil glands of *P. peltifer* turn brownish or even black when specimens are stored in low-proof ethanol or Faure's liquid (Raspotnig et al., 2001). This phenomenon, also known from several Astigmata (Kuwahara, 1976), is reported to be due to the presence of certain monoterpenes (especially neryl formate) in oil gland reservoirs and may serve as a first (morphological) indicator for a monoterpene-rich oil gland secretion.

In contrast, the remaining two detected compounds (2,3-dihydroxybenzaldehyde and 7-hydroxyphthalide) may not be of oil gland origin. In our investigation, the amounts of these components varied considerably in repeated gas chromatographic analyses of the same sample, suggesting that the substances were artifacts formed in the course of analysis. However, 7-hydroxyphthalide has been described as a component of the oil gland secretion in the astigmatid mite *Oulenzia sp*. (Shimizu and Kuwahara, 2001). Also, an unidentified component with molecular weight 138—showing a corresponding mass spectrum to the 2,3-dihydroxybenzaldehyde found in our study—has been reported from whole body extracts of the astigmatid mite *Dermatophagoides pteronyssinus* and has been classed with its oil gland secretion (Kuwahara et al., 1990).

Characteristic Oil Gland Secretion Profile in P. peltifer. Apart from γ-acaridial, the secretion profile of *P. peltifer* appeared to be consistent, irrespective of the locality of sampling, and thus representing a species-specific pattern. Profiles of juveniles were qualitatively similar to those of adults but differed in the relative abundance of components, especially in the composition of citral, thus holding a "hybrid" position among oil gland secretions of Oribatida: while oil gland profiles of adults and juveniles are not distinguishable in mixonomatans such as *C. gigantea* (Raspotnig et al., 2001) and also in certain Desmonomata such as Trhypochthoniidae (e.g., Sakata and Norton, 2003), chemical profiles have been reported to change during the ontogenetic development in *Nothrus palustris* (Shimano et al., 2002) and possibly also in other Nothridae (Sakata et al., 2003). Also in higher Oribatida (Brachypylida), profiles of juveniles may differ considerably from adults (Norton, personal information). However, whether adult–juvenile polymorphism of oil gland secretions represents an evolutive trend cannot be decided with the current status of knowledge. In this respect, the composition of citral in the oil gland secretion of *P. peltifer*, changing from geranial-rich citral in juveniles to a more balanced neral–geranial ratio in adults, is of interest because citral is regarded to be a key component for phylogenetic analyses. Predominantly neral-rich citral is characteristic of oil glands of higher Astigmata, in contrast to the geranial-rich citral of basal Astigmata such as Histiostomatidae (Sato et al., 1993; Shimano et al., 2002), suggesting that astigmatid mites may have arisen from a geranial-rich citral-possessing oribatid ancestor (Shimano et al., 2002). However, in Oribatida and, as demonstrated in the present paper, even within stages of the same species, several types of citral may be present (see also Shimano et al., 2002; Sakata et al., 2003).

Phylogenetic Considerations. The oil gland secretion of *P. peltifer* contains the characteristic three chemical fractions (terpenes, aromatics, and hydrocarbons) that are well known from oil gland secretions of other Oribatida and from Astigmata. Among these fractions, four of the five so-called "astigmatid compounds" (sensu Sakata and Norton, 2001) are present; these components, as already mentioned, constitute an apomorphy of Oribatida (including Astigmata) higher than middle-derivative Mixonomata. By contrast, the C_{17}-hydrocarbons may be regarded as plesiomorphic characters of oil gland secretions. Short- and middle-chain hydrocarbons are widely distributed in oil glands of Astigmata and Oribatida, including the early-derivative groups such as Parhyposomata (Sakata and Norton, 2001). Even though the conformation of double bonds was not determined in our investigation, we might have been dealing with (*Z*)-8-heptadecene and (*Z*,*Z*)-6,9-heptadecadiene, both of which are found in other desmonomatan species (Sakata et al., 1995, 2003).

Apart from these common oil gland components, *P. peltifer* also contained an unexpected and novel component for oribatid oil glands: rhizoglyphinyl formate. This component seems to be rare among astigmatid mites as well, and

the only other natural sources hitherto known are the oil gland secretions of *D. pteronyssinus* (Sato et al., 1993) and *Rhizoglyphus sp.* (Tarui et al., 2002), respectively. Although, it is too early to evaluate the phylogenetic significance of rhizoglyphinyl formate, it may represent a highly derivative oil gland product of certain desmonomatan Oribatida, having arisen after the splitting-off of Mixonomata from the oribatid stem line.

Biological Role of Oil Gland Secretion. The biological role of the oil gland secretion of *P. peltifer* is still obscure. However, citral is known to possess antifungal properties (Cole et al., 1975), representing an important chemical protection if living in a humid, fungi-rich environment. In addition, in nymphs of *N. palustris*, geranial has been reported to function as an alarm pheromone (Shimano et al., 2002), and oil gland secretions of Astigmata are a source of diverse pheromones and protective agents (Kuwahara, 1991). Most recently, alarm pheromonal and defensive properties of oil gland-derived astigmatid compounds have been decribed for *C. gigantea* (Raspotnig et al., in press). Due to the characteristic construction of oribatid oil glands—large sac-like glands with a mechanism for the controlled emission of secretion—their most important biological roles may include defense and alarm pheromonal communication (Raspotnig et al., 2003).

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QUANTIFICATION OF INVERTASE ACTIVITY IN ANTS UNDER FIELD CONDITIONS

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Abstract—Invertases (EC 3.2.1.26) are hydrolases that cleave sucrose into the monosacccharides, glucose, and fructose. They play a central role in carbohydrate metabolism of plants and animals. Methods presented so far to quantify invertase activity in ants or other animals have been hampered by the variability in both substrates and products of the enzymatic reaction in animals whose carbohydrate metabolism is highly active. Our method is based on a spectrophotometric quantification of the kinetics of glucose release. We first obtained an equilibrium state summarizing reactions of any carbohydrates and enzymes that are present in the extract. Sucrose was then added to quantify invertase activity as newly released glucose. Invertase activities differed significantly among species of ants. Variances were lowest among individuals from the same colony and highest among different species. When preparations were made from ants of the same species, invertase activity was linearly related to the number of ants used for extraction. Our method does not require ants to be kept on specific substrates prior to the experiment, or expensive or large equipment. It, thus, appears suitable for dealing with a broad range of physiological, ecological, and evolutionary questions.

Key Words—β-Fructosidase, carbohydrate metabolism, saccharase, sucrose, sugar.

INTRODUCTION

Invertases (β-fructofuranosidase, EC 3.2.1.26, also termed β-fructosidase, saccharase, or sucrase) are glycoside hydrolases (EC 3.2.1.-) that catalyze the cleavage of sucrose (α-D-Glucopyranosyl-β-D-fructofuranoside) into the two monosaccharides, glucose, and fructose (Henrissat and Bairoch, 1993; Sturm and Tang, 1999; Naumoff, 2001). Carbohydrates ingested by heterotrophic organisms undergo

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several metabolic steps, in the first of which polymeric carbohydrates are cleaved into their monomers, which then can pass through membranes. Invertase, thus, appears to be a particularly important enzyme for both plants and animals.

Feeding on animals as well as plant material, ants can metabolize many different food sources. Early investigations have indicated that differences among species in the activity of digestive enzymes may reflect the feeding habits of the ants (Ayre, 1967; Ricks and Vinson, 1972). Many ants feed on carbohydrate-rich liquid food sources, such as honeydew of homopterans, plant sap, and extrafloral nectar (i.e., nectar secreted on vegetative organs that is not functionally involved in pollination, see Heil and McKey, 2003). As sucrose is common in all these food sources, invertase activity is required to make use of such food sources.

Given this general importance, surprisingly few studies have tried to quantify invertase activity in ants (Ayre, 1967; Ricks and Vinson, 1972) or other animals (Martínez del Rio, 1990; Zhang et al., 1993). This might be due to the particular methodical problems arising from the quantification of invertase in animals whose carbohydrate metabolism is highly active. Invertase usually is quantified via the release of glucose from sucrose. Both substrate and product of the enzymatic reaction can be present at varying concentrations in the extract used to quantify enzyme activity. Earlier investigations (Ayre, 1963, 1967; Ricks and Vinson, 1972; Zhang et al., 1993) have been based on endpoint determinations of glucose after adding sucrose to extracts of insects (or parts of insects) and likely are hampered by the presence of glucose and/or sucrose in the reaction preparation prior to the enzymatic reaction. These problems might explain why even recent studies of digestive enzymes in ants have chosen indirect methods, such as feeding selected di- or oligosaccharides to living ants and quantifying monosaccharides as putative reaction products in the ants themselves (Boevé and Wäckers, 2003). The drawback of this method is that selected carbohydrates must be fed to cultivated ants, which have no access to other food sources. Otherwise, direct uptake of the monosaccharides, which are quantified as putative reaction products, would alter the outcome of the experiment. Moreover, carbohydrate metabolism does not stop at the level of monosaccharides, which are further metabolized in living organisms. Quantifying intermediate products can at best reveal semi-quantitative results concerning the velocity of the first step, since their quantities are dependent on both the reactions that lead to their production and by later reactions for which they serve as substrates.

To overcome these problems, we developed a method for quantifying invertase in extracts of ants, which is based on repeated and specific quantification of glucose by a spectrophotometrical method. This allows kinetics to be measured, instead of relying on an endpoint determination. Consequently, an equilibrium is obtained prior to the enzymatic reaction, which reflects reactions of any carbohydrates and enzymes that may be present in the extract. Sucrose is added after the

equilibrium has been reached to then quantify invertase activity as newly released glucose.

METHODS AND MATERIALS

Insects and Preparation. The experiment was carried out with ants freshly caught in their natural habitats in the vicinity of Puerto Escondido, Oaxaca, Mexico. Because these ants have different feedings strategies, they were likely to exhibit quantitative differences in invertase activity. Various glands that are located in the head or in the gaster may contribute differently to an ant's overall invertase activity (Ayre, 1967). Ants were, therefore, dissected in "Insect Ringer" solution (10.4 g NaCl, 0.32 g KCl, 0.48 g $CaCl_2$, and 0.32 g $NaHCO_3$ in 1 l water) in order to remove the largest parts of the content of head and gaster. Care was taken to include maxillary and postpharyngeal glands and the midgut, while poison glands and acid glands were discarded or, at least, emptied. Thoraxes and all chitin components were discarded. The parts putatively containing invertase were weighed and transferred to a 50 mm sodium phosphate buffer (pH 6.0). Preliminary experiments with different species of *Atta*, *Camponotus*, and *Crematogaster* revealed that this pH was close to the optimum for all ant species investigated. However, different pH conditions should be examined for other ant species in order to conduct the assays at an optimal pH. The contents of 1–10 individuals (equivalent to 8–28 mg fresh material) were combined and put into 300 μl buffer to which 50 μl solution of proteinase inhibitor (1 Complete Mini Tablet, Roche Diagnostics, Mannheim, Germany in 1.5 ml water) had been added. The material then was homogenized with sand and cleared by micromembrane filtration ("Rotilabo Spritzenfilter," 13 mm, 0.2 μm Nylon, Carl Roth, Karlsruhe, Germany). After clearing, the extracts were stored for ca. 1 hr at 4°C.

Quantification of Invertase. The quantification of enzyme activity is based on quantifying glucose released from sucrose with the Glucose (HK) Assay Kit (Sigma-Aldrich, Steinheim, Germany. The kit combines hexokinase and glucose-6-phosphate dehydrogenase). Ten microliters of extract and 100 μl HK reaction solution (prepared according to manufacturer's protocol) were introduced into cuvettes (70 μl micro disposable cuvettes, Plastibrand®, Brand, Wertheim, Germany) and placed immediately into a photometer (Genesys 20 Spectrophotometer, ThermoSpectronic, Cambridge, UK). A mixture of 10 μl buffer and 100 μl HK reaction solution served as a blank. Absorption was quantified at 340 nm every 5 min until a steady state was reached, usually by 30–45 min. In cases of very high initial absorptions (>0.3) and/or high increases in absorption, samples could be further diluted with HK reaction solution, since high initial values resulted from high amounts of glucose (absolute values) and/or sucrose and invertase (high increases) and, thus, were generally correlated with high invertase activities. After the steady state had

been reached, 20 μl sucrose solution (250 mg sucrose in 1 ml water) were added, and absorption was further quantified at regular intervals for 1.5–2 hr. Invertase activity was calculated from the slope of the linear increase in absorption after adding sucrose. For each ant species, 5–7 quantifications were conducted with different ants (usually representing different colonies). For each sample (i.e., ant colony), two extractions were conducted with different numbers of ants, and two replicates per extract were used for analysis; means were calculated from these four measurements.

Standard Curves. Standardization was conducted by quantifying defined amounts of glucose (D-Glucose, Sigma-Aldrich; three replicates per glucose concentration) under the same experimental conditions, and by subjecting purified invertase to the same experimental procedure. Of a 0.01 units ml^{-1} solution of invertase in TRIS–HCl buffer, pH 4.5 (invertase from baker's yeast, ca. 300 units mg^{-1} solid, Sigma-Aldrich, pH according to supplier's suggestion), different volumes (0, 2.5, 5, 10, and 15 μl) were diluted with TRIS–HCl buffer to a volume of 20 μl and mixed with 200 μl HK reaction solution. Sucrose (20 μl of a 0.25 g ml^{-1} solution) was added after 15 min, and absorption was quantified every 10 min for at least the next 30 min (three replicates per concentration of invertase). Ant invertase activity, thus, can be expressed as ng glucose released per min and per mg ant fresh material, or as units (with purified invertase as standard).

RESULTS AND DISCUSSION

The use of purified invertase under the experimental conditions as described above resulted in a linear increase of absorption over time after addition of the enzyme's substrate, sucrose (Figure 1A). Increases in absorption were then linear for at least 1.5 hr (personal observations), and the slopes of these curves were highly correlated with invertase activity. The resulting calibration curve is linear from 0 to ca. 400 n units μl^{-1} and then tends to reach saturation (insert in Figure 1A).

Different ant species were used to quantify invertase activity in tissue of freshly caught animals. In contrast to the curves observed for purified invertase, ant extracts first showed an increase in absorption, which resulted from the presence of the substrate (sucrose) as well as the product (glucose) of the enzymatic reaction in the extract (Figure 1B). Depending on the species and the number of ants used, 15–45 min were required to reach the equilibrium, after which sucrose could be added. Addition of sucrose solution first resulted in a slightly reduced absorption due to dilution effects by the added volume of sucrose solution (Figure 1B). After 10–15 min, the resulting curves showed almost linear slopes, and invertase activity calculated from these slopes differed significantly among the species tested (Table 1). Variability among ant species was much higher than within a species, and

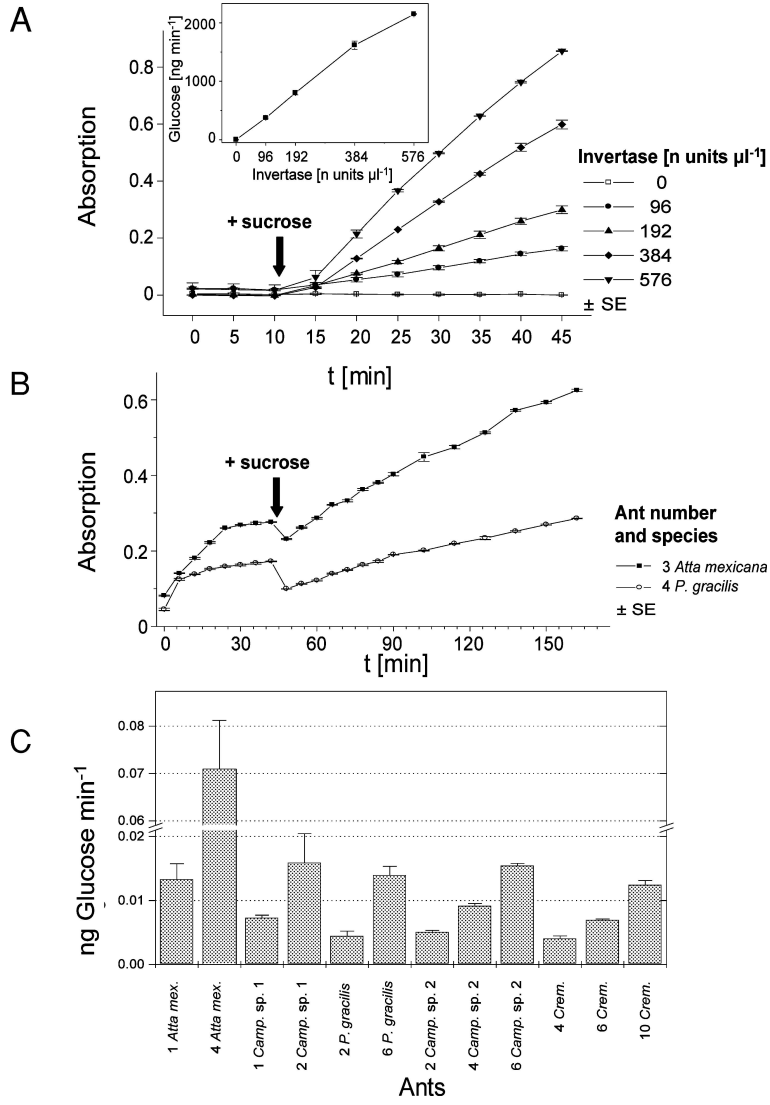

FIG. 1. Quantification of invertase and validation of the method. Time courses in absorption are given separately for purified invertase (A) and extractions of two ant species (B). Note the time at which sucrose was added. Use of the slopes of the curves representing different amounts of purified invertase for quantification of activity revealed an almost linear calibration curve (insert in panel A). Use of different numbers of ants (C) revealed linear dose–activity relations. *Atta mex.*, *Atta mexicana; Camp.*, *Camponotus; Crem., Crematogaster*; *P. gracilis*, *Pseudomyrmex gracilis*.

TABLE 1. MEAN INVERTASE ACTIVITIES IN DIFFERENT ANT SPECIES

	Atta mex.	*Camp.* 1	*P. gracilis*	*Camp. 2*	*Crem. spec.*
Activity (mean)	46.2	24.4	28.6	14.6	17.3
SE	5.4	3.9	6.3	0.9	2.3
N	6	4	5	6	5

Note. Invertase activity is given in ng glucose released per mg ant tissue per min; means marked with different letters are significantly different ($P < 0.05$ according to LSD *post hoc* analysis). *Atta mex*., *Atta mexicana; Camp*., *Camponotus; Crem., Crematogaster*; *P. gracilis*, *Pseudomyrmex gracilis*.

in general the values obtained from ants belonging to the same colony (genetically similar and usually having fed on the same food sources) were more similar than those obtained from ants from different colonies. When varying numbers of ants were extracted, an almost linear ant number—invertase activity relationship was found (Figure 1C).

The method described here allows the easy quantification of invertase activity in extracts of the digestive tracts of ants, and it should be applicable to other arthropods. The method is easy, does not require expensive or elaborate equipment, and can be applied under field conditions. Because the method measures enzyme activity, it is not suitable for identifying the presence of isoenzymes or for determining whether the enzyme is synthesized by the animal itself or, rather, is the product of endosymbiotic microorganisms. However, the method reliably measures the activity of a digestive enzyme and provides an analysis of the digestive capacity of the insect—or insect organ—used for extraction.

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PHYTOHORMONES MEDIATE VOLATILE EMISSIONS DURING THE INTERACTION OF COMPATIBLE AND INCOMPATIBLE PATHOGENS: THE ROLE OF ETHYLENE IN *Pseudomonas syringae* INFECTED TOBACCO

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Abstract—Interactions between the phytohormones ethylene, salicylic acid (SA), and jasmonic acid (JA) are thought to regulate the specificity of induced plant defenses against microbial pathogens and herbivores. However, the nature of these interactions leading to induced plant volatile emissions during pathogen infection is unclear. We previously demonstrated that a complex volatile blend including (*E*)-β-ocimene, methyl salicylate (MeSA), and numerous sesquiterpenes was released by tobacco plants, *Nicotiana tabacum* K326, infected with an avirulent/incompatible strain of *Pseudomonas syringae* pv. *tomato* (*Pst* DC3000). In contrast, a volatile blend, mainly consisting of MeSA and two unidentified sesquiterpenes, was released by plants infected with *P. syringae* pv. *tabaci* (*Pstb*) in a virulent/compatible interaction. In this study, we examined the interaction of multiple pathogen stresses, phytohormone signaling, and induced volatile emissions in tobacco. Combined pathogen infection involved the inoculation of one leaf with *Pst* DC 3000 and of a second leaf, from the same plant, with *Pstb*. Combined infection reduced emissions of ocimene and MeSA compared to plants infected with *Pst* DC 3000 alone, but with no significant changes in total sesquiterpene emissions. In the compatible

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interaction, *Pstb* elicited a large ethylene burst with a peak emission occurring 3 days after inoculation. In contrast, the incompatible interaction involving *Pst* DC3000 displayed no such ethylene induction. *Pstb-induced* ethylene production was not significantly altered by *Pst* DC3000 in the combined infection. We postulated that *Pstb*-induced ethylene production may play a regulatory role in altering the typical volatile emission in tobacco in response to *Pst* DC3000 infection. To clarify the role of ethylene, we dynamically applied ethylene to the headspace of tobacco plants following infection with *Pst* DC3000. Consistent with *Pstb*-induced ethylene, exogenous ethylene reduced both ocimene and MeSA emissions, and selectively altered the ratios and amounts of induced sesquiterpene emissions. Our findings suggest that ethylene can regulate the magnitude and blend of induced volatile emissions during pathogen infection.

Key Words—Ethylene, jasmonic acid, *Nicotiana tabacum*, plant volatiles, *Pseudomonas syringae*, salicylic acid.

INTRODUCTION

The interactions between plants and microbial pathogens are specific, complex, and dynamic, often resulting in either disease (compatible interaction) or resistance (incompatible interaction). In an incompatible interaction, a plant mounts a rapid response to a virulent pathogen that leads to localized cell death at the site of infection; this in turn prevents further pathogen infection. Incompatible interactions are usually associated with a hypersensitive response (HR), which includes localized cell death and the appearance of necrotic flecks at the site of infection. In contrast, many compatible interactions result in slow, "normosensitive" (normal/expected sensitive response) cell death that spreads beyond the site of infection by a virulent pathogen. Because the complexity of plant responses, most studies on plant-pathogen interactions have focused on a single pathogen in a particular plant (Jeger and Spence, 2001). However, complexes of diseases commonly occur within a plant during its lifetime. In many cases, the induction of plant responses by one type of stress interferes with plant response against the other. These interactions should set the framework for future research in plant pathology in order to promote long-term plant health and crop sustainability (Jeger and Spence, 2001). So far, most studies on plants with multiple stresses have focused on the pre-inoculation of plants with one pathogen and the subsequent examination of plant responses to a secondary pathogen infection (Sticher et al., 1997; van Loon et al., 1998). In this study, we examine a different situation, namely the attack of multiple bacterial pathogens at the same time.

Despite the complexity of host responses to different pathogens, the interactions of three phytohormones ethylene, salicylic acid (SA), and jasmonic acid (JA) are believed to regulate the specificity of plant defense responses (Dong, 1998; Reymond and Farmer, 1998). Ethylene regulates a variety of growth and

developmental processes, but it also mediates responses to a range of biotic and abiotic stresses in higher plants (Bleecker and Kende, 2000; Ciardi and Klee, 2001). Increased ethylene production from infected plant tissue has been reported for numerous pathogens (Boiler, 1991), and the exogenous application of ethylene can induce the accumulation of defense-related enzymes (Ecker and Davis, 1987). Ethylene is an important signaling component in plant-pathogen interactions, but its role in pathogenesis and resistance is occasionally ambiguous (Knoester et al., 2001). Accumulating evidence demonstrates that ethylene signaling plays a significant role in resistance against some fungal, bacteria, and viral diseases. For example, tobacco expressing the dominant *Arabidopsis etrl-1* gene, a mutant allele that confers ethylene insensitivity to plants (Schaller and Bleecker, 1995), is susceptible to what is otherwise non-pathogenic soil fungi, the *Pythium* spp. (Knoester et al., 1998; Geraats et al., 2002). Ethylene also increases disease symptom development. For example, increased ethylene levels stimulate the expansion of lesions caused by tobacco mosaic virus (TMV) in tobacco (Knoester et al., 2001). In tomato, the ethylene-insensitive mutant *Never ripe* shows a significant reduction in disease symptoms compared to the wild type after inoculation with virulent strains *of Fusarium oxysporum*, *Pseudomonas syringae* pv. *tomato*, and *Xanthomonas campestris* pv. *vesicatoria* (Lund et al., 1998).

SA has been recognized as a central molecule in the signal transduction pathway leading to systemic acquired resistance (SAR) to pathogens (Sticher et al., 1997). It is well established that endogenous SA accumulates at the site of HR in tobacco after inoculation with TMV (Malamy et al., 1990). Additionally, exogenous SA induces the expression of pathogenesis-related (PR) genes and decreases disease symptoms both in tobacco and *Arabidopsis* (White, 1979; Uknes et al., 1992, 1993). JA, a ubiquitous wound signal known to activate various defenses against herbivores, is also involved in the activation of disease resistance and various defense-associated responses in plants (Pieterse et al., 1998; Kenton et al., 1999; van Wees et al., 2000). Cross-talk occurs among SA, JA, and ethylene-dependent signaling pathways in fine-tuning the defense reactions, but is complicated and poorly understood. There are examples indicating that SA, JA, and ethylene-dependent defense pathways can affect each other's signaling in positive or negative ways, with some interactions occurring at the level of phytohormone production (Lund et al., 1998; Reymond and Farmer, 1998; Pieterse and van Loon, 1999; van Wees et al., 2000; Pieterse et al., 2001; O'Donnell et al., 2003).

The complex array of chemical responses that plants display during pathogen attack includes the induced emissions of volatile organic compounds (Croft et al., 1993; Buonaurio and Servili, 1999; Cardoza et al., 2002; Huang et al., 2003). In plant-insect interactions, both JA and ethylene are involved in herbivore-induced plant volatile emissions (Schmelz et al., 2003a, b, c). Plant volatiles have

established roles in host recognition by many pest insects (Bernays and Chapman, 1994) as well as functioning as an indirect defense by attracting natural enemies of the insect herbivores (Turlings et al., 1990; De Moraes et al., 1998). Compared to insect-induced plant volatile emissions, the role of pathogen-induced volatile emissions is less clear. However, multiple studies have implicated that volatile emissions from pathogen-infected plants may be in part responsible for the early suppression of pathogen growth (Croft et al., 1993; Wright et al., 2000; Cardoza et al., 2002). Unlike insect-induced volatile emissions, the involvement of phytohormone signals in triggering pathogen-induced plant volatile emissions is largely unexplored.

We previously reported that a complex volatile blend including (*E*)-β-ocimene, linalool, methyl salicylate (MeSA), indole, caryophyllene, β-elemene, α-farnesene, and two unidentified sesquiterpenes are emitted by tobacco plants, *Nicotiana tabacum* K326, infected with the avirulent/incompatible strain of *Pseudomonas syringae* pv. *tomato* (*Pst* DC3000). In contrast, a volatile blend consisting primarily of MeSA and two unidentified sesquiterpenes was released by plants infected with *P. syringae* pv. *tabaci* (*Pstb*) in a virulent/compatible interaction (Huang et al., 2003). Here, we examine the interactions between compatible and incompatible *P. syringae* strains and their impact on the host plant simultaneously. Specifically, we compare differences in volatile emissions among plants infected with *both Pst* DC3000 and *Pstb*, but on different leaves, and plants infected with either of the two; we then measure endogenous SA and JA levels at different times after dual inoculation with these two strains. We further examine possible differences in ethylene production among plants infected with *Pst* DC3000, *Pstb*, or both of them but on different leaves. Based on results from these experiments, we postulated that *Pstb*-induced ethylene production may play an important regulatory role in altering the typical volatile emissions in response to *Pst* DC3000 infection. To clarify the role of ethylene, we dynamically applied ethylene to the headspace of tobacco plants inoculated with *Pst* DC3000 to study changes in volatile emissions by plants after the infections. Possible interactions among ethylene, SA, and JA were also studied by measuring endogenous SA and JA levels in the plants after ethylene application.

METHODS AND MATERIALS

Plant Material. Seeds of tobacco *(Nicotiana tabacum* strain K326) were sown in a commercial soil mix (MetroMix 300, Scotts-Sierra Horticultural Company, Marysville, OH, USA) and grown in an environmental chamber (E15, Control Environmental Limited, Manitoba, Canada) at 25°C, and a relative humidity of 60 to 70%. Illumination with 400W metal halide and high-pressure sodium lamps provided a photoperiod of 12:12 hr (L:D). After 16 days, the soil was gently

washed from the roots of seedlings with tap water, and each seedling was transferred to a 1.0 l plastic cup containing a nutrient solution described by Baldwin and Schmelz (1994) with modifications as follows (mM): KNO_3, 1; NH_4NO_3, 0.5; $Ca(NO_3)_2.4H_2O$, 0.5; $MgSO_4.7H_2O$, 0.75; KH_2PO_4, 0.5; NaCl, 0.25; K_2S0_4, 0.25; Fe-Na-EDTA, 0.06; H_3BO_3, 0.05; $MnCl_2.4H_2O$, 0.015; $ZnSO4.7H_2O$, 0.002; $CuSO_4.5H_2O$, 0.00025; $Na_2MoO_4.2H_2O$, 0.0002. The nutrient solution in each cup was replenished every 10 days. Plants were used for experiments about 30 days after seeds were planted and had 7 to 8 leaves including the newly emerged leaf.

Bacterial Cultures and Plant Inoculations. P. syringae pv. *tomato* DC3000 (*Pst*DC3000) (Whalen et al., 1991) was grown in King's medium B broth (King et al., 1954), supplemented with 50 μg/ml rifampicin for 18 hr at 28°C on a shaker at 200 rpm. *P. syringae* pv. *tabaci* (*Pstb*, Department of Plant Pathology, University of Florida, FL) was grown the same way, but with no rifampicin. Both *Pst* DC3000 and *Pstb* were stored in 15% glycerol at −70°C for later use.

One day prior to plant inoculations, the bacterial strains were cultured in King's medium B as described above. Bacterial cells were collected by centrifugation at 4000 g for 15 min and resuspended in 10 mM $MgCl_2$. The density of bacterial cell suspensions was determined as colony-forming units/ml (CFU/ml) at 600 nm with a Spectro 22 spectrophotomer (Labomed, Inc., Culver city, CA) (1 $OD_{600nm} = 10^9$ CFU/ml) and adjusted to 4×10^6 CFU/ml in 10 mM $MgCl_2$ for *Pst* DC3000 and 4×10^7 CFU/ml for *Pstb*. All bacterial suspensions were supplemented with 400 μl/l of Silwett L-77 (OSI Specialties, Inc., Friendly, WV) to help cells adhere to the leaf surface. Plants were inoculated by dipping the whole plants or selected leaves in a bacterial suspension for 20 sec.

Volatile Collection and Analysis from Plants Inoculated with Bacterial Pathogens. To study the effects of interaction between the avirulent strain *Pst* DC3000 and the virulent strain *Pstb* on volatile emissions of tobacco plants, the 4th leaf of a plant was dipped in *Pst* DC3000 suspension *(Pst* DC3000-C), while the 5th leaf on the same plant was dipped in *Pstb* suspension (*Pstb*-C, Figure 1). The 4th leaf from another plant dipped in *Pst* DC3000 suspension served as one control (*Pst* DC3000), and the 5th leaf from another plant dipped in *Pstb* suspension served as the other control (*Pstb*). Thus, in the combined infection, collected volatiles represent the combined emissions from *Pst* DC3000- and *Pstb*-infected leaves. This design was selected because no systemic induction of volatiles was detected from uninfected leaves on infected plants in a preliminary study. Within 1 hr after inoculation, treated plants were transferred from the original hydroponic cups to glass dishes (40 mm height × 80 mm diam.) containing 130 ml of hydroponic solution. Individual plants along with the dish were then placed inside a bell-shaped glass chamber (17.8 cm height × 16.5 cm diam. at base; Analytical Research Systems Inc., Micanopy, FL) (Figure 2). Air, purified by passage through a charcoal column, entered through a hole in the base of the collection chamber at approximately 2 l/min, passed over the plants, and volatiles

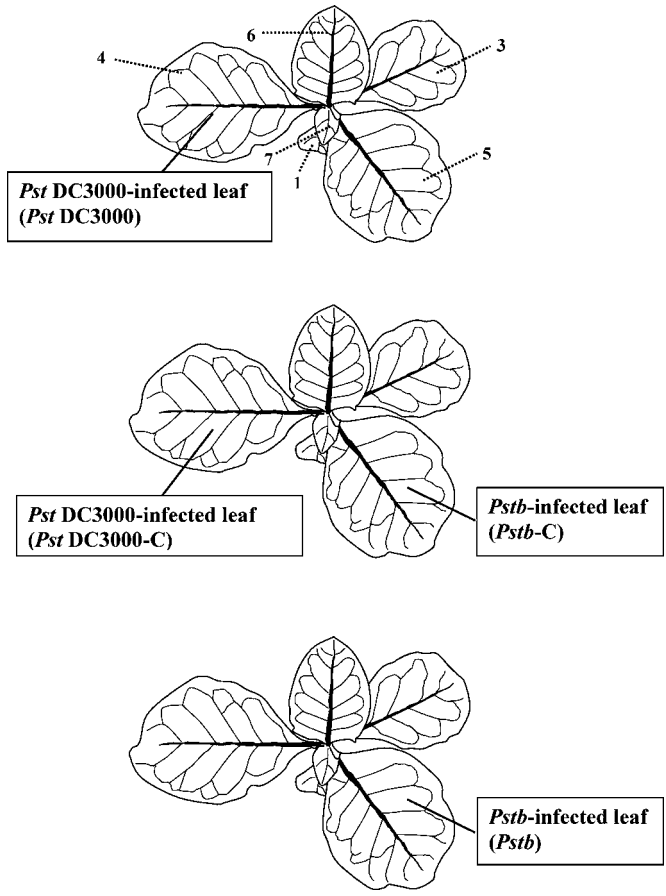

FIG. 1. Experimental design in the treatment of tobacco plants with avirulent *Pst* DC3000, virulent *Pstb*, and the combination of the two strains. Leaves are numbered from the bottom. The 2nd leaf is behind the 3rd and 6th leaves so it is not labeled. The 4th leaf of a plant was dipped in *Pst* DC3000 suspension (*Pst* DC3000-C), while the 5th leaf on the same plant was dipped in *Pstb* suspension (*Pstb*-C). The 4th leaf from another plant dipped in *Pst* DC3000 suspension served as one control (*Pst* DC3000) and the 5th leaf from another different plant dipped in *Pstb* suspension served as the other control (*Pstb*).

were sampled by pulling air from the top of the chamber at a rate of 500 ml/min through a trap containing 25 mg of Super Q adsorbent (Alltech Assoc., Deerfield, IL). The remainder of the air was vented out the bottom of the system. Thus, the quantity of volatiles analyzed represents 1/4 of the total plant volatile emissions. Volatiles were collected continuously in 24-hr periods for 3 days after inoculation.

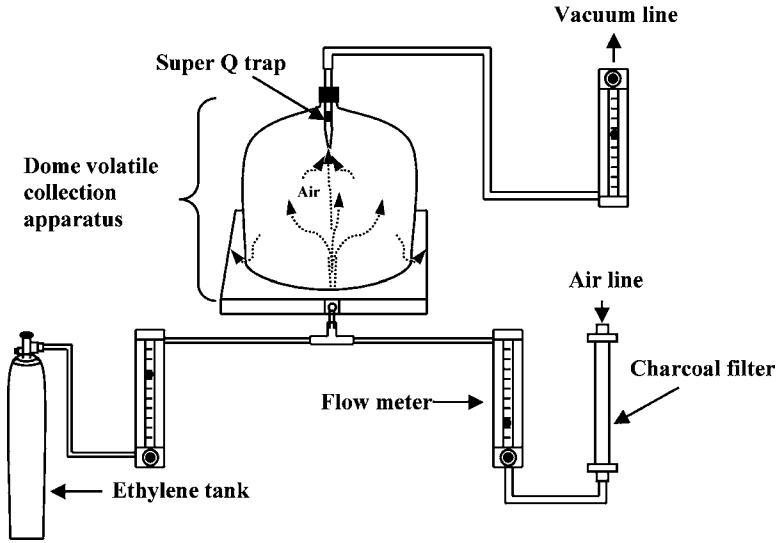

FIG. 2. Plant volatile collection and dynamic ethylene application system for analysis of plant response to bacterial infection. Clean air entered through a hole in the base of the collection chamber, passed over the plants, and volatiles were sampled by pulling air from the top of the chamber through a trap containing Super Q adsorbent. The remainder of the air was vented out the bottom of the system. In the experiment where ethylene was dynamically applied to the headspace of tobacco plants following infection with *Pst* DC3000, both clean air and air with ethylene from the tank were regulated to various flow rates to give final ethylene concentrations of 0, 10, 30, and 100 nl/1.

Compounds were eluted from the collection trap and analyzed as described by Huang et al. (2003). Each experiment was replicated 4 times.

Analysis of JA and SA. Plants were treated as described for the induction of volatiles (Figure 1). Healthy plants with one leaf dipped in 10 mM $MgCl_2$ were used as blank controls. Leaves exposed to each treatment were sampled at 0, 3, 6, 12, 24, and 48 hr post-inoculation, ground under liquid nitrogen, and stored at -70°C prior to hormone analysis. Five replicates for each time point were obtained for plants under the different inoculation treatments. The extraction and quantitative analysis of JA and SA followed the protocol of Engelberth et al. (2003).

Ethylene Analysis. To investigate whether plants produce ethylene in response to *Pst* DC3000 or *Pstb* infection, plants were inoculated by submerging their aerial portions either in a suspension of *Pst* DC3000 or *Pstb*. Control plants were mock-inoculated the same way but without bacteria cells. Treated leaves were detached from each plant at 0, 1, 2, 3, and 4 days post-inoculation of *Pst*

DC3000, while plants inoculated with *Pstb* were sampled at 0, 1, 2, 3, 4, 5, 6, and 7 days post-inoculation. Each detached leaf was placed into an 80-ml test tube sealed with a rubber septum. After 45 min, a 1-ml gas sample was withdrawn from the headspace with a syringe and analyzed by gas chromatography with an HP-5890 GC with injector, oven, and flame ionization detector temperatures of 150, 80, and 250°C, respectively, and an HayeSep Q column (80/100 mesh, 6 feet × 0.125 inches × 0.085 inches i.d., Alltech, Deerfield, IL) using nitrogen carrier gas flow rate of 100 ml/min. Quantification was based on an external standard curve constructed from 1-ml injections of known ethylene standards (Kao and Yang, 1983). Individual leaves were weighed, and ethylene production was calculated as nl g^{-1} hr^{-1}. Three replicates for each time point were obtained for plants under the different inoculation treatments. This experiment was repeated twice. Ethylene production was also measured in plants that were inoculated with avirulent and virulent strains simultaneously as described for the induction of volatiles (Figure 1).

Exogenous Application of Ethylene to Pst DC 3000-infected Plants. Based on *Pst* DC3000- and *Pstb*-induced ethylene emission patterns, we designed an experiment to assess the potential role of ethylene in regulating volatile emissions of plants in response to *Pst* DC3000 infection. In contrast to traditional application of ethylene that is added into a sealed container where a plant has no air exchange from outside, ethylene was dynamically applied to the headspace of tobacco plants following infection with *Pst* DC3000 by mixing air containing ethylene (1 μl/1; Boc Gases, Riverton, N.J.) with purified air. Both were regulated to give final ethylene concentrations of 0, 10, 30, and 100 nl/1 of air passed through a hole in the base of the collection chamber (Figure 2). The total air flow into each collection chamber was proximately 500 ml/min, 90% of which was sampled by pulling air through the filter trap. To study possible effects from ethylene alone, healthy plants were dynamically tested with ethylene at 100 nl/1. Collections and analyses of volatile organic compounds were performed as described above. To examine possible effects of ethylene on endogenous JA and SA levels in *Pst* DC3000 infected plants, plants were dipped in a) 10 mM $MgCl_2$ alone (control), or b) *Pst* DC3000 suspension alone (*Pst* DC3000), or c) *Pst* DC3000, then with 100 nl/1 ethylene to its headspace (*Pst* DC3000 + ethylene), or d) 10 mM $MgCl_2$ with 100 nl/1 ethylene applied in the headspace (ethylene). Different plants in each treatment were sampled at 0, 6, 12, 24 and 48 hr post-inoculation. Sample preparation and analysis for JA and SA were performed as described above. Five replicates for each time point were obtained for plants under the different inoculation treatments.

Statistical Analysis. All data were subjected to log (x + 1) transformations because the original data did not meet the assumptions of normality and homogeneity of variance required by ANOVA. Data generated from all the experiments were analyzed as a two way factorial design using Proc GLM except for the

experiment of ethylene measurement in the combined infection where randomized complete block design was used (SAS Institute, 1999). Significant ANOVAs were followed by Tukey's HSD test to separate means.

RESULTS

Interactions between Avirulent and Virulent Strains on Volatile Emissions. When one leaf of a plant was infected with *Pst* DC3000 and another leaf from the same plant with *Pstb*, the amount of (*E*)-β-ocimene emitted from plants with combined infection was significantly reduced ($P < 0.05$) and 30%, 31%, and 27% of that from plants with a single leaf infection (SLI) of *Pst* DC3000 on day 1, 2 and 3, respectively (Figure 3). Similarly, the combined infection produced only 27% and 18% of the MeSA found in the *Pst* DC3000 SLI on days 1 and 2, respectively (Figure 3A, B). In contrast, total sesquiterpene emission was not significantly altered by the combined infection except for SQ1 and 2 on day 2. Most of the sesquiterpenes in the combined infection were attributable to established patterns from the *Pst* DC 3000-infected leaves on day 1 and from the *Pstb*-infected leaves on day 3 (Figure 3C).

Interaction of Avirulent and Virulent Strains on Endogenous JA and SA. Leaves infected with *Pst* DC3000, both from plants with single leaf infection (SLI) and combined plant infection (CPI), produced both free and conjugated SA in significantly higher amounts than those infected with *Pstb* (whether from *Pstb* or *Pstb*-C plants) or controls (Figure 4A, B). Both free and conjugated SA levels increased rapidly and in similar amounts in the *Pst* DC3000-infected leaf from the CPI plant and the SLI plant infected with *Pst* DC3000 alone, except at 12 hr post-inoculation, when there was a statistically significant difference in the level of free SA ($P < 0.05$). The leaf infected with *Pst* DC3000 did not affect either free or conjugated SA levels in the other leaf infected with *Pstb* on the same plant compared to the leaf infected with *Pstb* on the SLI plant. Interestingly, JA was induced in higher amounts from leaves infected with *Pstb* alone ($P < 0.05$) when its free SA levels were low at 6 hr post-inoculation (Figure 4C). Likewise, Free SA levels in all *Pst* DC3000 infected leaves at this time point were high when JA levels were either lower than controls or not significantly different from controls.

Ethylene Production. Inoculation of tobacco plants with the virulent strain *Pstb* resulted in a large burst of ethylene emission compared to control plants treated with surfactant alone (Figure 5A). Ethylene was detected in higher amounts than controls at 2 days post-inoculation ($P < 0.03$). Substantial production occurred at 3 days post-inoculation; the average production rate was 252 nl g^{-1} hr^{-1}, almost 345-fold higher than control ($P < 0.001$). The *Pstb* induced ethylene burst declined but remained significantly elevated through day 6. Unlike *Pstb*, plants treated with *Pst* DC3000 did not release increased levels of ethylene (Figure 5B).

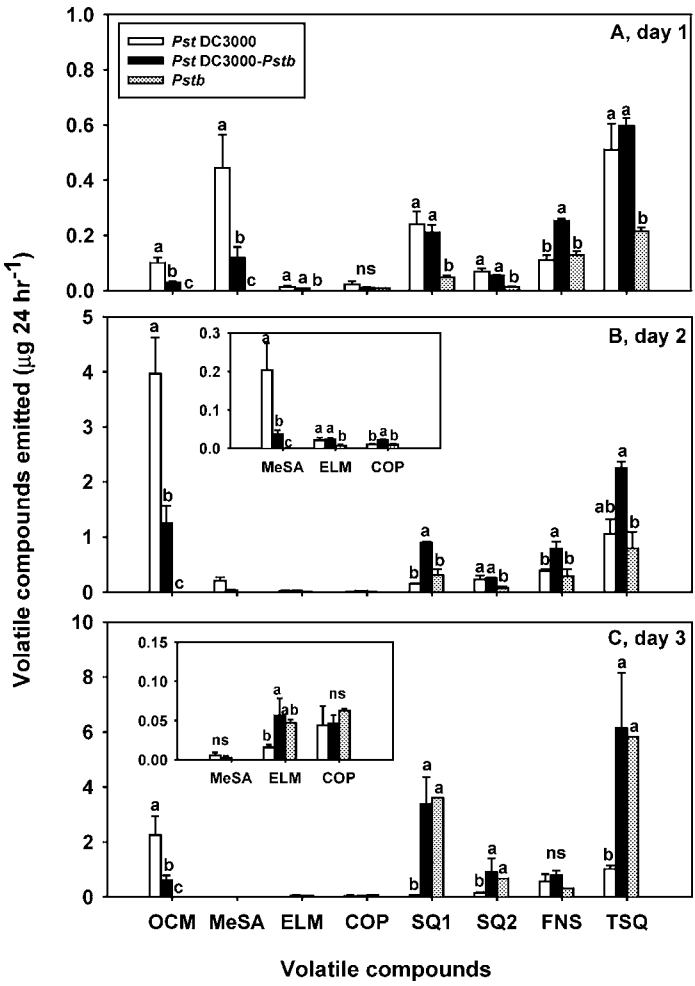

FIG. 3. Mean (+SE) volatile emissions (μg/24 hr^{-1}) from tobacco plants ($N = 4$) infected with both *Pst* DC-3000 at a titer of 4×10^6 CFU/ml and *Pstb* at a titer of 4×10^7 CFU/ml but on the different leaves (Figure 1) compared to control plants infected with either one of them alone. Volatiles shown were collected in 24-hr intervals, beginning immediately after inoculation and continuing for 3 days. Volatile compound abbreviation: OCM, (E)-β-ocimene; MeSA, methyl salicylate; ELM, β-elemene; COP, caryophyllene; SQ1, unknown sesquiterpene 1; SQ2, unknown sesquiterpene 2; FNS, (E)-α-farnesene; TSQ, total sesquiterpenes. Inserts shows an expanded view of amounts of MeSA, ELM, and COP emitted. Bars not sharing the same letters represent significant differences ($P < 0.05$, Tukey's HSD test).

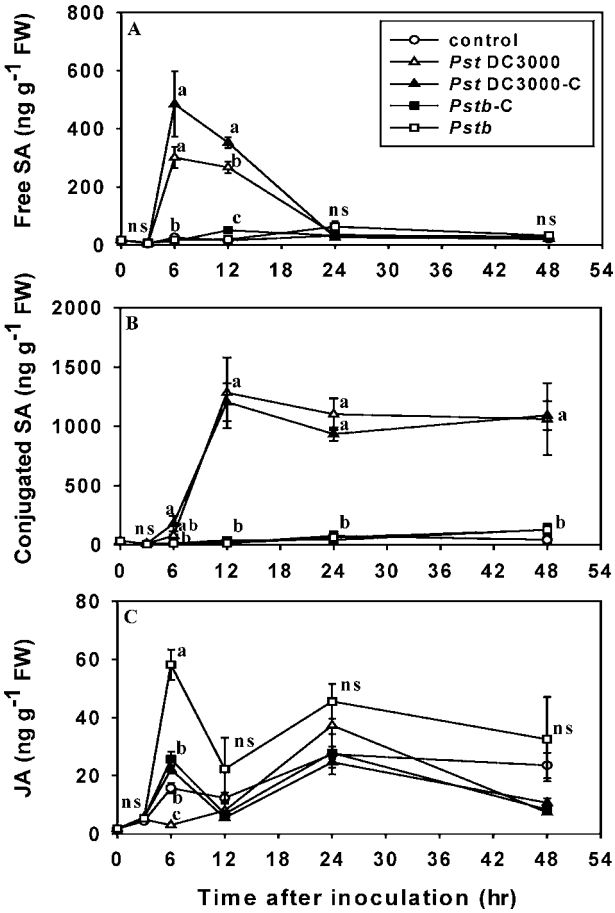

FIG. 4. Mean (±SE) A) free SA, B) conjugated SA, and C) JA levels (ng g^{-1} FW, $N = 5$) in individual tobacco leaves following inoculation of leaves with surfactant alone (control, ○), *Pst* DC3000 alone(△), *Pstb* alone (□), or plants with both *Pst* DC3000 (*Pst* DC3000-C, ▲) and *Pstb* (Pstb-C, ■) but on the different leaves (Figure 1). Hormonal analysis was performed only on the inoculated leaves. The titer of bacteria suspensions were 4×10^6 CFU/ml for *Pst* DC3000 and 4×10^7 CFU/ml for *Pstb*. Within each time point, symbols not sharing the same letters represent significant difference ($P < 0.05$, Tukey's HSD test).

Leaves infected with *Pst* DC3000 from combined plant infection (CPI) released very low levels of ethylene and were not significantly different from leaves infected with *Pst* DC3000 alone (Figure 5C). Likewise, leaves infected with *Pstb*, both from plants with single leaf infection (SLI) and CPI, produced similar but large amounts of ethylene.

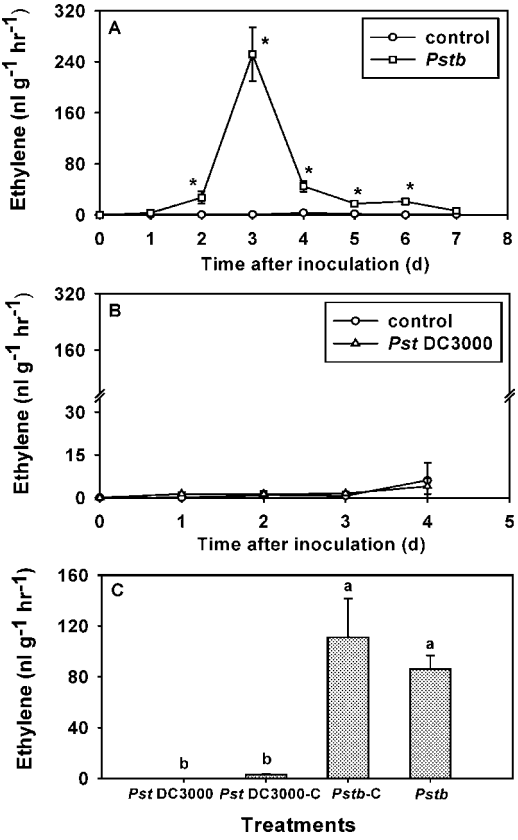

FIG. 5. Mean (±SE) ethylene emissions (nl g^{-1} hr^{-1}) from tobacco plants ($N = 3$) infected with either (A) *Pstb* (□) at a titer of 4×10^7 CFU/ml or (B) *Pst* DC-3000 (Δ) at a titer of 4×10^6 CFU/ml. (C) Mean (+SE) ethylene emission (nl g^{-1} hr^{-1}) from tobacco plants ($N = 5$) after 3 days of infection with both *Pst* DC-3000 and *Pstb* (using the inoculums above) but on the different leaves (Figure 1) compared to plants infected with either one of them alone. Control plants (O) (from A and B), were mock treated with the leaf surfactant Silwett L-77 alone. Asterisks denote significant increases in ethylene above control plants treated with the leaf surfactant alone ($P < 0.05$, Tukey's HSD test). Bars not sharing the same letters represent significant differences ($P < 0.05$, Tukey's HSD test). The experiment was repeated as least twice with similar results.

Interactions between Ethylene and Pst DC3000 on Volatile Emissions and Endogenous Hormones. Control plants with ethylene application emitted only trace amounts of sesquiterpenes and no ocimene or MeSA. Compared to plants infected with *Pst* DC3000 alone, ocimene emission was greatly inhibited in all

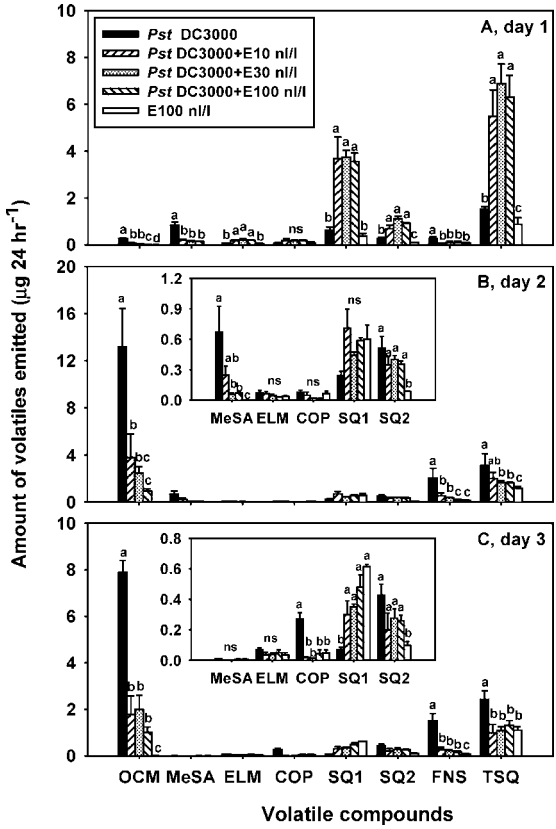

FIG. 6. Mean(+SE) volatile emissions from tobacco plants ($N = 4$) on A) day 1, B) day 2 and C) day 3 infected with *Pst* DC3000 at a titer of 4×10^6 CFU/ml and treated with ethylene at either 0, 10, 30, and 100 nl/1. A separate uninfected control for the highest ethylene treatment (100 nl/1) was also performed. Volatiles were collected in 24-hr intervals, beginning immediately after inoculation and continuing for 3 days. Volatile compound abbreviation: OCM, (*E*)-β-ocimene; MeSA, methyl salicylate; ELM, β-elemene; COP, caryophyllene; SQ1, unknown sesquiterpene 1; SQ2, unknown sesquiterpene 2; FNS, (*E*)-α-farnesene; TSQ, total sesquiterpenes. Inserts shows an expanded view of amounts of MeSA, ELM, COP, SQ1, and SQ2 emitted. Bars not sharing the same letters represent significant differences ($P < 0.05$, Tukey's HSD test).

ethylene treatments, even 10 nl/1 (Figure 6). Likewise, ethylene application significantly reduced MeSA emission from *Pst* DC3000 treated plants (Figure 6A, B). In contrast, the largest effect of ethylene on sesquiterpene emissions occurred on day 1 with a sharp increase in SQ1, SQ2 and elemene emissions, and a decrease in (*E*)-α-farnesene emission. Similar increases and decreases in the *Pst* DC3000-infected

plants treated with ethylene were also found on day 2, but with fewer changes and decreases in total sesquiterpene emissions compared to those treated with *Pst* DC3000 alone. With the exception of (*E*)-β-ocimene emission (Figure 6A, B), the different quantities of ethylene applied did not result in linear dose-dependent increases or decreases in volatile production, suggesting that the ethylene sensitivity threshold was below 10 nl/1. The combination of ethylene and infection resulted in a sustained decrease in free SA below that from plants infected with *Pst* DC3000 alone at 6 and 12 hr post-inoculation, yet there were no significant difference in conjugated SA levels throughout the whole experimental period (Figure 7A, B). Unlike SA, additions of ethylene during infection did not significantly increase the levels of JA. However, healthy plants, which were continuously exposed for 6 hr to ethylene, showed a small yet significant JA increase compared to the untreated control (Figure 7C).

DISCUSSION

Previously, we reported that tobacco plants release qualitatively and quantitatively greater amounts of volatiles in the incompatible interaction with *Pst* DC3000 compared to those in the compatible interaction with *Pstb* (Huang et al., 2003). Recognizing that both pathogen strains induce volatile emissions in tobacco, we were prompted to examine plant responses to multiple stresses initiated by both avirulent and virulent strains of *P. syringae*, which broadly represent two opposite, but common, outcomes of pathogen infection: disease and resistance. To investigate pathogen-pathogen interactions in the mediation of induced plant volatile emissions, we co-infected individual plants with one leaf inoculated with *Pst* DC3000 and the other with *Pstb*. Interestingly, the combined infection significantly reduced emissions of ocimene and MeSA compared to plants infected with *Pst* DC3000 alone, but sesquiterpene emissions were not dramatically affected except for SQ1 on day 2 (Figure 3). After examining free SA, conjugated SA, and JA, we found no convincing evidence that these changes were linked to alterations in the induced volatile emissions unique to the combined infection (Figure 3, 4).

The phytohormone ethylene is an important regulator of plant responses to pathogen infection (Bleecker and Kende, 2000; Ciardi and Klee, 2001). In the compatible interaction, *Pstb* triggered tobacco plants to produce an extremely large ethylene burst from 2 to 6 days after inoculation concomitant with the occurrence of disease symptoms; whereas the incompatible interaction involving *Pst* DC3000, which elicited strong HR, displayed no such ethylene induction. This outcome contrasts with the interactions of tobacco and tobacco mosaic virus (TMV), in which the increase in ethylene during an incompatible interaction is greater and more rapid than during a compatible interaction (De Laat and van Loon, 1982). However, there is also ample evidence for a positive correlation

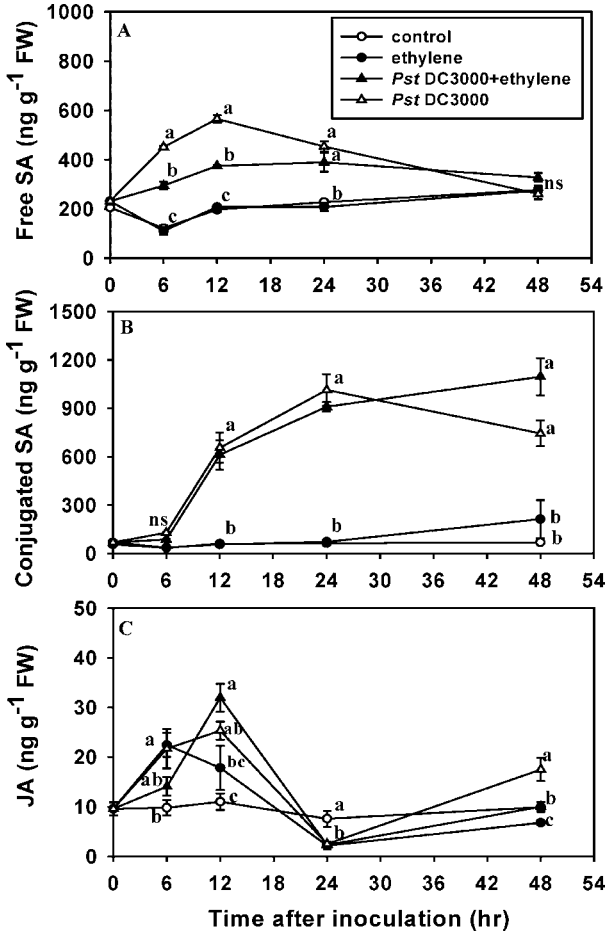

FIG. 7. Mean (±SE) A) free SA, B) conjugated SA, and C) JA levels (ng g^{-1} FW, $N = 5$) following inoculation of tobacco plants with the leaf surfactant alone (control, ○), 100 nl/l ethylene (●), *Pst* DC3000 + 100 nl/l ethylene (▲) or *Pst* DC3000 alone (Δ). Ethylene application was described in details in the Material and Method section. The titer of bacteria suspensions were 4×10^6 CFU/ml. Within each time point, symbols not sharing the same letters represent significant difference ($P < 0.05$, Tukey's HSD test).

of many plant diseases with ethylene production (Goto et al., 1980; Ben-David et al., 1986; Elad, 1990; Boller, 1991; Weingart and Völksch 1997). It is now becoming clear that ethylene plays an important role in the development of disease symptoms in response to certain pathogenic organisms (O'Donnell et al., 2001);

whereas ethylene does not appear to be essential for a resistance response in plants challenged with avirulent bacteria (Bent et al., 1992; Ciardi and Klee, 2001). Additionally, some fungi and bacteria can produce ethylene as a pathogenicity factor (Lund et al., 1998; Knoester et al., 2001). *Pstb* is believed to trigger ethylene production in plants, as this pathogen did not produce ethylene when isolated in culture (Weingart and Völksch, 1997). Importantly, ethylene induction by *Pstb* was not significantly altered in the combined infection (Figure 5C).

Therefore, we proposed that ethylene production induced by *Pstb* may play an important regulatory role in altering *Pst* DC3000-induced volatile emissions in tobacco. The role of ethylene was examined by continuous application to the headspace of *Pst* DC3000-infected plants. Consistent with *Pstb*-induced ethylene, application of ethylene resulted in sharp decreases in both ocimene and MeSA emissions, and also dramatically shifted the sesquiterpene profile. This supports the hypothesis that altered volatile profiles from the combined infection are due to the ethylene signaling pathway activated by *Pstb* from the same plants. The suppression of ocimene and MeSA emissions occurred as early as 1 day after inoculation, immediately prior to significant *Pstb* induced ethylene emission, suggesting that either only small amounts of ethylene emissions would be enough for this volatile inhibition, or some other factors in the ethylene signaling pathway were also involved, most likely ethylene perception (Hoffman et al., 1999; Ohme-Takagi et al., 2000). Ethylene effects on sesquiterpene emissions appear to be more closely linked to actual ethylene production, as they occur later.

SA, JA, and ethylene signaling pathways can be linked to each other in positive and negative ways (Dong, 1998; Reymond and Farmer, 1998; Díaz et al., 2002). We provide an additional example of possible antagonism between JA and SA by showing that *Pst* DC3000-infected leaves had very high free SA levels, but low JA levels not significantly higher than controls treated with leaf surfactant alone. In contrast, *Pstb*-infected leaves had elevated JA levels, but low control levels of SA at 6 hr post-inoculation (Figure 4). Moreover, ethylene alone did not affect SA levels in healthy plants, but it significantly inhibited *Pst* DCS3000-induced free SA levels. There is also evidence that ethylene can affect JA levels in healthy plants (Figure 7). However, we found little clear evidence for cross-talk among SA, JA, and ethylene pathways in the changes of pathogen-induced volatile emissions upon ethylene application or the combined infection in tobacco, except that there was a correlation of less SA with less MeSA emission in *Pst* DC3000-infected leaves after ethylene application, which was not observed in the experiment where plants were infected with both *Pst* DC3000 and *Pstb* simultaneously on different leaves. It is likely that factors in addition to ethylene are involved in alteration and regulation of pathogen-induced volatile emissions in the combination because the effect of interaction of avirulent and virulent pathogens on volatile emissions is more complicated than that from just application of ethylene alone.

Similar to pathogen infection, insect herbivory also stimulates ethylene and volatile emissions. While insect-induced plant volatiles have important roles in the attraction of natural enemies, less is known about the regulation of herbivore-induced volatile emissions. JA has been long considered as an endogenous regulator for insect-induced volatile emissions (Hopke et al., 1994, Schmelz et al., 2003a, b, c); however, a role for ethylene is just beginning to emerge. For example, exogenous applications of the ethylene precursor, 1-aminocyclopropane-l-carboxylic acid, in lima bean enhanced JA-induced emissions of ocimene and hexenyl acetate, but not the homoterpene (*E*)-4, 8-dimethyl-l,3,7-nonatriene (Horiuchi et al., 2001). The addition of ethylene to intact corn seedlings greatly promoted volatile emissions of caryophyllene, bergamontene, farnesene, and indole triggered by insect derived elicitors and JA application (Schmelz et al., 2003 a, b). However, ethylene burst in tobacco, *Nictotiana attenuata*, induced by *Manduca sexta* larvae or the application of its oral secretion suppresses JA-induced nicotine accumulation, yet does not alter the induced volatile emissions, namely the monoterpene linalool and the sesquiterpene bergamotene (Kahl et al., 2000). In the present study, ethylene regulates both the magnitude and blend of pathogen-induced volatile emissions during pathogen infection, probably through regulation of enzymes in volatile biosynthetic pathways. It has been reported that an ethylene responsive enhancer element was identified at the 1-kb upstream genomic region of a myrcene/ocimene synthase gene in *Arabidopsis*, which suggests that ethylene may regulate genes encoding terpene synthases at the transcriptional level in plants (Montgomery et al., 1993). Likewise, pollination-induced ethylene production in petunia (*Petunia hybridd*) flowers strongly down regulates the transcription of benzoic acid/salicylic acid carboxyl methyltransferases (BSMT), which convert the corresponding free acids into volatile methyl esters (Negre et al., 2003). It seems possible that similar ethylene mediated transcriptional regulation may also occur in leaf tissues.

Compared to the well-defined role for volatile emissions as indirect defenses in plant-herbivore interactions, little is known about their role in plant-pathogen interactions. Emission of volatiles from pathogen-infected plants may serve as a direct defense against pathogen infections. For example, lipid-derived volatiles, (*Z*)-3-hexenol and (*E*)-2-hexenal, emitted from *Phaseoltis vulgaris* (L.) leaves during HR response to *P. syringae* pv. *phaseolicola* are highly bactericidal (Croft et al., 1993). Corn-derived volatile compounds, hexenal and octanal, strongly inhibited radial growth of the fungus, *Aspergillus parasiticus* on solid culture media (Wright et al., 2000). MeSA has been shown to inhibit fungal growth *in vitro* (Cardoza et al., 2002), as well as to function as an airborne signal that can activate the expression of defense-related genes in tobacco (Shulaev et al., 1997). Similarly, herbivore-induced ocimene was found to function as an airborne signal in inducing transcript accumulation of an array of defense-related genes in lima bean leaves (Arimura et al., 2002). As a volatile, ethylene is a well-known inducer of necrosis, ripening, chlorosis, and senescence in plants. In the compatible interaction with

virulent *P. syringae*, there was no ocimene emission, much less MeSA at later time, but a dramatic ethylene burst throughout the disease symptom development in tobacco. In contrast, there were large ocimene and MeSA emissions, but no such ethylene induction in the incompatible interactions with avirulent *P. syringae*. Moreover, application of ethylene strongly inhibited ocimene and MeSA emissions, but selectively affected sesquiterpenes from tobacco plants infected with *Pst* DC3000. Possibly, *Pstb* uses ethylene emissions to inhibit other volatile emissions from plants that might compromise their growth in tobacco. This study provides new insights on pathogen-plant-pathogen interactions, especially from attacks by virulent and avirulent strains simultaneously, and provides evidence that ethylene plays an important role in mediating pathogen-induced volatile emissions.

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VARIATION IN PLANT VOLATILES AND ATTRACTION OF THE PARASITOID *Diadegma semiclausum* (HELLÉN)

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Abstract—Differences in allelochemistry of plants may influence their ability to attract parasitoids. We studied responses of *Diadegma semiclausum* (Hellén), a parasitoid of the diamondback moth (*Plutella xylostella* L.), to inter- and intraspecific variation in odor blends of crucifers and a non-crucifer species. Uninfested Brussels sprout (*Brassica oleracea* L. gemmifera), white mustard (*Sinapis alba* L.), a feral *Brassica oleracea*, and malting barley (*Hordeum vulgare* L.) were compared for their attractivity to *D. semiclausum* in a Y-tube bioassay. Odors from all plants were more attractive to the parasitoid than clean air. However, tested against each other, parasitoids preferred the volatile blend from the three cruciferous species over that of malting barley. Wasps also discriminated between uninfested crucifers: mustard was as attractive as feral *B. oleracea*, and both were more attractive than Brussels sprout. Attractivity of uninfested plants was compared with that of plants infested by larvae of the host *P. xylostella*. Host-infested mustard and Brussels sprout were more attractive than uninfested conspecifics. Interestingly, the volatile blends of uninfested white mustard and infested Brussels sprout were equally attractive. We also compared the volatile composition of different plant sources by collecting headspace samples and analysing them with GC-MS. Similarities of volatile profiles were determined by hierarchic clustering and non-metric scaling based on the Horn-index. Due to the absence of several compounds in its blend, the volatile profile of barley showed dissimilarities from blends of crucifers. The odor profile of white mustard was distinctly different from the two Brassicaceae.

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Feral *Brassica oleracea* odor profile was different from infested Brussels sprout, but showed overlap with uninfested Brussels sprout. Odor blends from infested and uninfested Brussels sprout were similar, and mainly quantitative differences were found. *D. semiclausum* appears to discriminate based on subtle differences in volatile composition of odor blends from infested and uninfested plants.

Key Words—*Diadegma semiclausum*, *Plutella xylostella*, *Hordeum vulgare*, *Sinapis alba*, *Brassica oleracea*, olfactometer, headspace volatiles, GC-MS.

INTRODUCTION

Plant-derived infochemicals are important for parasitoids of herbivores in the process of host location (Vinson, 1976; Vet and Dicke, 1992; Tumlinson et al., 1993; Völkl and Sullivan, 2000). The dietary breadth of both herbivores and their parasitoids may influence the degree to which plant odors are used in this process (Vet and Dicke, 1992). In general, odors from uninfested and mechanically damaged plants are easier to detect by a parasitoid than cues from the host, but they represent a weak predictor of host presence (Vet et al., 1991). On the other hand, information from the host itself (i.e., feces, frass, silk) is a more reliable indicator of a host, but is usually more difficult to detect than plant-derived volatiles. The solution to this reliability-detectability problem may rely on herbivore-induced volatiles. These are emitted upon damage by the host and are both reliable and detectable. In natural ecosystems, host-parasitoid interactions take place in habitats composed of several to many plant species, where both the expectancy of the host's presence and the specificity of volatile infochemicals may show great between- and within-plant variation (Takabayashi et al., 1994; De Moraes et al., 1998; Vet, 1999; Gouinguené, 2001). Variation in odors among plant species and cultivars can be greater than between damaged and undamaged conspecific plants (Geervliet et al., 1997), and such differences can be reflected in the attractance of parasitoids to plants (Elzen et al., 1983, 1986; Fox and Eisenbach, 1992; Geervliet et al., 1996). The differential ability of plants to attract natural enemies may even be responsible for further dietary specialization of herbivores exploiting specific plant taxa like the Cruciferae (Yano, 1994). For example, opposing choices at the plant-level could benefit the herbivore and lead to enemy-free space shaping host-parasitoid interactions (Fox and Morrow, 1981; Fox and Eisenbach, 1992; Bigger and Fox, 1997; Gratton and Welter, 1999; Oppenheim and Gould, 2002). If host-parasitoid interactions in ecosystems are to be understood, foraging behavior of parasitoids must be studied in relation to inter- and intraspecific variation in plant-derived infochemicals.

Crucifers are characterized by the presence of glucosinolates and their volatile by-products. These compounds play a role in the host searching behavior of parasitoid species that forage on hosts associated with plants of this family (Read et al., 1970, 1985; Shiojiri et al., 2001; Smid et al., 2002). Other studies show

effects of inter- and intraspecific variation in volatiles of cruciferous plants on their attractivity to parasitoids (Geervliet et al., 1996; Liu and Jiang, 2003; Kalule and Wright, 2004). Fox and Eisenbach (1992) found that *Diadegma insulare*, a parasitoid of larvae of *Plutella xylostella* L., preferred wild crucifers to collards. Higher levels of parasitism of *P. xylostella* by *Cotesia plutellae* were observed on common cabbage than on Chinese cabbage, which was attributed to differences in attractivity of plant odors for the parasitoid (Liu and Jiang, 2003).

Breeding programs involving glucosinolate chemistry largely focus on mammalian toxicity, and the demands of consumers in the utilization of plant organs (Mithen, 2001). Members of the Brassicaceae have undergone extensive breeding, resulting in huge variation in both the composition and the allocation of plant chemicals in different plant organs (Benrey et al., 1998; Mithen, 2001). Besides, the efforts to select for resistance in breeding programs mainly focused on direct defenses, ignoring effects on the third trophic level (Dicke, 1999; Bradburne and Mithen, 2000). It is possible that crucifer breeding programs have changed the apparency of plants not only for herbivores, but also for those parasitoids that use plant-derived infochemicals in habitat and host location. Therefore, domesticated plants might have an altered capacity to attract natural enemies, but experimental evidence is needed to test this assumption (Loughrin et al., 1995; Benrey et al., 1998; Cortesero et al., 2000).

We chose members of the family *Brassicaceae* to study how inter- and intraspecific variation in odor blends influence their ability to attract larval parasitoids. We examined the responses of *Diadegma semiclausum*, a specialist parasitoid of the diamondback moth (*Plutella xylostella*), in olfactometer bioassays. The uninfested plants compared were Brussels sprout, a naturalized population of previously cultivated *Brassica oleracea*, white mustard, and malting barley. The latter species was included in comparisons as a species not related to crucifers and a non-host for *P. xylostella*. Comparisons of host-infested and uninfested plants involved Brussels sprout and mustard.

METHODS AND MATERIALS

Plants. Plants used were malting barley (*Hordeum vulgare* L. cv Video) (Cyperales, Gramineae), white mustard (*Sinapis alba* L. cv Carnaval) (Capparales, Brassicaceae), Brussels sprout (*Brassica oleracea* L. gemmifera cv Cyrus) (Capparales, Brassicaceae), and a naturalized population of *Brassica oleracea* L. This feral population was found in a roadside hollow in 2001, it probably "escaped" from a local farm, and it is unknown how long it has been growing in the wild (Harvey et al., 2003). Plants were reared in a greenhouse compartment under a 16L; 8D photoperiod, 20–28°C, and 40–80% R.H. Plants were sown in ca. 1.2 l pots filled with standard compost (Lentse Potgrond®) with no extra fertilizer

added. To standardize the biomass of the plants to about 25 g, a different number of plants per pot were grown for each species; eighteen for barley (3–4 wk old), nine for mustard (3–4 wk old), and one for Brussels sprout (6–7 wk old) and the feral *B. oleracea* (6–7 wk old). White mustard was tested, when the first flower buds had started to develop. Before testing, each plant was removed from the pot and below-ground plant parts were wrapped in aluminium foil. The plant was then used for behavioral assays or headspace sample collection.

Insects. Diadegma semiclausum (Hellén) (Hymenoptera, Ichneumonidae) was collected from Brussels sprout fields in a woodland area in the vicinity of Wageningen (The Netherlands) and was maintained on *Plutella xylostella* L. (Lepidoptera, Plutellidae) reared on Brussels sprout (8D: 16L photoperiod, 20 ± 2°C and 70% R.H). In the rearing cages, parasitized host larvae were allowed to pupate on paper strips, then transferred into a plastic cage with neither host nor plant material present. Wasps emerging from cocoons were provided *ad libitum* with water and honey. Mated females of 5–10 days of age with no oviposition experience were used in olfactometer bioassays. To obtain infested plants (mustard or Brussels sprout), 20 second or early third instar *P. xylostella* larvae were evenly distributed over a test plant 14–16 hr before the experiment.

Olfactometer Bioassay. To test behavioral responses of individual *D. semiclausum* females to plant odors, a glass Y-tube olfactometer (diam. 3.5 cm, length of stem section 22 cm) was used (for details see Takabayashi and Dicke, 1992). The two arms of the Y-tube were connected to glass vessels containing the odor source. The volume of the containers was five-liter in all comparisons except those involving the feral *B. oleracea*. As this plant had long petioles, we used 30 l containers to accommodate the plants. When an odor source was compared with clean air, a piece of cotton-wool humidified with water was placed into the empty container. While the 5 l containers were directly attached to the olfactometer, the two 30-l containers were attached with a silicon hose. Air was filtered over charcoal and led into each container at 4 l/min. The air was extracted at the base of the olfactometer at 8 l/min. The olfactometer was illuminated from above with high frequency fluorescent lights at an intensity of 30–35 μmol photons/m^2/sec. Wasps were individually tested in the olfactometer, and each wasp was used only once. In order to increase their motivation to search for hosts (Potting et al., 1999), females were transferred from the cage into the Y-tube on a piece of Brussels sprout leaf, damaged by the host but not containing the host itself or its products. The observation started by releasing the wasp at the base of the Y-tube, at 4 cm distance from the opening. Wasps were either walking or flying towards the odor source. A finish line was drawn 1 cm from the sieve at the end of each arm. A choice occurred when a wasp crossed the finish line and did not return to the junction for at least 15 sec. Wasps that did not make a choice within 10 min after release and wasps that did not reach the junction of the olfactometer within 5 min were considered non-responding individuals. Odor sources were replaced after testing

5–7 females, and at least 8 sets of new plants were used for each comparison. To control for possible asymmetries in the set-up, the odor source was moved from one arm of the olfactometer to the other after testing 3–4 females.

The attractiveness of the odor blends from the following uninfested plants were tested against clean air and each other. Brussels sprout, feral *B. oleracea*, mustard, and barley. Odor blends of host-infested Brussels sprout and mustard were compared to uninfested conspecifics, and uninfested mustard was compared to host-infested Brussels sprout. The tests were carried out from March–September 2003.

Collection and Analysis of Headspace Volatiles. Headspace volatiles were collected from all plant sources tested in Y-tube bioassays (except for the *Plutella*-infested mustard), and were analyzed by GC-MS. Four to five samples of each were taken in the period of June-August 2003. Plants prepared for sampling as described in section "*Plants*" were transferred into 30 l collection flasks. Pressurized air was filtered through silica gel, molecular sieves 4A and 13X (Linde), and activated charcoal before entering the flask. The air inlet, air outlet, filters, and sampling jar were connected with 0.8 cm diam. teflon tubing. After the plant was placed into the collection flask, the system was purged for 1 hr at an airflow rate of 500 ml/min to remove volatile contaminants. Subsequently, volatiles were collected in a glass tube containing 90 mg Tenax-TA (20/35 mesh) for 4–5 hr at a flow rate of 150–250 ml/min. Blanks were taken in duplicate from empty collection containers. The collected volatiles were released from the Tenax by heating the trap in a Thermodesorption Cold Trap Unit (Chrompack) at 250°C for 10 min and flushing with helium flowing at 12 ml/min. The released compounds were cryo-focused in a coldtrap (0.52 mm ID deactivated fused silica) at a temperature of −85°C. By ballistic heating of the cold trap to 220°C, the volatiles were transferred onto the analytical column (DB5, 60 m × 0.25 mm ID, 0.25-μm film thickness), which was connected to a Finnigan MAT 95 mass spectrometer. The temperature of the column oven was programmed from 40°C (4 min hold) to 250°C (4 min hold) at a rate of 4°C/min, and the initial helium velocity was 25 cm/sec. The mass spectrometer was operated in the 70 eV EI ionization mode and was scanning from mass 24 to 300 at 0.7 sec/decade. Compounds were identified by comparison of the mass spectra with those in the Wiley 7th/NIST98 library and in the Wageningen Mass Spectral Database of Natural Products and by checking the retention index. Emission rates were measured by quantifying peak areas. Compounds are presented as peak area per liter of trapped air per gram above-ground fresh weight.

Statistical Analysis. A binomial test was used to determine whether preferences of parasitoids were significantly different from a non-preference situation ($p = q = 0.5$, two-tailed, $\alpha = 0.05$). Non-responding wasps were excluded from the analysis. To illustrate the dissimilarities between the odor blends of plants, classification (hierarchical clustering) and ordination (non-metric scaling)

methods were used. Those compounds not present in the blank and detected in at least two replicate samples were included in the analysis. The detected quantities of individual compounds within a sample were considered as variables, and dissimilarities among the 24 plant samples were calculated based on the Horn-index (average link method) (see Krebs, 1989, eqn. 1). We chose this index because the calculated similarities among the volatile blends are supposedly little affected by the number of compounds included in the analysis (Krebs, 1989).

$$R_0 = \frac{\sum[(x_{ij} + x_{ik})\log(x_{ij} + x_{ik})] - \sum(x_{ij}\log x_{ij}) - \sum(x_{ik}\log x_{ik})}{[(N_j + N_k)\log(N_j + N_k)] - (N_j \log N_j) - (N_k \log N_k)} \quad (1)$$

Where R_0 is the Horn's index of similarity for samples j and k, x_{ij}, and x_{ik} are the detected amounts of compound i in sample j and sample k, where N_j is Σx_{ij} the total amount of volatiles in sample j and N_k is Σx_{ik} the total number of compounds in sample k. Analysis was performed by the Syntax 5.1 program package (Podani, 1997).

RESULTS

Olfactometer Bioassay. When volatiles from different plants were tested against clean humidified air, 60% to 95% of wasps made a choice. Wasps preferred volatiles from all plant sources over clean air ($P < 0.01$) (Figure 1).

When offered a choice between blends of uninfested plants, 61% to 88% of the tested females made a choice (Figure 2). Parasitoids discriminated clearly among volatiles from different species of uninfested plants. All cruciferous plants were preferred over malting barley ($P < 0.001$), with wasps discriminating between

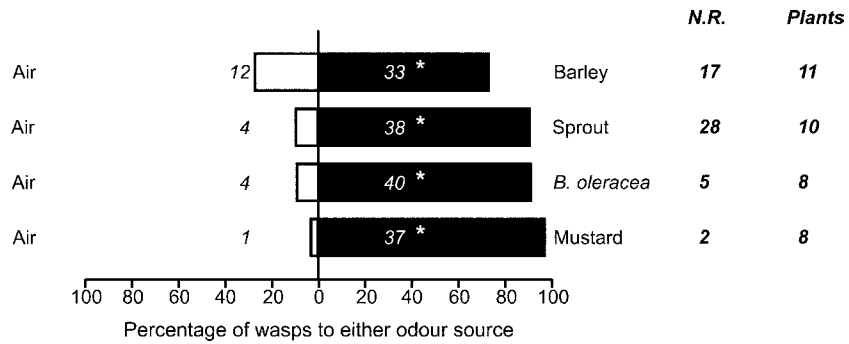

FIG. 1. Percentage of female *D. semiclausum* choosing either odor source when volatiles from uninfested plants are compared with clean air. Asterisks indicate significant preferences within tests (*$-P < 0.05$). Numbers next to graph are the number of non-responding (N.R.) individuals and the number of plants tested.

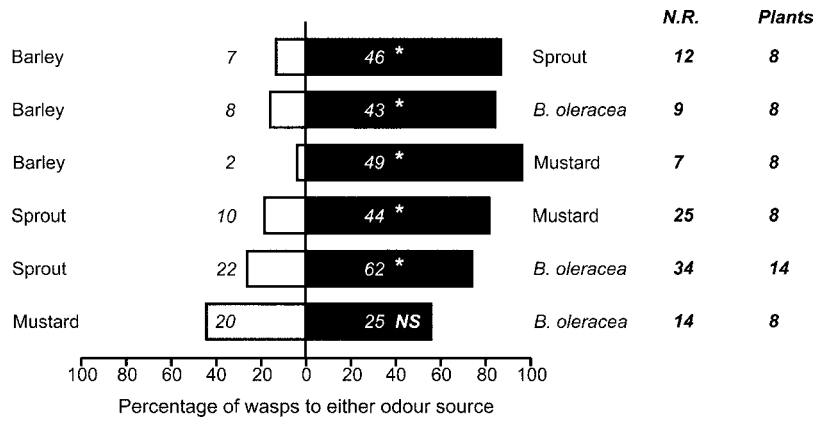

FIG. 2. Percentage of female *D. semiclausum* choosing either odor source from uninfested plants. Asterisks indicate significant preferences within tests ($^*-P < 0.05$). Numbers next to graph are the number of non-responding (N.R.) individuals and the number of plants tested.

the different cruciferous plants as well. Parasitoids preferred both mustard (82%) and the feral *B. oleracea* (74%) over Brussels sprout ($P < 0.001$) (Figure 2). The parasitoids were equally attracted to mustard and feral *B. oleracea*.

When parasitoids were exposed to volatiles from uninfested and host-infested cruciferous plants, 88% to 99% of individuals responded (Figure 3). Wasps discriminated between infested and uninfested conspecifics.

Parasitoids preferred both infested mustard (91%) and Brussels sprout (83%) to uninfested conspecifics. Interestingly, when females were offered a choice between host-infested Brussels sprout and uninfested mustard plants, no preference was observed.

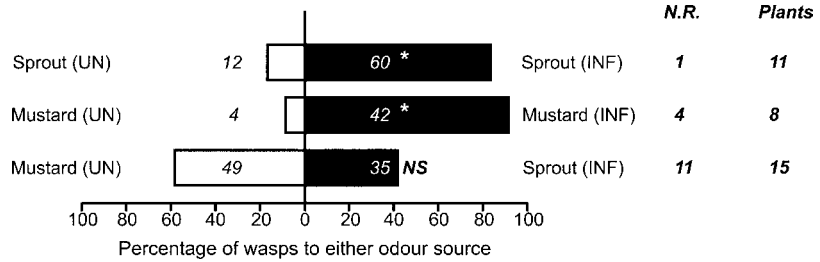

FIG. 3. Percentage of female *D. semiclausum* choosing either uninfested (UN) plants (Brussels sprout and mustard) or *P. xylostella*-infested plants (INF). Asterisks indicate significant preferences within tests ($^*-P < 0.05$). Numbers next to graph are the number of non-responding (N.R.) individuals and the number of plants tested.

Headspace Volatiles. In the 24 samples analyzed across treatments, 70 compounds were detected (Table 1). Compounds were identified as ketones, alcohols, aldehydes, esters, terpenoids, sulphides, nitrile, and others. In the headspace of barley, only 15 compounds were detected. Forty-six compounds were detected in the feral *B. oleracea* and in the mustard, 48 compounds were found in uninfested Brussels sprout and 59 in *Plutella*-infested Brussels sprout.

Characteristic GC traces of the plants tested are shown in Figure 4. Both quantitative and qualitative differences in headspace volatiles of tested plants were found. The smallest total amount of volatiles was emitted by barley, followed by mustard, feral *B. oleracea*, uninfested Brussels sprout, and host-infested Brussels sprout. Dissimilarities among the volatile profiles of different plants are presented in Figure 5. The volatile profile of barley showed the greatest dissimilarity among the treatments, with only 15 of the 70 compounds detected. Fourteen of these compounds were also present in the odor blend of at least one of the other plant sources. Compared to the crucifers, barley emitted few terpenoids. The dominant compounds produced by barley were the terpenoid linalool, which was also present in smaller amounts in the odor blend of infested Brussels sprout, 3-methyl-1-butanol, which was detected in smaller amounts both in the volatile blends of mustard and infested Brussels sprout, and the GLV (i.e., green leaf volatile) (*Z*)-3-hexen-1-ol, which was present in all samples.

The odor blend of the crucifers was dominated by the GLV (*Z*)-3-hexen-1-yl acetate, and the terpenoids sabinene, myrcene, limonene, and 1,8-cineole. The odor blend of mustard was more similar to the blends of the other crucifers than to that of barley, but it still grouped out from the blends of Brussels sprout and the feral *B. oleracea* (Figure 5). The 46 compounds detected in mustard greatly overlapped with those detected in the headspace of Brussels sprout and the feral *B. oleracea*. Four compounds, 2-methyl-1-butanol, 2-methyl-1-butyl acetate, 2-oxo-1,8-cineole, and germacrene D were detected in mustard alone. (*Z*)-3-hexen-1-yl 3-methylbutanoate and other compounds like 3-methyl-1-butanol, 3-methyl-1-butyl acetate, and indole were detected in mustard and infested Brussels sprout only. The compounds 3-methyl-1-butanol and 3-methyl-1-butyl acetate were also present in the odor blend of barley.

Infested Brussels sprout plants produced a similar odor blend to uninfested Brussels sprout and to a lesser extent to *B. oleracea*, but many of the compounds were emitted in considerably higher amounts by infested Brussels sprout. Four compounds, dehydroxylinalool oxide A, dehydroxylinalool oxide B, β-bisabolene, and cis-β-elemene were only present in the odor blend of feral *B. oleracea* (Table 1). (*E*,*E*)-α-Farnesene was found in high amounts in the feral *B. oleracea* and mustard only, whereas 3-methyl-2-pentanol was present in the volatile blend of the feral *B. oleracea* and that of infested Brussels sprout only.

The odor blends of infested and uninfested Brussels sprout plants showed the highest similarity. However, among the 59 compounds released by infested

TABLE 1. VOLATILE COMPOUNDS DETECTED IN THE HEADSPACE OF BARLEY, UNINFESTED MUSTARD (*S. ALBA*), BRUSSELS SPROUT, FERAL *B. OLERACEA* AND IN BRUSSELS SPROUT INFESTED BY LARVAE OF *P. XYLOSTELLA*[a]

Compound[b]	Barley ($N = 4$)	*S. alba* ($N = 5$)	*B. oleracea* ($N = 5$)	Brussels sprout ($N = 5$)	B. sprout +*Plutella* ($N = 5$)
Ketones					
1 3-pentanone$^{\Delta,\#,\circ}$	—	—	—	—	2.3 ± 1.6
2 2-hexanone	0.2 ± 0.2	0.1 ± 0.1	0.5 ± 0.2	1.5 ± 0.8	3.1 ± 1.7
3 1-cyclopropyl-2-propen-1-one$^{\circ}$	—	1.3 ± 0.5	0.9 ± 0.6	1.9 ± 1	10.7 ± 4.9
4 3-heptanone	—	1.3 ± 0.6	2.5 ± 0.4	3.3 ± 1.1	4.5 ± 1
5 2-heptanone	—	0.7 ± 0.2	1.1 ± 0.3	1.7 ± 0.4	1.9 ± 0.4
6 2-methyl-2-cyclopenlen-1-one (t)	1.7 ± 1	—	0.5 ± 0.5	0.8 ± 0.8	6.4 ± 4.8
7 3-octanone$^{\circ}$	—	—	2.6 ± 2.3	0.2 ± 0.2	2.4 ± 1.6
8 1,7-octadiene-3-one, 2-methyl-6-methylene	—	0.7 ± 0.5	2 ± 0.9	5.4 ± 2.7	8.6 ± 2.8
Alcohols					
9 1-penten-3-ol$^{\Delta,\square,\circ,\#}$	—	4.6 ± 0.9	1.2 ± 0.7	18.6 ± 11.4	117.5 ± 60.9
10 3-pentanol$^{\Delta,\square,\circ}$	—	0.3 ± 0.2	0.7 ± 0.2	2.6 ± 0.9	21.9 ± 11.5
11 3-methyl-3-buten-1-ol	—	—	—	—	0.7 ± 0.4
12 3-methyl-1-butanol$^{\circ}$	28.3 ± 6.2	6.5 ± 1.6	—	—	2.6 ± 0.8
13 2-methyl-1-butanol	—	1.1 ± 0.5	—	—	—
14 1-pentanol$^{\Delta,\square,\circ}$	0.4 ± 0.4	0.8 ± 0.1	1.3 ± 0.9	1.5 ± 0.7	8.2 ± 4
15 (*Z*)-2-penten-1-ol$^{\Delta,\square}$	—	—	0.2 ± 0.1	1.1 ± 0.7	12.5 ± 8.7
16 3-methyl-2-pentanol$^{\Delta,\square}$	—	—	1.3 ± 0.7	—	1 ± 0.8
17 (*Z*)-3-hexen-1-ol$^{\Delta,\square,\circ,\times,\#}$	17.5 ± 8.2	11.3 ± 2.8	9.5 ± 2.6	46 ± 22.5	358.8 ± 252.9
18 1-hexanol$^{\Delta,\square,\circ}$	1.6 ± 0.6	2.3 ± 0.4	3.9 ± 2.1	5.3 ± 2.5	28.3 ± 18.2
Esters					
19 3-methyl-1-butyl acetate$^{\circ}$	1.3 ± 0.6	6.5 ± 2.5	—	—	0.8 ± 0.4
20 2-methyl-1-butyl acetate	—	0.4 ± 0.2	—	—	—
21 (Z)-2-penten-1-yl acetate	—	1.7 ± 1	0.1 ± 0.1	24.4 ± 16.9	182.2 ± 112.3
22 pentyl acetate$^{\circ}$	—	1.3 ± 0.5	—	2.7 ± 1.7	17.6 ± 10.8

TABLE 1. CONTINUED

Compound[b]	Barley ($N = 4$)	*S. alba* ($N = 5$)	*B. oleracea* ($N = 5$)	Brussels sprout ($N = 5$)	B. sprout +*Plutella* ($N = 5$)
23 (*Z*)-3-hexen-l-yl acetate$^{\Delta,\circ,\times,\#,\blacktriangle,\bullet}$	—	231 ± 73.4	46.1 ± 17.4	381.7 ± 256.2	2175.8 ± l325.7
24 hexylacetate$^{\Delta,\circ,\#,\blacktriangle}$	—	11.5 ± 3.6	4.8 ± 1.9	18.2 ± 11.6	111.5 ± 76.8
25 (*Z*)-3-hexen-l yl-propanoate$^{\circ}$	—	—	—	—	2.2 ± 1.2
26 heptyl acetate$^{\circ}$	—	0.9 ± 0.5	0.3 ± 0.3	1.5 ± 1	4.7 ± 2.3
27 2-ethylhexyl acetate	5.5 ± 2.7	6.4 ± 1.5	11.3 ± 4.7	37.5 ± 10.5	33.9 ± 10.4
28 (*Z*)-3-hexen-l-ylbutanoate	—	0.2 ± 0.2	—	—	3.6 ± 2.8
29 methyl salicylate$^{\Delta}$	—	3.2 ± 1.8	0.9 ± 0.6	0.8 ± 0.6	1.5 ± 0.6
30 (*Z*)-3-hexen-l-yl 2-methylbutanoate	—	0.3 ± 0.2	—	0.4 ± 0.4	6.9 ± 3.7
31 (*Z*)-3-hexen-l-yl 3-methylbutanoate	—	3 ± 0.6	—	—	3.2 ± 2.3
Terpenoids					
32 α-thujene$^{\times,\Delta,\circ,\bullet}$	—	1.5 ± 0.8	27.9 ± 6.1	49.7 ± 17.8	83.8 ± 29
33 α-pinene$^{\Delta,\times,\#,\blacktriangle,\bullet}$	—	0.7 ± 0.3	6.1 ± 1.8	11.5 ± 2.3	19 ± 4
34 thuja-2,4,(10)-diene	—	—	1.4 ± 0.4	2.7 ± 0.4	4.4 ± 1.3
35 sabinene$^{\Delta,\circ,\times,\#,\blacktriangle,\bullet}$	—	31.2 ± 10.6	233.5 ± 45.1	345.7 ± 112.2	595 .9 ± 2 12.2
36 β-pinene$^{\Delta,\circ,\times,\#,\blacktriangle}$	—	2.4 ± 1	7.5 ± 1.5	15.1 ± 4.5	24.4 ± 7.2
37 myrcene$^{\Delta,\circ,\times,\#,\blacktriangle}$	8.8 ± 1.7	12.1 ± 5.4	59.9 ± 19.4	79.2 ± 36.7	150.4 ± 69.9
38 linalooloxide A, dehydroxy-	—	—	2.7 ± 1.8	—	—
39 linalooloxide B, dehydroxy-	—	—	3.5 ± 2.5	—	—
40 α-phellandrene	—	0.1 ± 0.1	—	0.7 ± 0.4	0.4 ± 0.3
41 α-terpinene$^{\Delta,\bullet}$	—	0.6 ± 0.6	0.6 ± 0.5	6.4 ± 2.7	8.9 ± 4.5
42 limonene$^{\Delta,\times,\#,\bullet}$	6.6 ± 1.3	18.9 ± 7.7	188.4 ± 49	257.2 ± 107.2	510.8 ± 209.5
43 β-phellandrene$^{\Delta,\times,\#}$	3 ± 1.2	1.2 ± 0.6	3.4 ± 1	4.9 ± 1.8	9.6 ± 3.6
44 l,8-cineole$^{\Delta,\circ,\times,\#,\blacktriangle}$	—	39.6 ± 14.6	41.2 ± 8.9	127.9 ± 40.6	255 ± 79.1
45 2-oxo-l,8-cineole	—	2.3 ± 1.6	—	—	—
46 (E)-β-ocimene$^{\times,\bullet}$	—	—	0.8 ± 0.5	1.4 ± 1.1	1.8 ± 0.8
47 γ-terpinene$^{\Delta,\circ,\bullet}$	—	0.6 ± 0.6	2.5 ± 0.6	7.5 ± 3.8	10.1 ± 4.2

TABLE 1. CONTINUED

48 trans-4-thujanol	—	0.2 ± 0.1	10.1 ± 3.2	14 ± 7.5	36 ± 15.5
49 α-terpinolene[•]	—	0.2 ± 0.1	1.4 ± 0.9	1.7 ± 0.8	2.5 ± 1.1
50 linalool oxide B	0.7 ± 0.7	—	0.9 ± 0.6	—	—
51 p-cymene[Δ]	—	0.1 ± 0.1	0.5 ± 0.2	0.9 ± 0.5	2.1 ± 1
52 linalool[Δ,□,×,#]	53 ± 9.1	—	—	—	3.9 ± 3.9
53 cis-4-thujanol	—	—	3.3 ± 2	3.4 ± 3.3	10.8 ± 4.8
54 (E)-3,4,8-dimethyl-1,3,7-nonatriene[Δ,,○,•]	—	1.9 ± 1.2	47.8 ± 27.1	1.4 ± 0.5	34.7 ± 17.8
55 terpinen-4-ol	—	—	—	0.6 ± 0.4	1.6 ± 0.8
56 α-terpineol	1.3 ± 0.7	3.2 ± 1.7	0.2 ± 0.2	0.4 ± 0.4	0.6 ± 0.4
57 cis-β-elemene,[Δ,×,#]	—	—	73.8 ± 57.5	—	—
58 germacrene D tent[Δ,•]	—	3.2 ± 1.2	—	—	—
59 (E,E)-α-famesene[○,×]	—	0.4 ± 0.4	41.6 ± 29.9	—	—
60 β-bisabolene	—	—	5.3 ± 4.6	—	—
61 carvone	—	—	—	1.1 ± 0.7	1.2 ± 1.2
Aldehydes					
62 (E)-2-hexanal[Δ,○,#,▲,•]	—	—	—	—	2.9 ± 2.1
Sulphides					
63 dimethyl trisulphide[Δ,□,○,×,#,▲]	—	0.5 ± 0.0	0.8 ± 0.3	1.2 ± 0.3	1.7 ± 0.5
Nitrogen containing					
64 benzyl cyanide[Δ]	—	—	—	0.8 ± 0.8	11.5 ± 5.1
65 indole[Δ,×]	—	1.7 ± 1.1	—	—	7.8 ± 4.7
Unknown[c]					
66 unknown[57b,68,82]	—	0.2 ± 0.2	—	2.5 ± 1.1	5 ± 1.9
67 unknown[81b,135,150]	7.9 ± 0.8	—	—	—	—
68 unknown[mtxt]	—	—	—	1.9 ± 0.8	1.6 ± 1.3
69 unknown[107,108b,150]	—	—	0.1 ± 0.1	1.3 ± 0.8	2.9 ± 1.3
70 unknown[79,107,150b]	—	—	—	2.9 ± 1.3	2.3 ± 1.1
Total amount	139.1 ± 23	420.1 ± 130	857.8 ± 255	1503.2 ± 647	4939.7 ± 2586

[a] Amounts of individual compounds are given as average peak area (±SE) per liter of trapped air per gram above ground biomass.
[b] References related to volatile analysis on crucifers. [Δ]-compound detected by Geervliet et al., 1997; [□]-compound detected by Agelopoulos et al., 1995; [○]-compound detected by Mattiacci et al., 1994; [×]-compound detected by Tollsten and Bergström, 1988; [#]-compound detected by Blaakmeer et al., 1994; [▲]-compound detected by Agelopoulos and Keller, 1994; [•]-compound detected by Shiojiri et al., 2001.
[c] characteristic mass peaks, b denotes the base peak.

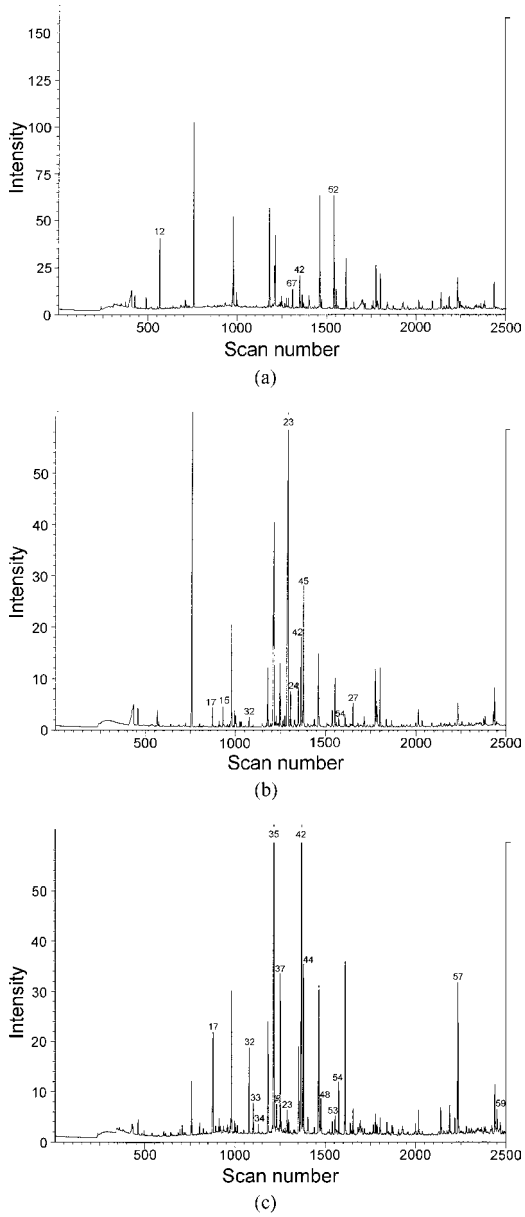

FIG. 4. Gas chromatograms of volatiles collected from (**a**) uninfested barley, (**b**) uninfested *S. alba*, (**c**) uninfested *B. oleracea*, (**d**) uninfested Brussels sprout, and (**e**) Brussels sprout infested by *P. xylostella*. Numbers next to peaks correspond to the compound numbers in Table 1.

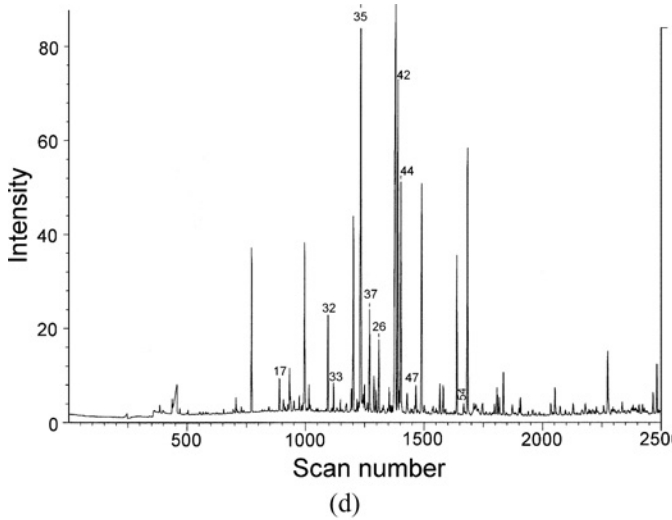

FIG. 4. Continued

Brussels sprout, 11 were not present in the odor blend of uninfested Brussels sprout (Table 1).

DISCUSSION

In this study, *D. semiclausum* preferred all plant species tested, including barley, over clean air. Yet, when uninfested plants of different species were tested

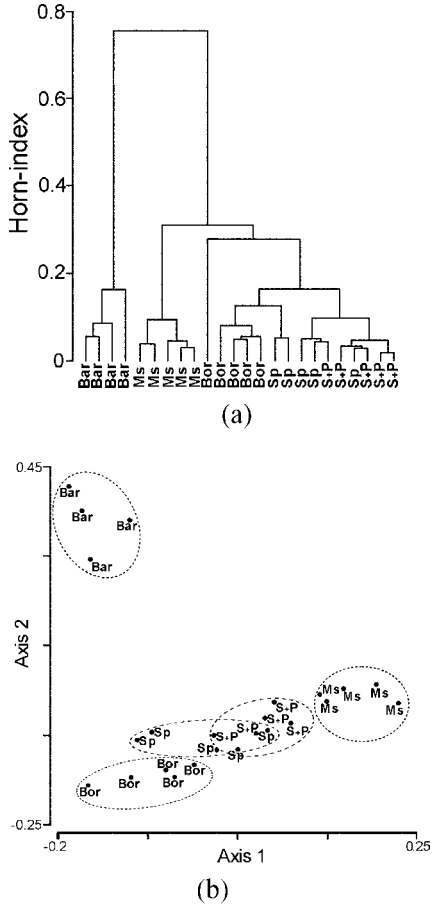

FIG. 5. Dissimilarities in volatile profiles of plants. (**a**) Hierarchic clustering and (**b**) non-metric scaling of dissimilarities in volatile profiles based on the Horn-index. "Bar"-barley, "Ms"-mustard, "Bor"-feral *B. oleracea*, "Sp"-uninfested Brussels sprout, "S+P"-Brussels sprout infested by *P. xylostella* larvae.

against each other, females preferred volatile blends of crucifers to barley. In a comparable study, *Cotesia kariyai* a parasitoid of the noctuid *Pseudaletia separata*, was attracted to several unrelated non-host food plants, which was explained by the presence of the same GLVs in the headspace of the different plant species (Takabayashi et al., 1991). Except for one compound, all the compounds produced by barley were also present in at least one of the crucifers. These included (*Z*)-3-hexen-1-ol, myrcene, and limonene. Linalool, the compound dominantly produced

by barley, was also detected in infested Brussels sprout. Therefore, the presence of those compounds in the odor blends of barley, which were present in the odor of crucifers, could explain the preference of wasps towards barley odors when compared with clean air.

Parasitoids also discriminated between odors of uninfested crucifers. Despite the lower amounts of total volatiles and the fewer compounds produced by both mustard and the feral *B. oleracea*, these plants were more attractive to *D. semiclausum* than Brussels sprout. Some alcohols and terpenoids emitted by mustard were present in this species only, and some alcohols, esters, and indole were detected both in mustard and infested Brussels sprout. The GLV (*Z*)-3-hexen-1-yl acetate is known to be produced by herbivore-infested Brussels sprout in greater quantities than by uninfested plants (Blaakmeer et al., 1994; Mattiacci et al., 1994; Geervliet et al., 1997). This was the dominant compound that mustard produced. However, this same compound was also the dominant compound in uninfested sprouts, and it was produced in larger quantities than in uninfested mustard. This finding indicates that (*Z*)-3-hexen-1-yl acetate alone was not responsible for the attraction of this parasitoid to mustard.

White mustard also contains specific aromatic hydroxy-benzyl and benzyl glucosinolates (McCloskey and Isman, 1993; Hopkins et al., 1998; Mewis et al., 2002). Aromatic derivatives of these glucosinolates were detected in the headspace of uninfested mustard (Tollsten and Bergström, 1988). We did not detect these derivatives in our samples, possibly due to differences in methodology for collecting and analyzing samples. Tollsten and Bergström (1988) used cut plants while we used potted plants.

Qualitative and quantitative differences in the odor blends of plants may enable natural enemies to discriminate between odor sources while searching for their prey or host (De Boer, 2004). *D. semiclausum* discriminated between odor blends of plants, and presence or absence of compounds in the blend combined with quantitative differences in blend composition could have played a role in this. While presence/absence of compounds in volatile blends could have been important for females to discriminate between odors of barley and crucifers, differences in quantity and ratios of compounds could be important in the discrimination between mustard, the feral *B. oleracea*, and Brussels sprout. This was suggested by the finding that the volatile profile of the feral *B. oleracea* was similar to that of uninfested Brussels sprout, with a few compounds detected exclusively in the feral *B. oleracea*. Yet, wasps were able to discriminate between these lines of *B. oleracea*. Furthermore, the greater amounts of plant volatiles produced by uninfested Brussels sprout compared to the feral *B. oleracea* combined with few qualitative differences, did not result in a stronger attraction of *D. semiclausum*. This indicates that subtle differences in volatile profiles of uninfested plants could be important in the attractivity of these plants for *D. semiclausum*.

Brussels sprout plants damaged by hosts are more attractive for the parasitoids *Cotesia glomerata*, *C. rubecula*, and *C. plutellae* than artificially damaged or intact Brussels sprout plants (Steinberg et al., 1992, 1993; Blaakmeer et al., 1994; Geervliet et al., 1996; Shiojiri et al., 2000). A recent study by Ohara et al. (2003) showed that *D. semiclausum* females were attracted by the odor blends of uninfested and infested cruciferous plants. The observed attraction was not due to the host or products directly associated with the host (i.e., feces, silk exuviae), but the damaged plant itself (Ohara et al., 2003). Our results also suggest that *D. semiclausum* assesses changes in volatile blends as a result of herbivore damage. The odor blend of infested Brussels sprout was similar to that of uninfested Brussles sprout, but many of the compounds were emitted in higher amounts by infested plants, which was in line with findings of other studies (Blaakmeer et al., 1994; Geervliet et al., 1997; Reddy and Guerrero, 2000; Smid et al., 2002). Compounds like (*Z*)-3-hexen-1-yl acetate, (*Z*)-3-hexen-1-ol, and limonene, which were also detected in our samples, could play a role in the attraction of the parasitoids *C. rubecula*, *C. glomerata*, and *C. plutellae*, and of the predatory lacewing *Chrysoperla carnea* (Geervliet et al., 1997; Reddy et al., 2002; Smid et al., 2002). The volatile profile of *Plutella*-infested Brussels sprout shares many similarities with the odor profiles from white cabbage and Brussels sprout infested by different *Pieris* spp. (Geervliet et al., 1997; Blaakmeer et al., 1994). Moreover, parasitoid species associated with *P. xylostella* and *Pieris spp.* differ in their ability to distinguish odor blends of plants that are infested by hosts or non-hosts (Shiojiri et al., 2001). It is not yet known what compounds may be involved in the ability of *D. semiclausum* to discriminate between blends of infested and uninfested Brussels sprout, or how the volatile composition of mustard is influenced by herbivore damage. These may be important factors to understand its searching behavior, and interactions with its host in the field. We currently study how inter- and intraspecific variation in plant volatiles influence the ability of this parasitoid to discriminate between plants infested by hosts and non-hosts.

Based on dissimilarities in the volatile profiles, wasps were able to discriminate between infested and uninfested Brussels sprout plants. Interestingly, such discrimination was not observed when infested Brussels sprout was compared with mustard, although the dissimilarity in odor blends compared to the dissimilarity of blends of infested versus uninfested Brussels sprout plants were greater. Hence, the lack of discrimination by *D. semiclausum* between infested Brussels sprout and mustard was not because of the great similarity of odor blends. Despite the considerable differences in blends, wasps did not discriminate, possibly because the "values" of information (i.e., expectancy of the host's presence) from the odors of infested Brussels sprout and mustard were similar. Based on studies of *Leptopilina heterotoma*, a parasitoid of *Drosophila* spp., Vet et al. (1998) hypothesized that parasitoids may actively not discriminate among subtle, quantitative

differences and rely on qualitative differences until they learn to discriminate via experiences. Data from a separate study suggest that such a mechanism may play a role in the searching behavior of *D. semiclausum* (Bukovinszky, 2004). While host location by inexperienced females on Brussels sprout is hindered by the greater preference of wasps to search on uninfested mustard, oviposition experience redirects odor preference in favor of host-infested Brussels sprout and enhances the efficiency of females to locate subsequent host-infested plants, irrespective of neighboring plant species (Bukovinszky, 2004).

Relative to other herbivores that are specialists on crucifers, *P. xylostella* has a broad host plant diet (Yano, 1994; Bigger and Fox, 1997). Mustard is a preferred food plant of *P. xylostella* and is used as a trap crop (Palaniswamy et al., 1986; Talekar and Shelton, 1993). *P. xylostella* develops faster and reaches greater body weight on white mustard than on Brussels sprout (R. Gols, unpublished data). Hence, if the occurence of *P. xylostella* on these plant species is different, it could be a viable strategy for *D. semiclausum* to have a preference towards volatile blends that reflects the food-plant preference of the host.

As plant breeding and biological control developed independently, we have limited information on what mechanisms are responsible for triggering different responses of parasitoids to different plant species and genotypes of the same plant species (van Lenteren et al., 1995; Bottrell et al., 1998; Cortesero et al., 2000). The finding that feral *B. oleracea* is more attractive than cultivated conspecifics might indicate that artificial selection in cultivated plants has altered their ability to attract natural enemies compared with their wild relatives. However, further studies are needed to explicitly test this hypothesis. Our results indicate that volatile traits responsible for the attraction of natural enemies to plants would be valuable to consider in breeding programs enhancing biological control. Although plant traits that increase attractivity for the parasitoid may also attract herbivores, such traits could still be valuable tools in developing pest-suppressive diversification strategies (i.e., companion planting, intercropping).

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FURTHER FIELD EVALUATION OF SYNTHETIC HERBIVORE-INDUCED PLANT VOLATILES AS ATTRACTANTS FOR BENEFICIAL INSECTS

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Abstract—Fifteen synthetic herbivore-induced plant volatiles (HIPVs) were field-tested for attractivity to beneficial insects in two experiments conducted in an open field and a hop yard in Washington State. Eleven insect species or families showed significant attraction to 13 HIPVs. The ladybeetle, *Stethorus punctum picipes*, was attracted to sticky traps baited with methyl salicylate (MeSA), *cis*-3-hexen-1-ol (He), and benzaldehyde (Be). The minute pirate bug, *Orius tristicolor*, was attracted to traps baited with MeSA, He, Be, and octyl aldehyde (Oa), and the bigeyed bug, *Geocoris pallens*, responded to MeSA, indole, and *trans*-2-hexen-1-al. The mymarid wasp, *Anagrus daanei*, was attracted to He, Oa, and farnesene. The chloropid fly, *Thaumatomyia glabra*, was highly attracted to methyl anthranilate. Insect families responding to HIPVs included Syrphidae (MeSA, He), Braconidae ((*Z*)-3-hexenyl acetate, He, *cis*-jasmone (J), methyl jasmonate (MeJA), methyl anthranilate (MeA)), Empididae (MeSA), Sarcophagidae (MeSA, Be, J, nonanal and geraniol), Tachinidae (Be), and Agromyzidae (MeSA). Micro-Hymenoptera (primarily parasitic wasp families) were attracted to MeSA, He, and indole. These results are discussed with respect to known properties and bioactivity of the tested HIPVs and to their potential as tools for recruiting natural enemies into agroecosystems.

Key Words—Herbivore-induced plant volatiles, attractants, natural enemies, conservation biological control.

INTRODUCTION

Plants respond to herbivore damage by producing volatiles that attract natural enemies of the herbivores responsible for the damage (Dicke et al., 1990a; Turlings

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et al., 1990; Vet and Dicke, 1992; Stowe et al., 1995; Takabayashi and Dicke, 1996). Despite the accumulation of a substantial volume of literature on the biology and chemistry of herbivore-induced plant volatiles (HIPVs) during the past 10–15 years (e.g., Dicke et al., 1990b, 1998; Whitman and Nordlund, 1994; Pare et al., 1999; Sabelis et al., 1999; Thaler, 1999, Kessler and Baldwin, 2001; Scutareanu et al., 2003, Van Den Boom et al., 2004, Engelberth et al., 2004), field demonstration of the potential of synthetic HIPVs as beneficial insect attractants did not occur until recently (James, 2003a,b). These studies showed that sticky traps baited with synthetic versions of two HIPVs (methyl salicylate (MeSA), (*Z*)-3-hexenyl acetate) attracted significantly greater numbers of some predatory insect species in the families Chrysopidae, Miridae, Geocoridae, Anthocoridae, Syrphidae, and Coccinellidae. Subsequently, controlled-release dispensers of methyl salicylate were evaluated in hops and grapes in Washington and showed evidence of recruiting increased numbers of specific beneficial insects to treated sites (James and Price, 2004).

Methyl salicylate and (*Z*)-3-hexenyl acetate are only two of many HIPVs that have been identified to date (Whitman and Nordlund, 1994; Pare and Tumlinson, 1996; Dicke, 1999). Many HIPVs are available from commercial sources and suitable for field-testing as attractants for beneficial insects. Following the success in field-testing MeSA and (*Z*)-3-hexenyl acetate as attractants (James, 2003a,b) and subsequent demonstration of MeSA as a tool to recruit populations of beneficial insects to baited crops and perhaps improve biological control of pests (James and Price, 2004), thirteen additional synthetic HIPVs were field-tested in two experiments conducted in 2003.

METHODS AND MATERIALS

Two field experiments based on the protocols used in James (2003b) were conducted at Washington State University's, Irrigated Agriculture Research and Extension Center at Prosser in 2003.

Experiment 1. This was conducted in a 1 ha open field from 30 April-3 September 2003. Yellow sticky cards (23 × 18 cm, Trece Incorporated, Salinas, CA) were stapled to 25 × 25 cm wooden platforms attached by wire to wooden poles ~2 m above the ground. Cards were baited with 2 ml glass vials containing 1 ml of candidate HIPV solution or left unbaited (controls). Vials were loosely plugged with cotton wool and suspended by wire at a height of ~2 cm above the center of each card. Eleven HIPVs were tested (*cis*-3-hexen-1-ol (98%), *trans*-2-hexen-1-al (97%), linalool (97%), benzaldehyde (99.5%), indole (99%), methyl salicylate (98%), (*Z*)-3-hexenyl acetate (98%), geraniol (98%), methyl anthranilate (commercial formulation, Goose Chase™ 26.4%), *cis*-jasmone (90%), and methyl jasmonate (95%). All were obtained from Sigma-Aldrich, St Louis, MO, USA except methyl anthranilate, which was a gift from Leonard Askham, Bug

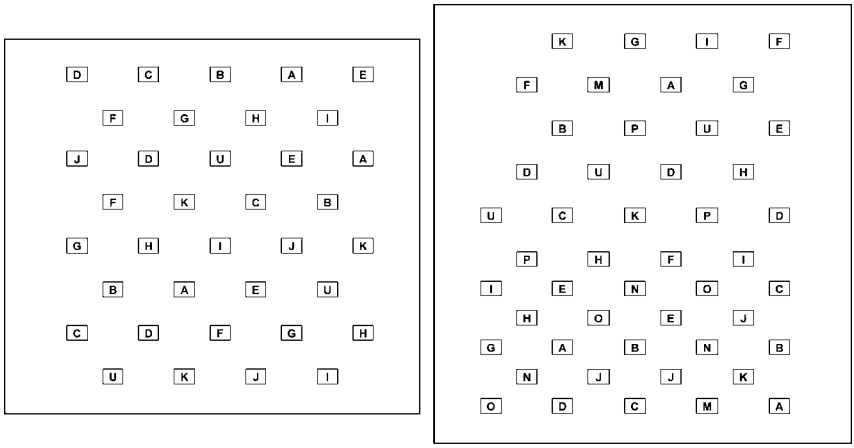

FIG. 1. Plot diagrams for Experiment 1 (A) and Experiment 2 (B) showing location details for traps and volatiles.). A – *cis*-3-hexen-1-ol, B – *trans*-2-hexen-1-al, C – linalool, D – benzaldehyde, E – indole, F- methyl salicylate, G – (*Z*)-3-hexenyl actetate, H – geraniol, I – methyl anthranilate, J – *cis*-jasmone, K – methyl jasmonate, M – nonanal, N – farnesene, O- - 3 – octanone, P – octyl aldehyde, U – unbaited.

Busters Ltd., Spokane, WA. All HIPVs were used undiluted (except indole, which was diluted 1:1 with hexenal) and solutions were replaced weekly. The cards were placed in a 6 × 6 grid with 15 m between the randomized treatments with three replicates of each treatment (see Figure 1 for details of trap/volatile locations). The field was cultivated and devoid of vegetation until June; thereafter it became weedy with Kochia (*Kochia scorparia* L) and Redroot pigweed (*Amaranthus retroflexus* L) dominating. Unsprayed vineyards bordered the field on two sides, alfalfa on one side and weedy waste ground on the other. Insects were abundant in these surrounding areas. Sticky cards were collected/replaced weekly and examined under a stereomicroscope. Trapped insects were identified (to species or family) and counted.

Experiment 2. This was conducted in a drip-irrigated, post-harvest hop yard at Washington State University-Prosser during 4 September-22 October 2003. The yard was bordered on three sides by weedy fields and on the fourth by a vineyard. Insects were abundant in the yard and the surrounding areas. Yellow sticky cards were tied vertically to wooden poles ∼2 m above the ground and baited with glass vials containing 1 ml of candidate HIPV solutions or left unbaited (controls). Fifteen HIPVs were tested; the eleven used in Experiment 1 plus nonanal (95%), farnesene (98%), 3-octanone (97%), and octyl aldehyde (99%). All were obtained from Sigma-Aldrich except methyl anthranilate (see Experiment 1). All HIPVs

were used undiluted (except indole which was diluted 1:1 with hexenal), and solutions were replaced weekly. Bait vials were taped vertically to the lower edge of the cards, which were placed in a 6 × 9 grid with at least 15 m between the randomized treatments (see Figure 1 for details of trap/volatile locations). All treatments were replicated three times. The hop yard was used for insecticide trials prior to the HIPV experiment, and large numbers of arthropod herbivores (primarily mites and aphids) were present in September. Weeds were also abundant in the yard. Sticky cards were collected/replaced weekly and examined under a stereomicroscope. Trapped insects were identified and counted.

Sixteen groups, families, or species of insects were sufficiently numerous to allow an assessment of attraction to HIPVs in one or both of the experiments. Abundance was judged by occurrence on unbaited sticky cards. Insects that were trapped regularly throughout the trapping period were recorded in this study. These were *Geocoris pallens* Stal. (Hemiptera: Geocoridae), *Orius tristicolor* (White) (Hemiptera: Anthocoridae), *Lygus hesperus* Knight (Hemiptera: Miridae), *Stethorus punctum picipes* (Casey) (Coleoptera: Coccinellidae), *Anagrus daanei* Triapitsyn (Hymenoptera: Mymaridae), *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), *Thaumatomyia glabra* (Meigen) (Diptera: Chloropidae), Syrphidae, Braconidae, Empididae, Sarcophagidae, Tachinidae, Agromyzidae, Therevidae, Dolichopodidae, and micro-Hymenoptera [parasitic families including Scelionidae, Encyrtidae, and Mymaridae (excluding *Anagrus* spp.)]. *Lygus hesperus* and *F. occidentalis* can be pestiferous, but are also predatory on aphids and mites. Agromyzidae (leaf-mining flies) are pests of many vegetable and flower crops. Voucher specimens of the species mentioned in this work are held at Washington State University-Prosser. Trapping data were analyzed using either the Mann-Whitney Rank-Sum Test or the Kruskal-Wallis ANOVA on ranks.

RESULTS

Experiment 1. Three insect species, three insect families, and micro-Hymenoptera were attracted to some HIPVs (Figures 2–3). The minute pirate bug, *O. tristicolor* was attracted to traps baited with MeSA or *cis*-3-hexen-1-ol, while the bigeyed bug, *G. pallens* was attracted to MeSA, *trans*-2-hexen-1-al, and indole (Figure 2). The chloropid fly, *T. glabria*, was highly attracted to methyl anthranilate with a season mean (±SE) of 4450 ± 645/trap compared to 1.2 ± 0.2/trap on unbaited traps. No other HIPV trapped a season mean of more than 1.4 *T. glabra* per trap. Syrphidae (hover flies), Empididae (dance flies), and Agromyzidae (leaf-mining flies) were attracted to MeSA-baited traps, and syrphids also responded to *cis*-3-hexen-1-ol (Figures 2–3). Micro-Hymenoptera were attracted to MeSA, indole and *cis*-3-hexen-1-ol-baited traps (Figure 3).

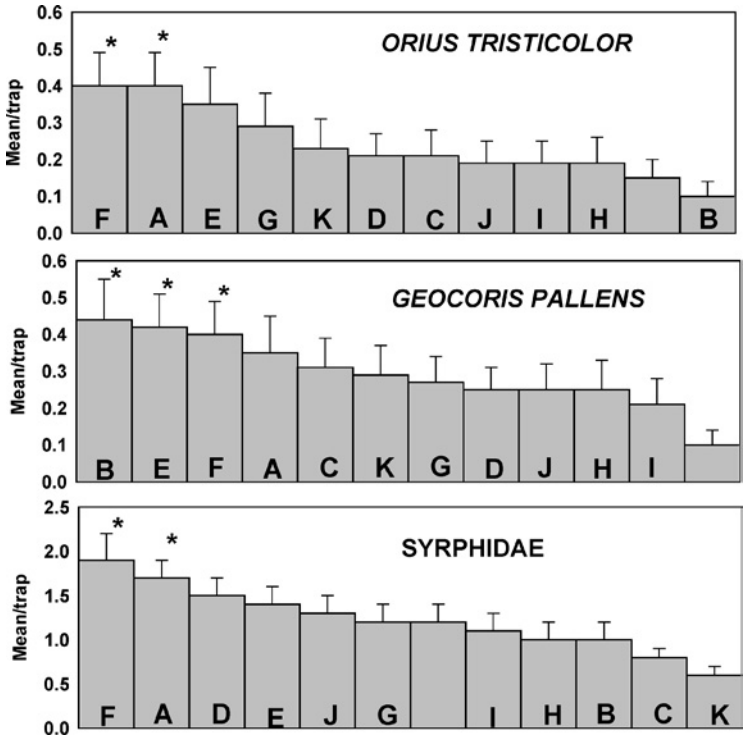

FIG. 2. Mean (±SE) captures of *O. tristicolor*, *G. pallens*, and Syrphidae in sticky traps baited with synthetic HIPVs during April-September 2003 (experiment 1). Unmarked columns represent unbaited (control) traps and asterisks indicate significant differences from unbaited traps ($P < 0.05$). See Figure 1 for HIPV identification.

Experiment 2. Three insect species and three insect families were attracted to some HIPVs in this experiment (Figures 4–5). The mymarid wasp, *A. daanei* was strongly attracted to traps baited with farnesene, octyl adehyde, and *cis*-3-hexen-1-ol (Figure 4). *Orius tristicolor* and *S. punctum picipes* were attracted to some HIPVs during the first 2–3 weeks of September (Figure 4), but not in late September and October. *Orius tristicolor* was attracted to traps baited with octyl aldehyde or benzaldehyde, while *S. punctum picipes* responded to traps baited with MeSA, *cis*-3-hexen-1-ol, or benzaldehyde (Figure 4). Wasps in the family Braconidae were attracted to traps baited with (*Z*)-3-hexenyl acetate, geraniol, methyl anthranilate, methyl jasmonate, *cis*-jasmone, and *cis*-3-hexen-1-ol (Figure 5). Flesh flies (Sarcophagidae) were attracted to benzaldehyde, nonanal, geraniol, and *cis*-jasmone baited traps, while tachinid flies were attracted to benzaldehyde (Figure 5).

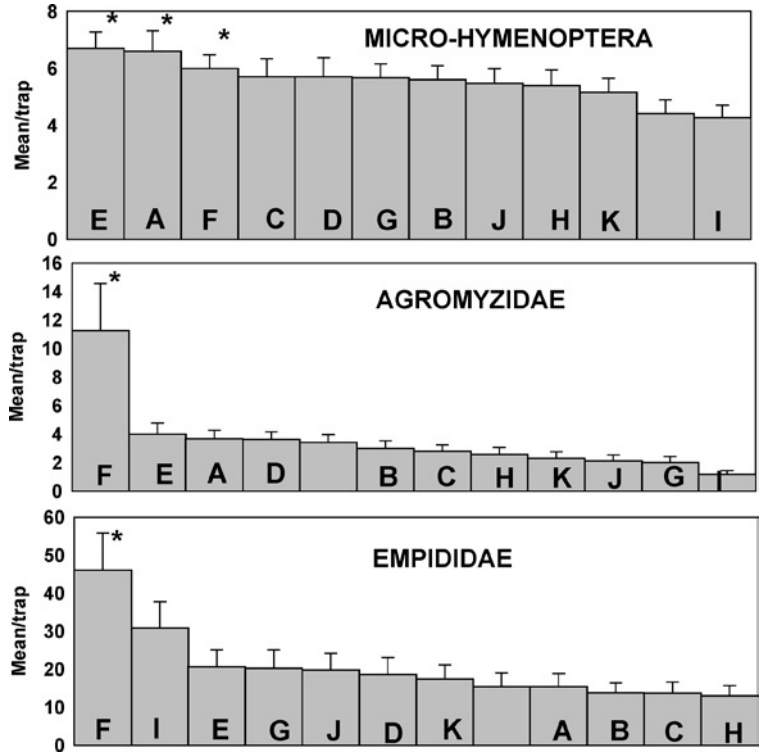

FIG. 3. Mean (±SE) captures of Empididae, Agromyzidae, and micro-Hymenoptera in sticky traps baited with synthetic HIPVs during April-September 2003 (experiment 1). Unmarked columns represent unbaited (control) traps and asterisks indicate significant differences from unbaited traps ($P < 0.05$). See Figure 1 for HIPV identification.

DISCUSSION

The data presented here together with results from previous studies (James, 2003a,b) indicate that synthetic HIPVs have potential as attractants for beneficial insects. Simple field attraction experiments formed the basis of these studies, and indicate that eleven HIPVs in addition to methyl salicylate and (*Z*)-3-hexenyl actetate are attractive to beneficial insects (James, 2003a,b). A summary of these compounds and the insects they attract in the field is presented in Table 1. Western flower thrips, *F. occidentalis*, Lygus bug, *L. hesperus*, long legged flies (Dolichopodidae), and stiletto flies (Therevidae), despite good abundance during both experiments, were not attracted to any HIPV (data not presented).

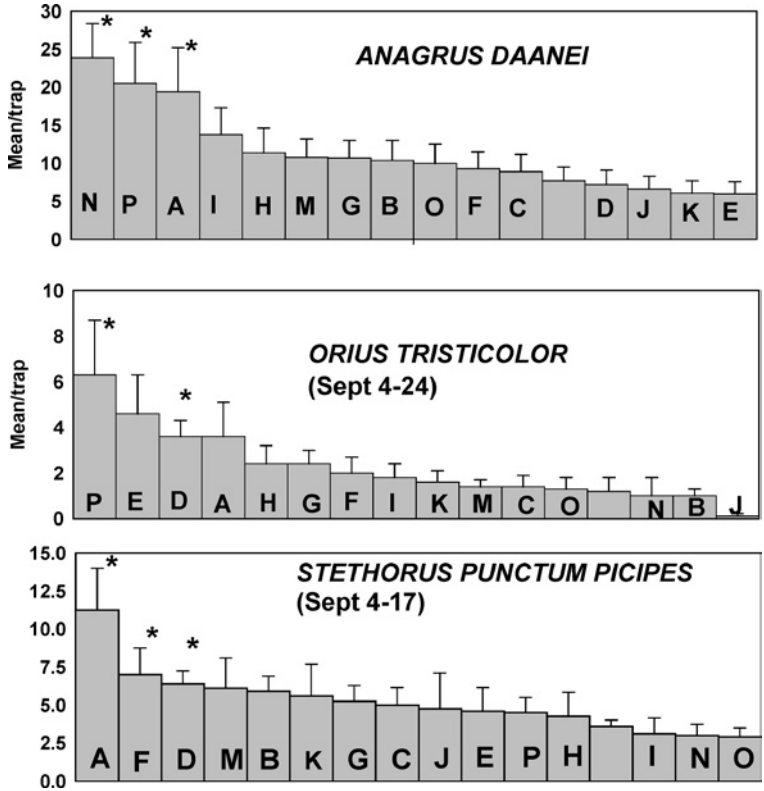

FIG. 4. Mean (±SE) captures of *A. daanei*, *O. tristicolor*, and *S. punctum picipes* in sticky traps baited with synthetic HIPVs during September-October 2003 (experiment 2). Unmarked columns represent unbaited (control) traps and asterisks indicate significant differences from unbaited traps ($P < 0.05$). See Figure 1 for HIPV identification identification.

Green leaf volatiles (GLVs) are typically released by plants immediately after wounding or herbivore attack and are ubiquitous in the environment. Nevertheless, they appear to serve as signals to some natural enemies including braconid and ichneumonid wasps (Whitman and Eller, 1990, 1992). In this study, the GLV, *cis*-3-hexen-1-ol was attractive to *O. tristicolor*, *S. punctum picipes*, *A. daanei*, braconid wasps, and micro-Hymenoptera, while *trans*-2-hexen-1-al was attractive to *G. pallens*. Another GLV, (*Z*)-3-hexenyl acetate, was shown earlier to be attractive to *O. tristicolor* and the predatory mirid, *Deraeocoris brevis* (James, 2003b). Recently, a priming role for GLVs was described in which chemical defense

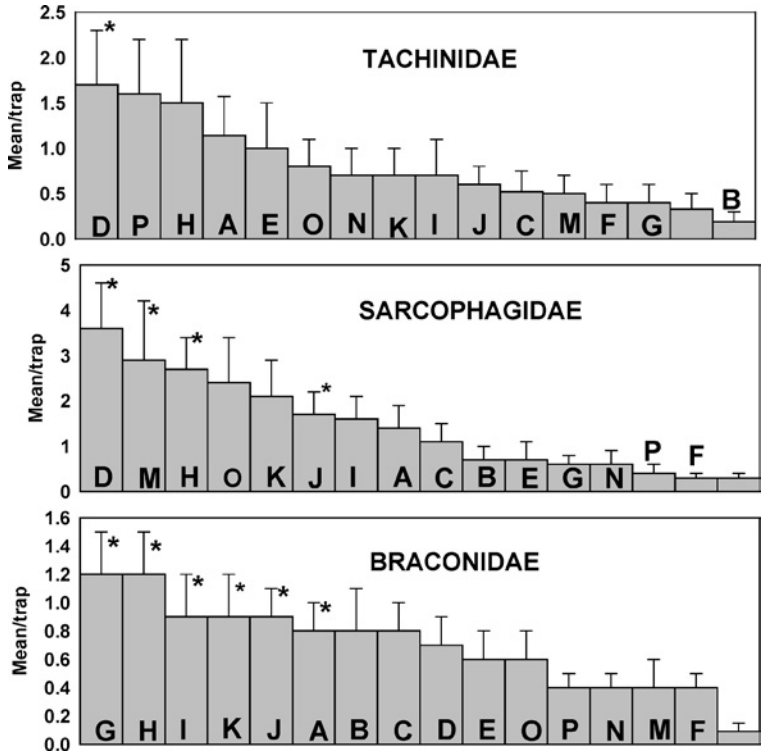

FIG. 5. Mean (±SE) captures of Tachinidae, Sarcophagidae, and Braconidae in sticky traps baited with synthetic HIPVs during September-October 2003 (experiment 2). Unmarked columns represent unbaited (control) traps and asterisks indicate significant differences from unbaited traps ($P < 0.05$). See Figure 1 for HIPV identification.

responses in plants near herbivore-damaged plants were enhanced by exposure to these volatiles (Engleberth et al., 2004).

Benzaldehyde is a common plant volatile associated with flowers and ripening fruit (Buchbauer et al., 1993; Leskey et al., 2001; Plepys et al., 2002). However, it is also an induced volatile in tea plants damaged by aphids (Han and Chen, 2002). Attractiveness of benzaldehyde to three natural enemy species (*Coccinella septempunctata* L. (Coleoptera: Coccinellidae), *Aphidius* sp. (Hymenoptera: Braconidae), and *Chrysopa sinica* Tjeder (Neuroptera: Chrysopidae) was demonstrated in electroantennogram and olfactometer tests (Han and Chen, 2002). In the current field study, benzaldehyde was attractive to *O. tristicolor*, *S. punctum picipes*, and flies in the families Tachinidae and Sarcophagidae. Han and Chen (2002) also showed geraniol and indole were HIPVs in aphid-damaged tea plants. Both compounds

TABLE 1. SUMMARY OF POSTIVE RESPONSES BY INSECTS TO SYNTHETIC HIPVS IN FIELD EXPERIMENTS CONDUCTED IN WASHINGTON STATE 2002–2003

Insect species, genus, family or group	Synthetic HIPV												
	A	B	G	F	E	I	J	K	H	M	P	D	N
Chrysopa nigricornis				√a,c									
Stethorus p. picipes	√		√	√b,c								√	
Orius tristicolor	√		√b	√c							√	√	
Geocoris pallens		√		√b,c	√								
Deraeocoris brevis			√b	√c									
Anagrus daanei	√										√		√
Thaumatomyia glabra						√							
Hemerobius sp				√c									
Syrphidae	√			√a,c									
Braconidae	√		√	√c		√	√	√					
Empididae				√c									
Sarcophagidae				√c			√		√	√		√	
Tachinidae												√	
Agromyzidae				√									
Micro-Hymenoptera	√			√c	√								

Note. A *-cis*-3-hexen-1-ol, B *-trans*-2-hexen-1-al, G -(Z)-3-hexenyl actetate, F-methyl salicylate, E-indole, I-methyl anthranilate, J-*cis*-jasmone, K-methyl jasmonate, H-geraniol, M-nonanal, P-octyl aldehyde, D-benzaldehyde, N-farnesene.
a = reported in James 2003a; b = James 2003b, c = James and Price, 2004.
Absence of positive data for an insect species or family does not necessarily mean no response. It may not have been present during one or more of the 4 studies.

also attracted beneficial insects in the current field study. Indole attracted micro-Hymenoptera and *G. pallens*, while geraniol attracted sarcophagid flies and braconid wasps. Indole is a component of the HIPV blend produced by caterpillar-damaged cotton and cabbage plants, utilized by hymenopteran parasitoids to locate their caterpillar hosts (Pare and Tumlinson, 1997; Dicke, 1999; Fukushima et al., 2002).

Farnesene is a common component of HIPV blends from a number of plants including cotton, corn, bean, pear, and apple (Du et al., 1988; Turlings et al., 1991; Boeve et al., 1996; Pare and Tumlinson, 1997; Scutareanu et al., 1997). It has also been shown in the laboratory to be attractive to parasitic wasps including *Aphidius ervi* (Haliday) (Hymenoptera: Aphididae) (Du et al., 1998), a ladybeetle, *Coleomegilla maculata* (Degeer) (Coleoptera: Coccinellidae) (Zhu et al., 1999), a lacewing, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) (Zhu et al., 1999), and a pirate bug, *Anthocoris nemoralis* (Fabricius) (Hemiptera: Anthocoridae) (Scutareanu et al., 1997). In the current field study, the parasitic mymarid wasp, *Anagrus daanei*, was the only insect attracted to farnesene. This wasp along with other *Anagrus* spp. is an important egg parasitoid of grape leafhoppers (*Erythroneura* spp.) in Washington State and California.

In a study by Birkett el al. (2003), 3-octanone and octanal (octyl aldehyde) were shown to be volatiles emitted by bean leaves. Substantially greater amounts of 3-octanone were emitted by whitefly-infested than by clean leaves, indicating that it is an HIPV. Octanal was also more common from whitefly-infested leaves but to a lesser extent. Octanal was reported as a minor component of the HIPV blend released by cabbage infested with caterpillars of pierid butterflies (Dicke, 1999). No response by insects to 3-octanone was detected in the field experiments, but two species, *A. daanei* and *O. tristicolor*, showed significant attraction to octanal. This is the first report of beneficial insects responding to this compound. Nonanal was also shown by Birkett et al. (2003) and Dicke (1999) to be an HIPV from whitefly-infested bean leaves and caterpillar-infested cabbages, respectively. In these field experiments, it attracted sarcophagid flies. De Greet and Poland (2003) showed nonanal was attractive to the herbivorous beetle, *Hylastes opacus* Erichson (Coleoptera: Scolytidae).

The two jasmonates, *cis*-jasmone and methyl jasmonate (MeJA), play a role in plant-plant communication. Methyl jasmonate is released by some herbivore-attacked plants and is a signal to neighboring plants that respond by producing predator-attracting volatiles (Bruin and Dicke, 2001). A direct role as attractants for predators and parasitoids of herbivores has not been well described for MeJA and *cis*-jasmone. Park et al. (2001) showed that the parasitic wasp, *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae) responded to MeJA in electroantennogram studies. Braconid wasps showed some attraction to *cis*-jasmone and MeJA-baited traps in this study, and sarcophagid flies responded to *cis*-jasmone.

Methyl anthranilate is a common secondary plant metabolite occurring in flowers (Knudsen et al., 1993) and in fruits of *Vitis labrusca* L. (Stern et al., 1967). It is currently marketed in the United States as a non-lethal bird repellent (Aronov and Clark, 1996). Kairomonal activity of methyl anthranilate was observed in the flowers of *Cimicifuga simplex* for butterflies (Pellmyr, 1986), and Murai et al. (2000) showed it is an attractant for two herbivorous thrips species and a thrips parasitoid, *Ceranisus menes* Walker (Hymenoptera: Eulophidae). Tscharntke et al. (2001) reported methyl anthranilate as a component of the HIPV blend produced by black alders attacked by psyllid insects, *Psylla alni* L. (Homoptera: Psyllidae). In the current field tests, methyl anthranilate was attractive to the predaceous frit fly, *T. glabra*, which was attracted by the hundreds, often within minutes of setting out the vial. A high degree of attraction of this species to methyl anthranilate was first reported by Landolt et al. (2000). *T. glabra* is widely distributed in western North America and considered beneficial because of its predation as larvae on root-feeding aphids (Summers and Newton, 1989). The only other insects to respond to methyl anthranilate were braconid wasps.

Attraction of *S. punctum picipes*, *G. pallens*, *O. tristicolor*, and syrphids to MeSA-baited traps was reported in earlier field-trapping studies (James, 2003b)

and also in a subsequent experiment evaluating controlled-release dispensers of MeSA for recruitment of predators to vineyard plots (James and Price, 2004). Attraction of these beneficial insects to MeSA here provides further evidence for the potency of this compound. More evidence for syrphid attraction to MeSA was also obtained in a short-term trapping experiment conducted in November 2003 in rose bushes with unusually high populations of flies present. Methyl salicylate-baited traps captured a mean of 43 $\pm$ 8 flies/trap/week compared to 5 $\pm$ 1 flies/trap/week on unbaited traps (James, unpubl data). James and Price (2004) also reported recruitment of micro-Hymenoptera ('parasitic wasps') and dance flies (Empididae) to MeSA-treated plots. Both of these groups were also attracted to MeSA in the current study. Additional evidence for MeSA-attraction of empidid flies was gained in the rose bush study: a mean of 122 $\pm$ 18 flies/trap/wk was captured on MeSA-baited traps compared to 30 $\pm$ 5 flies/trap/wk on unbaited traps. Herbivorous leaf-mining flies (Agromyzidae) were attracted to MeSA in the current study. This is the first (and so far, only) pestiferous insect group shown to be attracted to MeSA in our field experiments. Molleman et al. (1997) reported attraction of pestiferous Lepidoptera species to MeSA in pear orchards in The Netherlands.

The possibility that some insects may respond differently to HIPVs according to physiological condition, season, or other endogenous and/or exogenous factors, cannot be excluded. Some evidence for this possibility was obtained for *S. punctum picipes* and *O. tristicolor*. The responses shown by both species to some HIPVs in the first half of September, disappeared later in the month and in October (means for *S. punctum picipes* during September 17-October 22 were 1-3/trap/wk for all baited and unbaited traps and 0.5–2.0/trap/wk for *O. tristicolor*). Both species undergo adult hibernal reproductive dormancy in Washington State, and the behavioral/physiological switch to overwintering that occurs during autumn may alter responses to prey-finding stimuli like HIPVs. Interestingly, *O. tristicolor* failed to respond to MeSA during the autumn experiment, in contrast to the summer experiment and to earlier studies conducted during summer (James, 2003b; James and Price, 2004). Similarly, sarcophagid flies responded to MeSA in vineyard plots (James and Price, 2004) but did not respond to this HIPV during the autumn experiment.

Of the 15 HIPVs tested in the two field experiments, only two (linalool and 3-octanone) failed to attract any insect family or species. The simple sticky card/HIPV bait method used in this and earlier studies (James, 2003 a,b) appears to be sufficient to at least coarsely-screen synthetic HIPVs for attraction to beneficial insects. Presumably, if some attraction occurs, it is an indication of a degree of bioactivity that may be explored more rigorously with further experimentation. For example, demonstration of MeSA as an attractant for beneficial insects in sticky card studies (James, 2003 a,b) led to the use of this compound in controlled-release devices aimed at establishing the potential of enhancing recruitment and

retention of natural enemies in crop ecosystems (James and Price, 2004). The use of horizontally-oriented sticky cards in experiment 1 did not appear to improve trapping efficiency over the use of vertically-oriented cards, used in earlier work and in experiment 2. As in the earlier studies, no regard was given to volatilization rates of the HIPVs used, which in most cases were likely to be considerably above 'natural' levels. Thus, some HIPVs may have been repellent to some degree to some insects (see Whitman and Eller, 1992). Another factor that may have influenced insect attraction, was the presence of naturally-occurring HIPV's, particularly during the first month of experiment 2, when large populations of herbivores (mites and aphids) were present. Synergistic effects of naturally-occurring HIPVs on the attractivity of synthetic HIPV's during September, may have contributed to the greater responses by *S. punctum picipes* and *O. tristicolor* in this month. The possibility of positive or negative interactions between naturally-occurring and synthetic HIPV's adds another variable to field experiments, and this needs to be investigated further. Identification of a positive response by an insect to a synthetic HIPV in field screening tests should be viewed only as a first step towards detailed and controlled studies.

A number of other HIPVs that are commercially available remain to be field-tested, including other GLVs, ocimene, myrcene, pinene, limonene, nerolidol, etc. In addition, there are a number of HIPVs that are not available commercially (e.g., (*E*)-dimethyl-1,3,7-nonatriene), which should also be tested. The evidence for successful recruitment of beneficial insects in hops and grapes using controlled-release dispensers of MeSA presented by James and Price (2004), suggests that synthetic HIPVs may have potential in integrated pest management by increasing populations of predators and parasitoids. The HIPVs indicated in this study to be attractive to beneficial insects should be evaluated further for their potential as aids to crop pest management.

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SYNOMONE OR KAIROMONE? – *Bulbophyllum apertum* FLOWER RELEASES RASPBERRY KETONE TO ATTRACT *Bactrocera* FRUIT FLIES

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Abstract—*Bulbophyllum apertum* flower (Orchidaceae) releases raspberry ketone (RK) in its fragrance, which attracts males of several fruit fly species belonging to the genus *Bactrocera*. Besides RK as a major component, the flower contains smaller amounts of 4-(4-hydroxylphenyl)-2-butanol, plus two minor volatile components, veratryl alcohol and vanillyl alcohol. Within the flower, the lip (labellum) had the highest concentration of RK with much smaller quantities present in petals; other flower parts had no detectable RK. Male fruit flies attracted to the flower belong to RK-sensitive species – such as *Bactrocera albistragata*, *B. caudatus*, *B. cucurbitae* (melon fly), and *B. tau*. Removal and attachment of the pollinarium to a fly's thoracic dorsum occurred when a male of *B. albistragata* was toppled into the floral column cavity, due to an imbalance caused by it shifting its body weight while feeding on the see-saw lip, and then freeing itself after being momentarily trapped between the lip and column. During this process, the stiff hamulus (the pollinia stalk protruding prominently towards the lip) acted as a crowbar when it was brushed downwards by the toppled fly and lifted the pollinia out of the anther. If the fly was big or long for the small triangular lip, it would not be toppled into the column cavity and would just walk across the column, during which time the pollinarium could be accidentally removed by the fly's leg, resulting in a failed transport of the pollinarium. This suggests an unstable situation, where the orchid relies only on a particular pollinator species in the complex ecosystem where many RK-sensitive species inhabit. Wild males of *B. caudatus* (most common visitors) captured on *Bulbophyllum apertum* flowers were found to sequester RK in their bodies as a potential pheromonal and allomonal ingredient. Thus, RK can act either as a floral synomone (pollinarium transported) or kairomone (accidental

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removal of pollinarium leading to total pollen wastage), depending on the body size of the male fruit flies visiting the flowers.

Key Words—*Bulbophyllum apertum* (synonym *Bu. ecornutum*), Orchidaceae, fruit flies, *Bactrocera* species, Tephritidae, floral fragrance, raspberry ketone, synomone, kairomone, pheromone, pollination, coevolution.

INTRODUCTION

Bulbophyllum (Orchidaceae) is probably the largest orchid genus, with over 1,000 species grouped under ca seventy sections, and has flowers that have a specialized hinged lip (labellum) mechanism that tips an attracted fly precisely against the column, thus facilitating and ensuring cross pollination. Most species of *Bulbophyllum* produce foul smelling and carrion-like scent to attract flies (Van der Pijl and Dodson, 1969; Dressler, 1981). Studies of some *Bulbophyllum* species have shown the flowers are pollinated by flies belonging to four dipteran families – Calliphoridae, Lonchaeidae, Milichiidae, as well as Tephritidae-without knowledge of the chemical component(s) responsible for fly attraction (Christensen, 1994). However, "a small and active fly" (probably a tephritid fruit fly) was reported to visit and 'fertilize' two species of *Bulbophyllum – Bu. macranthum* and *Bu. stritellum*, as well as *Dendrobium superbum* (synonym – *D. anosmum*) by Ridley in 1890. Fruit flies belonging to the genus *Strumeta* (currently *Bactrocera* Macquart [Diptera: Tephritidae]) appear to be the exclusive pollinators of *Bu. baileyi* F. Müel, the flowers of which release in the morning a fruity scent that is responsible for attracting flies (Symthe, 1969). A "pleasant odor" was also reported for flowers of *Bu. giellerupii* J. J. Smith and described as attractive to *Dacus* (currently *Bactrocera*) fruit flies for pollination (Howcroft, 1983). However, none of the chemical components of all *Bulbophyllum* floral fragrances were identified until the start of the millennium. Recently, the ginger orchid, *Bu. patens* King, was shown to release a ginger essence – zingerone, as a floral synomone that attracts several fruit fly species (Tan and Nishida, 2000); and the fruit fly orchid, *Bu. cheiri* Lindley, releases several phenylpropanoids, of which methyl eugenol is the major component, that attracts male flies of *Bactrocera papayae* Drew and Hancock (not a distinct species from *B. dorsalis*) that assist in pollination (Tan et al., 2002; Nishida et al., 2004).

Males of many *Bactrocera* species are attracted to either methyl eugenol (ME) or raspberry ketone (RK), both of which are plant attractants. The RK-sensitive species are also attracted to cue-lure - a synthetic analogue of RK that contains an acetyl group. However, zingerone is the only compound that attracts, though weakly, male flies of both ME- and RK-sensitive species (Tan and Nishida, 2000). Male flies of pest and non-pest species are important pollinators of *Bu. patens* and *Bu. cheiri*, which secrete specific floral fragrance containing zingerone and

ME, respectively. They are grouped under the section Sestochilus that has 60–70 species worldwide (Vermeulen, 1991). As the total number of RK-sensitive fruit fly species (>6) in Malaysia, is higher than that of the ME-sensitive species (3), it is important to search within the section Sestochilus for a *Bulbophyllum* species that secrete RK in order to further understand the coevolution between *Bulbophyllum* orchids and *Bactrocera* fruit flies.

Bulbophyllum apertum Schltr. (name published in 1906 before its currently used synonym *Bu. ecornutum* J. J. Sm. [J. J. Vermeulen, personal communication, 2003]) is an epiphyte with a widespread distribution in the tropics from Thailand to Moluccas, where it is found in podzolic forests and in shrubby forests on limestone hills at 400–1300m elevation. Its inflorescence is one-flowered with pale green, yellowish, or pale pink petals and sepals with or without purple markings; and has a bright orange hamulus – an appendage loosely attached to the pollinia (Vermeulen, 1991). The flower has 'no particular smell,' and the function of the hamulus is unknown (Ramussen, 1985). Nevertheless, the small and non-resupinate flower (Figure 1), with a floral fragrance resembling that of RK, is visited by male fruit flies belonging to several pest and non-pest species (Tan, 2000 a,b). The objectives of this paper are: a) to determine the species of attracted flies and to observe the behavior of fruit fly visitors; b) to observe the role of hamulus in pollinarium removal; c) to analyze the floral fragrance and confirm the presence of RK; and d) to determine the content of RK in various floral organs and fruit fly visitors.

METHODS AND MATERIALS

Plants and Flowers. Observations of the orchid flowers and plants were conducted in the Tenom Orchid Center (on the fringe of a tropical rainforest) Tenom, Sabah, East Malaysia. The *Bu. apertum* (subspecies verrucosum) plants were from the Nabawan population in Sabah. Flowers were individually collected, weighed, and immersed in ethanol (redistilled) for chemical analyses.

Observation of Fruit Flies Attracted to Bu. apertum *Flowers.* Fly attraction to the flowers was observed before and after they bloomed. Pollinarium removal by a fruit fly visitor was carefully observed with special attention paid to the role of the hamulus in this process. After observation, flies visiting the flower were collected in clear plastic bags whenever possible and identified to species.

Extraction of Floral Fragrance. Flowers of *Bu. apertum* were plucked in late morning on the day they bloomed (at 0600–0800 hr), as a day-old (1 d.o.), and on subsequent mornings as 2 and 3 d.o. under natural conditions, where fruit fly feeding could occur. Each flower was immersed in sufficient ethanol in a 5 ml glass vial and used for GC-quantifications. For individual floral organs (column, lip, petals, and lateral and medial sepals), each floral part was carefully removed from a freshly bloomed flower (within 3 hr after it bloomed and not

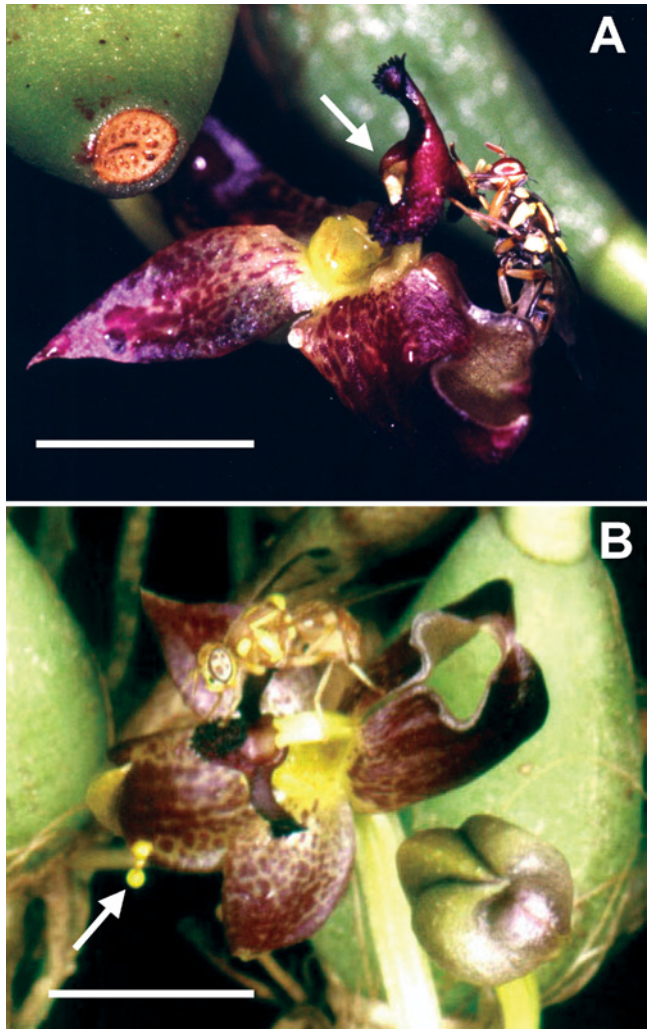

FIG. 1. (A) Flower of *Bulbophyllum apertum* with a male fruit fly of *Bactrocera caudatus* feeding on the triangular lip, which is in an open position and twisted by the fly [bar = 1 cm]. Note: a) Arrow points to hinge between the lip and column-foot, and b) the fruit fly is longer than the floral lip. (B) Flower of *Bulbophyllum apertum* with a misplaced pollinarium on the medial sepal, and a male melon fly (*Bactrocera cucurbitae*) feeding on the triangular purple lip, which is in a closed position, tilted towards the column [bar = 1 cm]. Note: a) arrow points to a pollinarium (pollinia + hamulus) freshly deposited after being removed accidentally by the fly's leg, b) the melon fly is longer than floral lip, and c) a floral bud at bottom right.

exposed to fruit flies), weighed and soaked in 0.5 ml ethanol in a 1 ml glass vial for quantification of RK. For GC-MS analysis, a combined extract of four flowers was concentrated under reduced pressure (ca. 20 mmHg, below 35°C) and partitioned between a mixture of hexane, benzene, and methyl acetate (1:1:2 v/v/v), and 1% sodium bicarbonate in water. The organic layer was washed with saturated sodium chloride in water and dried over anhydrous sodium sulfate and concentrated in partial vacuum, and a portion was used for GC-MS.

Extraction of Volatiles from Fruit Flies. Two *B. caudatus* male flies (main visitors) were captured after feeding for over 20 min on *Bu. apertum* flowers. The whole body was soaked in 0.5 ml ethanol in a 1 ml glass vial.

Chemical Analysis. GC-MS analyses were performed on an Hewlett Packard 5989B mass spectrometer coupled with an HP 5890 series II plus gas chromatograph, using an HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness) and programmed from 60° (2 min holding) to 290°C at a rate of 10°C/min; the GC was equipped with a total ion monitor. GC-quantification of volatile chemicals was performed on an HP 4890A gas chromatograph using HP-1MS and HP-5MS capillary column (15 m × 0.25 mm, 0.25 μm film thickness) and programmed from 60° (1 min holding) to 280°C at a rate of 10°C/min; the GC was equipped with a total ion monitor and a flame ionization detection.

Quantification of Volatiles. 1) Flowers. Quantification of the volatile components in a whole flower and each floral organ was performed using GC under the conditions described above by comparing the FID-intensities with those of the standard samples.

2) Flies. Portions of the ethanol fly extract were subjected to GC-MS analysis and GC-quantification as described above for flowers.

3) Authentic Samples. Raspberry ketone (4-(4-hydroxyphenyl)-2-butanone) (**1**), vanillyl alcohol (**3**), and veratryl alcohol (**4**) were purchased from Tokyo Chemical Industries Co. Ltd. Rhododendrol (racemic, 4-(4-hydroxyphenyl)-2-butanol; synonymous with frambinol or betuligenol) (**2**) was synthesized by reduction of **1** using lithium aluminium hydride in ether.

RESULTS

Fruit Fly Species. Species of male fruit fly visitors to *Bu. apertum* flowers were identified as *B. albistragata* (de Meijere), *B. caudatus* (Fabricus), *B. cucurbitae* (Coquillett) (the melon fly), and *B. tau* (Walker), hereafter collectively referred to as RK-sensitive species. These fruit flies respond to cue-lure baited traps (Tan and Lee, 1982). Usually a fruit fly, rarely two, was seen on a flower at any one time under natural conditions. Except for *B. albistragata*, most male visitors appeared to be too big for the relatively small and moveable floral lip of the *Bu. apertum* flower (Figure 1A). Some of the larger flies were observed to feed on the lip until satiation while standing on a lateral sepal (Figure 1A). No other

insects were observed on the orchid flowers, with the exception of an occasional ant walking across a petal or sepal.

Pollinarium Removal and Pollination. In one instance, a male *B. albistragata* effectively removed a pollinarium. The fly first landed near a flower, climbed onto it, and then began to probe and lap with its proboscis the surface of a petal. It eventually climbed on top of the see-saw lip (normally on a higher plane than the column - a characteristic of a non-resupinate flower). The fly continued to feed and move slowly along the short lip, and suddenly, it was toppled into the column cavity by the lip due to an imbalance during shifting of the fly's weight from one side of the floral hinge to the other (facing the column). The fly became momentarily trapped between the closed lip and column. While the fly struggled to free itself from a tight situation, the pollinarium was dislodged. During this process, the viscidium (a sticky area at the base of the hamulus) was touched, and pushed downwards (during tilting of the fly), then upwards (as the fly moved backward to free itself), sticking to the fly's thoracic dorsum. The fly with the pollinarium stuck onto its thorax immediately took off. The stiff hamulus (the bulbous end that has a sticky viscidium protruded prominently in the direction of the floral lip from the anther) acted like a crowbar to lift the pollinia out of the anther. In several other cases, where the fly was too big for the lip mechanism to work effectively, the fly either just lapped on the lip surface while standing on one of the lateral sepals without alighting on the lip (Figure 1A), or if it did go onto the lip, it simply walked across the column during an imbalance. During these visits, mainly by *B. caudatus*, the pollinarium was either not removed or was accidentally removed. In the latter case, one of the fly's legs either trampled on or brushed downward onto the hamulus, thereby removing the pollinarium (four instances of eleven observed). Figure 1B shows a pollinarium freshly deposited on the medial sepal after being removed accidentally by a melon fly's leg.

One or two relatively large seedpods were observed in the study site on several occasions over a period of seven years among two clumps of *Bu. apertum* pseudobulbs on a tree trunk and side branches over 3 m above ground. The seedpods were positive evidence that successful pollination of flowers had occurred in the previous season.

Floral Components of Bu. apertum. Figure 2 shows the GC trace of an extract of the floral lip of *Bu. apertum* where the attractant chemicals were concentrated. The major volatile component **1** was identified as raspberry ketone (RK, 4-(4-hydroxyphenyl)-2-butanone). RK was accompanied by its corresponding alcohol - rhododendrol (**2**). Vanillyl alcohol (**3**) and veratryl alcohol (**4**) were also detected as minor constituents. The identification was done by comparing their mass spectra and GC-retention times (on HP-1 and HP-5) with those of the authentic samples:

Compound **1** (raspberry ketone). MS: *m/z*(%) 164(71, M^+), 149(9), 121(15), 107(100), 91(13), 77(14), 43(19). Compound **2** (rhododendrol). MS: *m/z*(%) 166(67, M^+), 148(36), 133(100), 121(7), 107(95), 94(7), 77(19), 45(12).

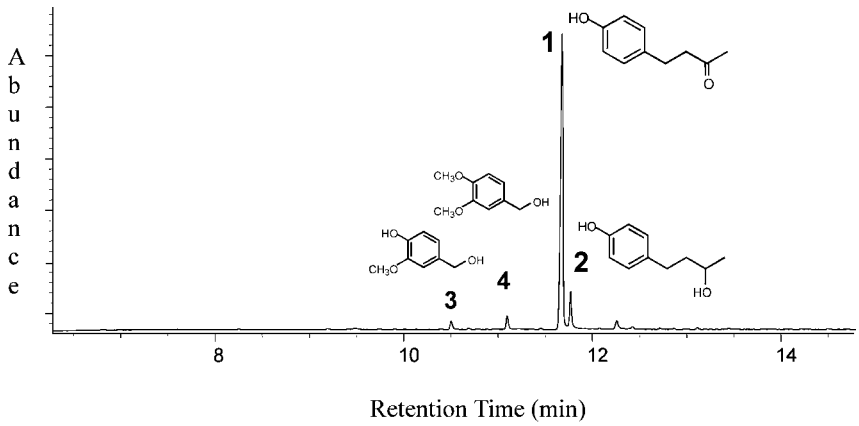

FIG. 2. Gas chromatogram of an extract from the lip of the *Bulbophyllum apertum* and structure of compounds. 1: Raspberry ketone, 2: Rhododendrol, 3: Vanillyl alcohol, 4: Veratryl alcohol.

Compound **3** (vanillyl alcohol). MS: *m/z*(%) 154(100, M^+), 137(38), 125(37), 122(26), 93(38), 65(33). Compound **4** (veratryl alcohol). MS: *m/z*(%) 168(100, M^+), 151(31), 139(45), 137(30), 109(20), 97(12), 93(13), 65(17).

Quantities of Raspberry Ketone in Floral Parts and Male Fruit Fly Bodies. Figure 3 shows the GC quantification and fresh weight of floral parts – column, lip, petals, and lateral and medial sepals. RK was almost exclusively in the lip, with small quantities (<0.5 μg) in the petals, and none or undetectable amounts in the other three floral parts. The quantity of rhododendrol in the lip was more or less proportional to that of RK (approx. 15% of **1**). Both vanillyl and veratryl alcohols were detected in low quantities (less than 1 μg/lip) in two of the ten lip specimens.

The mean ($\pm$SD) floral RK content for 1 d.o. was 7.8 $\pm$ 5.0 μg, 2 d.o. 9.2 $\pm$ 6.2 μg, and 3 d.o. 5.9 $\pm$ 3.8 μg. Although a slight decrease in RK was noted for the 3 d.o. flowers, there was no significant difference in RK content of the three different ages of flowers.

The total RK sequestered in the body tissue of *B. caudatus* after feeding on a *Bu. apertum* flower was 5.0 μg per fly.

DISCUSSION

Bu. apertum flowers show a large variation in their average RK content both within and among flower groups of different ages. Besides possible varietal differences, RK may be dependent on environmental factors such as temperature

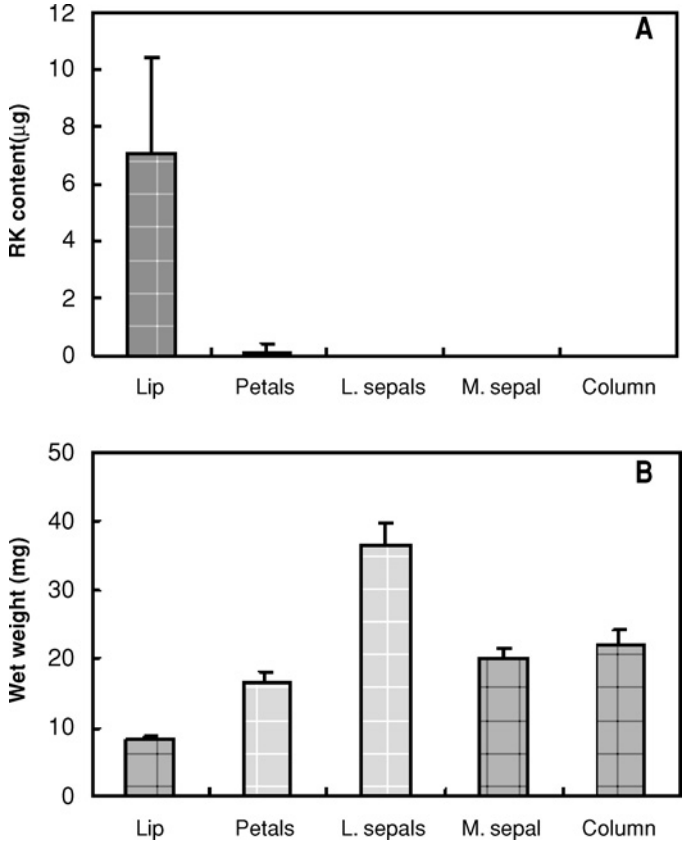

FIG. 3. Quantities of raspberry ketone in each floral organ (lip, lateral sepals, median sepal, petal, and column) ($N = 11$) (A), and fresh weight of floral organs ($N = 5$) (B) in each part of a *Bulbophyllum apertum* flower.

and light intensity that affect the synthesis/production of RK by individual flowers, as well as on the natural release rate by each flower and whether RK has been taken up by flies. These factors warrant further investigation.

The fact that RK is found almost exclusively in the lip of *Bu. apertum* explains why an attracted fly will eventually be led to feed on the lip surface. The see-saw lip seems designed to topple an attracted fly into the column cavity, and the precise removal of the pollinarium, which sticks onto the fly's thoracic dorsum, depends on the efficiency of this see-saw lip mechanism, and plays a vital role in pollination. However, this mechanism works only on flies with certain body sizes. If a fruit fly has a relatively large body, it can stand on one of the lateral

sepals and feed on the lip surface until satiation without climbing onto the lip, thus avoiding being toppled into the column cavity. Furthermore, if a large fly alights on the lip, it can just walk across the column during an imbalance of the see-saw lip; sometimes in this process it accidentally removes the pollinarium with its leg and deposits it on another part of the flower (Figure 1B). Although more cases of accidental removal of pollinarium were observed, leading to total pollen wastage, successful pollination must have also occurred as indicated by the presence of seedpods among some clusters of pseudobulbs.

The function of the hamulus as pollinia stalk is unclear (Rasmussen, 1985; J. J. Vermeulen, 2003, personal communication). The hamulus tends to break just below the bulbous swelling when touched after removal from the anther (Rasmussen, 1985). We propose that the hamulus, being stiff, acts like a crowbar that forces the pollinia out of the anther when the opposite end (bulbous swelling/viscidium) is pushed downwards by the toppled fly. When the pollinarium sticks to the dorsum of a fly, the pollinia on the hamulus protrude like small dumb-bells standing on one end. This is a different mechanism from *Bu. patens* and *Bu. cheiri*, where the pollinarium has no hamulus, but a flexible and soft pollinia stalk. When removed and transported during pollination, the pollinia become attached closely on the thoracic dorsum of a fruit fly (Tan and Nishida, 2000; Tan et al., 2002).

In two RK-sensitive species, *B. cucurbitae* and *B. tryoni* (Nishida et al., 1993; Tan and Nishida, 1995), RK is consumed and positively sequestered into the rectal (pheromonal) gland. We also found that it is sequestered into the body of wild *B. caudatus* after feeding on the *Bu. apertum* flower. The quantity of RK sequestered by the wild male (5.0 μg/male) was sufficient to provoke an aversive effect against a predatory Asian house gecko, *Hemidactylus frenatus*, when topically applied to houseflies (5.1 μg/fly) (Tan, 2000c). Thus, *Bu. apertum* seems to effectively endow a defensive benefit to its potential pollinator.

An attractant to monitor and control RK-sensitive fruit fly pest species has been developed commercially in the form of 'Cue lure' an analog of RK. This lure is not found in nature. It is spontaneously converted to RK in the presence of moisture (Metcalf, 1990). Males of the melon fly that were fed cue lure sequestered RK into the rectal glands (Nishida et al., 1990); wild male melon flies fed on cue lure mated more frequently than wild males not exposed to cue lure; and mass-reared flies that fed on cue lure were more successful in mating competition than control flies (Shelly and Villalobos, 1995). However, the advantage of feeding on cue lure is temporary - lasting less than three days (Shelly and Villalobos, 1995). Therefore, in order to maintain the sexual advantage, wild and polygamous flies may need to visit and feed on a natural RK source regularly. In the tropics, natural floral sources of RK have been previously detected in *Dendrobium superbum* flowers, which have fixed lips and where attracted *B. cucurbitae* male flies were observed to feed only on petals and sepals (i.e., there was no pollinarium removal and, thus, the flies played no role in pollination) (Nishida et al., 1993). Here,

we report the first case of a *Bulbophyllum* species emitting RK that potentially mediates a mutualistic association, in which the orchid flower gets its pollinarium transported during cross-pollination, and the fruit fly benefits by improving its reproductive performance as well as defense. Another species, *Bu. emiliorum*, also has a distinct scent of RK in the morning, to the human nose and its floral fragrance is currently under investigation.

There has been no information on the pollination of *Bu. apertum* by natural pollinators. Self-pollination of *Bu. apertum* has never been observed and appears unlikely (Ramussen, 1985). RK-sensitive fruit fly species seem to act as pollinators in the cross-pollination of the *Bu. apertum* flowers. However, flower visitation by species of male fruit flies whose bodies are apparently too long for effective pollinarium pickup may lead to wastage of pollinia via accidental removal, and the flower thereby loses all its pollen contained in the pollinia without gaining any ecological benefit. Only the smaller male fruit flies are effective pollinators for *Bu. apertum*. Consequently, the floral fragrance containing RK acts as a kairomone, which is a chemical signal where the perceiving organism, here the male fruit fly benefits, and the orchid emitter loses. This may create a situation that selects for *Bu. apertum* to evolve a more efficient lip mechanism -i.e., to produce larger flowers with longer lips to accommodate the long body length of other male fruit flies. Conversely, pollinarium removal by a male *B. albistragata* showed that the *Bu. apertum* flower is well adapted to attracting a relatively small-sized fruit fly that effectively pollinates the flowers, leading ultimately to the formation of seedpods. In this latter orchid-fruit fly association, both organisms benefit and the RK in the floral fragrance acts as a synomone.

From an overall behavioral and chemoecological viewpoint, RK in the floral fragrance of *Bu. apertum* can act as either a kairomone or synomone depending on the body size of the fruit fly visiting this orchid. In contrast, the floral fragrances of *Bu. patens* and *Bu. cheiri*, which contain zingerone and methyl eugenol, respectively, always act as synomones regardless of the fruit fly species attracted (Tan and Nishida, 2000; Tan et al., 2002). In the wild, the other three described species of tephritid fruit flies, *B. caudatus*, *B. cucurbitae*, and *B. tau* that have relatively longer body lengths than *B. albistragata* are more abundant. Therefore, it remains to be seen whether *Bu. apertum* will eventually adapt to the more abundant male fruit flies that have body lengths that are longer than the orchid's lip or maintain the *status quo* and remain dependant on the less abundant, smaller sized fruit flies such as *B. albistragata*, with consequently a lower chance of successful pollination in the tropical rain forest ecosystem.

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HYDROQUINONE IS NOT A PHAGOSTIMULANT FOR THE FORMOSAN SUBTERRANEAN TERMITE

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Abstract—It has been suggested that hydroquinone found in the labial glands of a number of termite species acts as a primary phagostimulating factor. We tested hydroquinone as a phagostimulant using workers from three colonies of the Formosan subterranean termite, *Coptotermes formosanus*, under both laboratory and field conditions. Hydroquinone at concentrations ranging from ca. 0.002–20.0 ng/cm^2 did not increase visitation by *C. formosanus* workers to treated over control filter papers, and was actually repellent at a 20 ng/cm^2 dose. No phagostimulant response to hydroquinone was observed in two colonies. In the third, there was a significant increase in feeding on filter paper treated with a 2 ng/cm^2 dose, but was significantly lower at a 20 ng/cm^2 dose. Furthermore, sand treated with a gradient of hydroquinone, did not evoke increased tunneling activity compared with controls. GC-MS analysis of *C. formosanus* workers indicated that hydroquinone was present at an average of 41 pg/worker. It was also determined that within one week about 11% hydroquinone in aqueous solution oxidized to 1,4-benzoquinone. Our findings indicate that hydroquinone alone does not act as a phagostimulant but instead may act as a repellent at higher concentrations. The attractant/arrestant of the Formosan termite may have multiple components of which hydroquinone, at low doses, could be one.

Key Words—Formosan subterranean termite, *Coptotermes formosanus*, hydroquinone, phagostimulant.

INTRODUCTION

Termite workers foraging for food use trail pheromones to guide other members of their caste to foraging sites (Pasteels and Bordereau, 1998). Once a suitable food source is located, the termites tend to stay and feed at that site.

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Reticulitermes santonensis and *Schedorhinotermes lamanianus*, have a highly polar, heat-resistant, and nonvolatile component of their labial glands, which when released onto food promoted aggregation and feeding, and was designated a phagostimulating pheromone (Kaib and Ziesmann, 1992; Reinhard and Kaib, 1995, 2001a,b; Reinhard et al., 1997). Using feeding bioassays conducted with *R. santonensis* and *S. lamanianus*, Reinhard and Kaib (2001b) reported feeding-stimulating activity in extracts of labial glands from 11 termite species from five families including one from the Formosan subterranean termite, *Coptotermes formosanus*. Based on these observations, they suggested that such a general feeding-stimulating signal might have evolved in many termite species. The feeding-stimulating component in the labial gland extract was subsequently identified as 1,4-dihydroxybenzene[2] or hydroquinone and varied in concentration from 0.02 ng in *Kalotermes flavicollis* to 10.0 ng in *Mastotermes darwiniensis*, with 0.03 ng in *C. formosanus* (Reinhard et al., 2002a). Doses of 5 and 10 ng hydroquinone applied to 2.5 cm^2 filter paper disks resulted in a phagostimulating effect in *M. darwiniensis*, as did a concentration of 100 ng/cm^2 in field tests carried out in Malaysia with *Coptotermes curvignathus* (Reinhard et al., 2002a).

The Formosan subterranean termite was introduced into the United States during the middle of the 20th century and has since become a serious pest of homes and living trees in several southern states. With increasing restrictions on the use of conventional insecticides, there is need to find environmentally safe technologies to manage this pest. Baiting was suggested a long time ago (Esenther and Beal, 1978) as a means of controlling infestations, and with the introduction of chitin synthesis inhibitors, it has become the treatment of choice (Su, 1994). However, a major constraint in the use of this technology is the failure of the baits to attract termites. Several additives have been used in bait matrices (primarily cellulose containing materials) to make these attractive (Chen and Henderson, 1996; Rojas and Morales-Ramos, 2001). Reinhard et al. (2002b) suggested the use of hydroquinone in bait systems for termite management as both laboratory and field tests, indicated increased feeding by *M. darwiniensis* and *C. acinaciformis* in response to the treatment. In addition, hydroquinone was also reported to act as an attractant over a short distance.

We have also observed that a substrate on which termites have been feeding for some time acts as an attractant/arrestant for conspecific individuals (unpublished information). Therefore, we undertook this study to evaluate hydroquinone as an attractant-arrestant phagostimulant for *C. formosanus* both under laboratory and field conditions.

[2]Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

METHODS AND MATERIALS

Insects. Three field colonies of *C. formosanus* from existing bucket traps were selected. Colonies S-67 and S-6B were from the grounds of Southern Regional Research Center and U-70 from the University of New Orleans (UNO), Lakefront campus, both in New Orleans, LA USA. After collection, the termites along with fresh spruce wood pieces were placed in plastic boxes and kept in an incubator maintained at 28 ± 1°C, 80 ± 5% RH and constant darkness.

Attraction/Phagostimulation Assay. A thin layer of sand (15 g) was poured into individual plastic Petri dishes (90 × 13 mm) and moistened with 2.5 ml distilled water. Two, 2.5 cm diam Whatman #1 filter papers were weighed and each lightly marked with a pencil either as T or C for treated or control, respectively. Hydroquinone (Aldrich, Milwaukee, WI) was dissolved in distilled water to obtain concentrations of 0.01, 0.1, 1.0, 10.0, and 100.0 ng/50 μl (hydroquinone was readily soluble at these concentrations). The filter paper disks were treated either with 10 μl of one of the hydroquinone solutions or water and placed at opposite ends of the Petri dish. Twenty workers were released into each dish, and the dishes were placed under a CCD camera (Emcal Scientific, Poway, CA) under low intensity red light. Distribution of the workers was recorded every 15 min for 3 hr, using a Panasonic AG-6740 time-lapse video recorder. After 3 hr, an additional 80 workers were released into each Petri dish (to obtain greater feeding) and placed in the incubator. After 24 hr, the filter papers were removed, washed, oven dried, and weighed. The assay was repeated six times for workers from each of the three colonies at all five concentrations of hydroquinone. Average consumption was determined for each treatment and each colony.

Tunneling Bioassay. A plastic container (75 × 75 × 30 mm) was connected to a glass T-tube via a piece of Tygon tubing inserted into a hole drilled into the side (Figure 1A). Each free end of the T-tube was connected with a Tygon connector to a 15 cm tube (plastic pipette, 5 mm ID) filled with fine sand. To one tube, a hydroquinone solution was added with a glass syringe through two small holes 5 cm apart (Figure 1B) at the proximal end to produce a gradient of 0.1 to 1.0 to 10.0 ng in 230 μl water, with the lowest concentration being closest to the T-tube. In the other, the sand was treated with distilled water and acted as control. Both tubes were covered with aluminum foil. Termite workers (100) were released into the square container, and tunneling activity through the sand in plastic tubes was monitored every 15 min for 2 hr and then every hour for the next 4 hr. The assay was repeated four times for each of the three colonies.

Field Test. In an initial test, paper towels treated with 125 ng/cm^2 hydroquinone, were strongly repellant, so in the actual field trials a weaker solution was used. In this test 20 × 20 cm paper towels (Bounty®, Procter and Gamble, Cincinnati, OH) were weighed and treated with either 800 ng hydroquinone (2 ng/cm^2) in 2.6 ml distilled water or just water. The towels were folded, enclosed

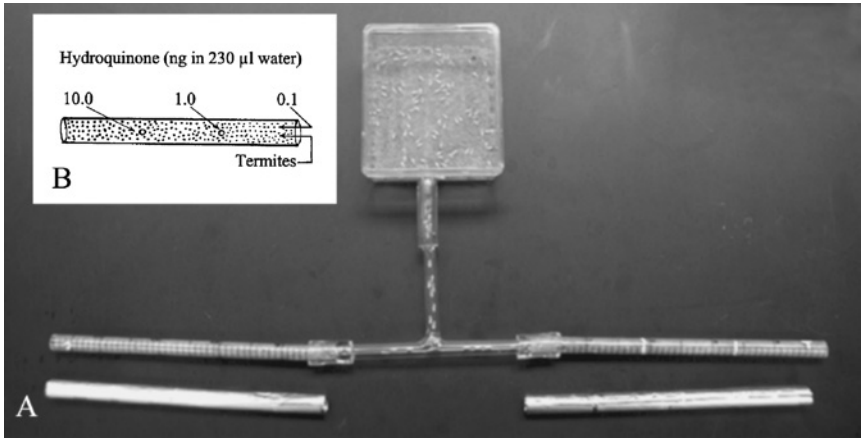

FIG. 1. Setup for tunneling assay. A. The square plastic container with the termites is connected to two tubes through a T-tube. Two aluminum foil covers were slipped over the tubes to exclude light. B. Each tube is filled with sand treated either with water or with hydroquinone in an increasing gradient.

in Tygon tubing (10 cm × 2.2 cm ID) and placed in three different bucket traps (three treated and three control tubes/trap) on the UNO campus after removing the wood. After 72 hr, the tubes and enclosed towels were removed. Each towel was carefully washed, oven dried, and weighed. Average consumption was calculated. The test was replicated four times for each of the three colonies.

Determination of Endogenous Hydroquinone. Freshly collected workers from the three test colonies were used for extraction of hydroquinone. Three samples of 300 workers from each of the three colonies were placed in 4 ml vials and frozen in liquid nitrogen. Water (2 ml) was added to each vial, and the tissue was sonicated (power level 9) with an ultrasonicator (Sonic Dismembrator 60, Fisher Scientific), three times each for 10 sec at 0°C. The aqueous samples were centrifuged for 30 min at 12,000 rpm (4°C), and the supernatant (minus the fat layer) filtered (0.45 μm Millex-GV), lyophilized, and then extracted three times with ether (3 ml) following the addition of $MgSO_4$. The ether was filtered, concentrated to dryness, and the oily residue was extracted with acetonitrile (3 × 100 μl). The acetonitrile solution was concentrated to dryness with nitrogen and dissolved in 45 μl ether. The samples were trimethylsilyl (TMS)-derivatized with N,O-bis-(trimethylsilyl)-acetamide (BSA) (5 μl) in a sonicating bath for 1 hr. A 2 μl aliquot of each sample was analyzed by GC-MS (6890 GC and 5973 MS, Agilent Technologies, Palo Alto, CA). Analysis conditions were: 70 eV EI source, HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm *d*p), He carrier gas (1 ml/min), temperature programmed from 60°C with a 1 min hold to 110°C at 10°C/min

followed by a ramp to 160°C at 3°C/min. Quantitation was based on *m/z* 239 using a standard curve of TMS-derivatized hydroquinone.

Hydroquinone Oxidation Test. Hydroquinone (30 μg in 50 μl distilled water) was applied to 2.5 cm diam filter paper disks (Whatman #1) placed in three 100 × 15 mm plastic Petri dishes (5/dish) in addition to one dish containing water treated control paper disks, and stored at room temperature and in darkness. Five disks were removed after 0, 1, or 4 days and individually extracted with 3 × 100 μl ether. Extracts (1 μl) were tested by GC-MS as described above but with a temperature program from 30°C with a 1 min hold to 210°C at 10°C/min. Quantitation was done using single ion monitor (SIM) at *m/z* 110 and 108 for hydroquinone and benzoquinone, respectively.

Data Analysis. All the data were analyzed using an unpaired *t*-test, Graph Pad Prism version 4.0 (Graph Pad Software Inc. 2003, San Diego, CA).

RESULTS

In the choice test, the workers showed no significant preference for either treated or control disks at hydroquinone concentrations between 0.002 and 2.0 ng/cm^2, but in all three colonies a concentration of 20 ng/cm^2 had a significant repellant effect (Table 1). In two of the three colonies, there was no evidence that hydroquinone affected feeding behavior as the quantities of treated and control paper consumed did not differ significantly. In the third colony, there was evidence that hydroquinone acted as a phagostimulant at a concentration of 2.0 ng/cm^2 but was a feeding deterrent at 20 ng/cm^2 (Table 2).

In the tunneling assay, the distance tunneled in treated and control arms, as a function of time, did not differ significantly in any of the colonies tested (Table 3). For both control and treated tubes, the average distance tunneled in 6 hr was about

TABLE 1. PERCENT DISTRIBUTION OF *C. formosanus* WORKERS FROM THREE COLONIES ON HYDROQUINONE VS. CONTROL FILTER PAPER DISKS[a]

Treatment dose (ng/cm^2)	S-67		U-70		S-6B	
	C	T	C	T	C	T
0.002	6.3 ± 1.7	5.3 ± 0.9	6.7 ± 2.8	3.6 ± 1.7	5.2 ± 1.8	6.8 ± 2.6
0.02	3.3 ± 0.5	6.3 ± 1.8	4.6 ± 2.1	3.3 ± 0.4	3.9 ± 1.3	8.7 ± 2.5
0.2	8.4 ± 1.6	5.9 ± 1.2	9.6 ± 5.1	6.3 ± 3.6	10.0 ± 2.6	6.5 ± 2.7
2.0	6.6 ± 0.8	5.4 ± 1.8	8.1 ± 3.6	3.7 ± 1.4	4.7 ± 1.0	4.7 ± 1.3
20.0	11.8 ± 2.1	6.2 ± 1.0*	11.3± 3.0	1.7 ± 0.3**	11.2 ± 2.5	2.6 ± 0.3**

[a]Choice test. Filter paper disk (2.5 cm diam) treated with hydroquinone (T) or water (C). Location of the number of workers out of 20 that were on either of the filter paper disks, recorded every 15 min for 3 hr. Average % distribution ±SE, $N = 6$ for each of the 3 colonies. Means significantly difference at $P < 0.05$(*) and $P < 0.01$(**).

TABLE 2. AVERAGE CONSUMPTION (mg) OF CONTROL VS. HYDROQUINONE TREATED FILTER PAPERS BY WORKERS OF *C. formosanus* FROM THREE COLONIES[a]

Treatment dose (ng/cm^2)	S-67		U-70		S-6B	
	C	T	C	T	C	T
0.002	5.8 ± 1.0	3.4 ± 0.6	3.6 ± 0.4	4.2 ± 0.7	6.3 ± 0.9	5.9 ± 0.6
0.02	4.7 ± 0.9	5.2 ± 0.3	3.9 ± 0.7	4.6 ± 1.1	7.0 ± 0.7	6.2 ± 0.6
0.2	4.4 ± 0.9	3.8 ± 0.6	4.1 ± 0.4	2.9 ± 0.8	7.9 ± 1.4	7.6 ± 1.0
2.0	3.0 ± 0.3	5.4 ± 0.8*	2.4 ± 0.6	3.7 ± 0.8	8.5 ± 1.3	6.1 ± 1.2
20.0	5.7 ± 0.9	3.2 ± 0.5*	3.3 ± 1.3	3.0 ± 0.8	6.3 ± 0.4	6.2 ± 0.4

[a] Choice test with filter papers (2.5 cm diam) treated with hydroquinone (T) and water (C) presented to 100 workers over a period of 24 hr. Consumption is the average ±SE of 6 replicates for each colony. Means significantly different at $P < 0.05$(*).

79 mm. After 24 hr, workers of U-70 and S-6B had tunneled the entire length of the tube in all cases and come out at the open end (data not presented). Workers of colony S-67 had also tunneled through the tube but did not come out.

In the initial field test, hydroquinone at 125 ng/cm^2 of paper toweling strongly repelled the termites resulting in their abandoning the traps. In the subsequent test, there was no significant difference in consumption of the paper towels between the treated (2 ng/cm^2) and control groups for any of the three colonies tested (Table 4).

Hydroquinone was detected in the whole body extracts of all workers for the three test colonies (Figure 2). Levels ranged from 1–159 pg/worker across all samples with a colony average of 41 ± 37 pg/worker. Hydroquinone was fairly stable over the period used in the bioassays. After one day under conditions similar to assays with termites, recovery of hydroquinone was similar to 0 day, with no increase in the oxidation product (1,4-benzoquinone). Subsequently, the stability of hydroquinone was tested by observing the amount of 1,4-benzoquinone by GC-MS obtained from treated paper disks under conditions similar to the bioassay. The benzoquinone/hydroquinone ratio, after exposure to atmospheric conditions

TABLE 3. AVERAGE DISTANCE (mm) TUNNELED BY *C. formosanus* WORKERS FROM THREE COLONIES THROUGH SAND TREATED WITH A CONCENTRATION GRADIENT OF HYDROQUINONE (C) VS. CONTROL SAND (T)[a]

Time hours	S-67		U-70		S-6B	
	C	T	C	T	C	T
1	12.2 ± 2.6	14.2 ± 3.2	13.0 ± 1.8	11.7 ± 1.7	10.7 ± 2.2	15.0 ± 1.2
2	25.2 ± 3.4	33.2 ± 1.9	27.7 ± 2.5	29.2 ± 1.3	28.0 ± 1.3	28.3 ± 2.6
3	36.7 ± 3.3	43.7 ± 3.6	47.7 ± 2.5	44.7 ± 3.1	37.0 ± 4.8	41.0 ± 3.7
6	69.7 ± 5.9	69.5 ± 3.0	85.2 ± 4.3	80.7 ± 3.9	80.7 ± 5.2	86.7 ± 3.1

[a] Total length of the tube with sand was 150 mm. Average distance ±SE, $N = 4$.

TABLE 4. AVERAGE WEIGHT OF CONTROL AND HYDROQUINONE TREATED PAPER TOWELS CONSUMED BY *C. formosanus*, IN FIELD TRAPS OVER A PERIOD OF THREE DAYS[a]

Colony	Control	Treated
U-70	695.7 ± 574.4	128.3 ± 49.5
U-101	1471.0 ± 376.6	1607.0 ± 279.7
Undesignated	1450.0 ± 156.9	1572.0 ± 382.9

Note. Consumption of hydroquinone treated (2 ng/cm^2) and control paper towels placed in traps for three days. Average mg ± SD, $N = 4$.

for up to 4 days, had not significantly changed (data not shown) although the total recovery had decreased.

DISCUSSION

Through a series of publications it was suggested that labial glands of a number of termite species contain a phagostimulating factor (Reinhard and Kaib, 1995; 2001a,b; Reinhard et al., 1997). The factor was subsequently identified as

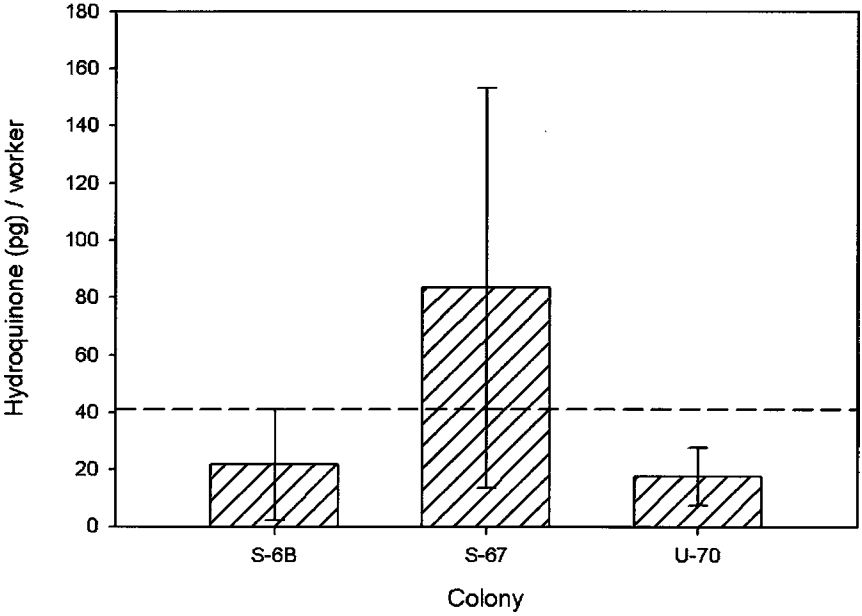

FIG. 2. Amounts of hydroquinone (mean ± SD, $N = 3$) from whole body extraction of three colonies of *C. formosanus* workers. Broken line indicates colony average.

hydroquinone (Reinhard et al., 2002a). It was also shown that hydroquinone was present in the labial gland extract from 15 termite species including *C. formosanus*. They further reported that the compound significantly stimulated feeding by *M. darwiniensis*, on filter papers treated at 2 and 4 ng/cm^2. Consequently, we tested hydroquinone as an attractant/phagostimulant for *C. formosanus*, in comparable tests carried out both in the laboratory and the field. Hydroquinone at concentrations ranging from 0.002–20 ng/cm^2 did not evoke preferred visitation by *C. formosanus* workers to treated filter paper over control paper. On the other hand, the compound was repellent at the 20 ng dose. In rare instances, we observed an initial attraction to hydroquinone treated filter papers with the workers quickly dispersing uniformly. Similarly, no significant phagostimulant response was observed except for workers from one of the test colonies and at only the 2.0 ng dose. However, what is puzzling is that in our experiments, hydroquinone acted as a strong repellent at higher concentrations. Recently, it was reported that in choice tests with *C. formosanus*, hydroquinone did not induce increased consumption at lower doses, whereas at higher doses (1 ng/mg filter paper) the compound acted as a feeding deterrent (Cornelius, 2003). Sand treated with a gradient of hydroquinone, did not evoke increased tunneling activity. In the field test, using a dose comparable to the one used by Reinhard et al. (2002a) for *C. curvignathus*, we found that the termites quickly abandoned the traps. When we used a concentration that had been shown effective for *M. darwiniensis* in the laboratory, there was no difference in feeding between control and treated paper towels.

Analysis of *C. formosanus* workers indicated that hydroquinone indeed was present at an average of 41 pg/worker (colony range: 20–80 pg). This is in conformity with the findings of Reinhard et al. (2002a). They reported an average of 30 pg hydroquinone per labial gland of *C. formosanus*. The stability of hydroquinone in aqueous solution was tested under conditions much more rigorous than the assay, and only a small increase in the amount of oxidation product (1,4-benzoquinone) was observed.

Our results indicate that response of termites to hydroquinone may be species or even colony specific. We have observed that extracts of substrate on which termites were allowed to feed for some time is active as an attractant/arrestant (Raina: unpublished results). The identification of active component(s) in the extract is in progress. It may well be multi-component, of which hydroquinone at very low concentrations could be one of the components. It is also possible that the active factor may be derived from the interaction of termite excretions, primarily from labial glands, on the cellulose-based substrate. Identification of such an attractant will be valuable for use in baits to increase their efficiency.

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INHERITANCE OF RESISTANCE TO MAMMALIAN HERBIVORES AND OF PLANT DEFENSIVE CHEMISTRY IN A *Eucalyptus* SPECIES

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Abstract—Hybridization in plants provides an opportunity to investigate the patterns of inheritance of hybrid resistance to herbivores, and of the plant mechanisms conferring this resistance such as plant secondary metabolites. We investigated how inter-race differences in resistance of *Eucalyptus globulus* to a generalist mammalian herbivore, *Trichosurus vulpecula*, are inherited in their F_1 hybrids. We assessed browsing damage of three-year-old trees in a common environment field trial on four hybrid types of known progeny. The progency were artificial intra-race crosses and reciprocal inter-race F_1 hybrids of two geographically distinct populations (races) of *E. globulus*; north-eastern Tasmania and south-eastern Tasmania. Populations of trees from north-eastern Tasmania are relatively susceptible to browsing by *T. vulpecula*, while populations from south-eastern Tasmania are more resistant. We assessed the preferences of these trees in a series of paired feeding trials with captive animals to test the field trial results and also investigated the patterns of inheritance of plant secondary metabolites. Our results demonstrated that the phenotypic expression of resistance of the inter-race F_1 hybrids supported the additive pattern of inheritance, as these hybrids were intermediate in resistance compared to the pure parental

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hybrids. The expression of plant secondary metabolites in the F_1 hybrids varied among groups of individual compounds. The most common pattern supported was dominance towards one of the parental types. Together, condensed tannins and essential oils appeared to explain the observed patterns of resistance among the four hybrid types. While both chemical groups were inherited in a dominant manner in the inter-race F_1 hybrids, the direction of dominance was opposite. Their combined concentration, however, was inherited in an additive manner, consistent with the phenotypic differences in browsing.

Key Words—*Trichosurus vulpecula*, hybrid, plant secondary metabolites, generalist herbivore, additive inheritance, *Eucalyptus globulus*, hybridization, Formylated phloroglucinol compounds, tannins, essential oils, 1,8-cineole.

INTRODUCTION

Understanding the genetic control of plant resistance to herbivores is crucial in understanding the evolutionary processes behind plant/herbivore interactions (Marquis, 1990; Mauricio and Rausher, 1997), and hybridization offers an ideal opportunity to investigate some of these processes (Bailey et al., 2004). The investigation of the differential resistance of hybrid plants to herbivores, compared to the pure parental plants, has provided insight into the mode of inheritance of resistance in plants. Studies have shown that responses of herbivores to hybrids can vary greatly among plant species and also among different herbivores within one plant species. Various patterns of hybrid resistance have been observed. Hybrid plants may be more susceptible to herbivores than the parental plants (hybrid susceptibility pattern; Floate et al., 1993; Fritz et al., 1998; Dungey and Potts, 2003), more resistant than the parental plant (hybrid resistance pattern; Boecklen and Spellenberg, 1990; Eisenbach, 1996), and exhibit resistance that is intermediate to the parental plants, which indicates additive inheritance of resistance (additive pattern: Aguilar and Boecklen, 1992; Fritz et al., 2001; Scott et al., 2002). In addition to these patterns, hybrids have also exhibited resistance similar to one of the parental plants (dominance pattern; Hjälten, 1997; Orians and Floyd, 1997; Fritz et al., 1998; Hjältén et al., 2000). If hybrids resemble the susceptible parent, then there is dominance for susceptibility, while if the hybrid resembles the resistant parent, there is dominance for resistance. Finally, hybrids can exhibit resistance that is not different from either of the parental plants (no difference pattern; Fritz et al., 1996, 1998; Orians and Floyd, 1997).

Research exploring the response of herbivores to hybrids has focused predominantly on invertebrates, and this has been highlighted in several reviews on the ecological and evolutionary processes of hybridization in plants (Strauss, 1994; Fritz, 1999; Fritz, et al., 1999; Orians, 2000). In comparison, the susceptibility of hybrids to mammalian herbivores has received considerably less attention (but see Vila and D'Antonio, 1998; Scott et al., 2002; Dungey and Potts, 2002; Anderson

and Paige, 2003). Additionally, while many studies have focused on the phenotypic patterns of resistance of hybrids to herbivores, relatively few have investigated the inheritance patterns of the defensive plant characteristics conferring resistance, such as plant secondary metabolites (Orians, 2000 for review; Byrd et al., 1999; Orians et al., 2000; Fritz et al., 2001; Hallgren et al., 2003).

During the past two decades, aspects of the ecological interactions of *Eucalyptus* species and the mammalian herbivores that feed on them have been investigated (e.g., Landsberg, 1987; Hume and Esson, 1993; Lawler et al., 2000; Close et al., 2003). There is clear inter - and intraspecific variation in browsing damage to some eucalypt species by mammalian herbivores (Lawler and Foley, 1999; Lawler et al., 2000; O'Reilly-Wapstra et al., 2002; Scott et al., 2002; Close et al., 2003). Plant secondary metabolites play an important role as the mechanisms behind this variation in resistance (McArthur and Sanson, 1993; Lawler et al., 2000; Wallis et al., 2002; Marsh et al., 2003). Recently, the role of plant genotype in influencing the resistance of a eucalypt species, *Eucalyptus globulus*, to browsing by a generalist marsupial folivore, the common brushtail possum (*Trichosurus vulpecula* Kerr 1792), was documented (O'Reilly-Wapstra et al., 2002; Dungey and Potts, 2002), and inter-race differences accounted for the majority of the genetic-based variation observed in O'Reilly-Wapstra et al. (2002). A group of metabolites known as formylated phloroglucinol compounds (FPCs) (Eschler et al., 2000) were found to be the most significant defensive leaf trait conferring resistance in juvenile coppice foliage of *E. globulus* (O'Reilly-Wapstra et al., 2004). The mode of inheritance of plant resistance and plant defensive traits within this system and in other eucalypt/mammalian herbivore systems, however, remains unclear.

Hybrid resistance to herbivores is determined not only by the genetic background of the plant but is also influenced by variation in environmental factors (Fritz, 1999), such as access to nutrients (Orians and Floyd, 1997). To understand the consequences of herbivore feeding on hybrid plants, it is necessary to differentiate the genetic and environmental causes of the hybrid resistance. Many investigations have been conducted in natural plant systems and, in some cases, these studies have not been controlled for environmental variation, and the genetic status of the hybrid plants was often unknown (Aguilar and Boecklen, 1992; Whitham et al., 1994; Gange, 1995). Managed plant systems growing hybrids of known pedigree in common environment field trials are ideal for investigating the inheritance of resistance in hybrids, without confounding effects of environmental influences.

In this study, we aimed to investigate the inheritance of resistance to browsing by *T. vulpecula* and the inheritance of the defensive plant compounds within *E. globulus*. We assessed the browsing damage by *T. vulpecula* on known F_1 progeny of two genetically differentiated geographical races of *E. globulus* from north-eastern Tasmania and south-eastern Tasmania (see Dutkowski and Potts,

1999 for race classifications of *E. globulus*). The pedigreed progeny used were grown in a common environment field trial and, therefore, differences in susceptibility to browsing is most likely due to host genetics, not environmental effects. The progeny were artificial intra-race and reciprocal inter-race F_1 hybrids involving trees from north-eastern Tasmania and south-eastern Tasmania. Populations of trees from north-eastern Tasmania are relatively susceptible to browsing by *T. vulpecula* (O'Reilly-Wapstra et al., 2002) and contain lower concentrations of FPCs (O'Reilly-Wapstra et al., 2004). Conversely, populations from south-eastern Tasmania are relatively more resistant to browsing (O'Reilly-Wapstra et al., 2002) and have higher concentrations of FPCs (O'Reilly-Wapstra et al., in press). Therefore, this genetic material provided an ideal opportunity to investigate three main questions:

1. How are the racial differences in resistance of *E. globulus* to *T. vulpecula* inherited in their F_1 hybrids in a common environment field trial?
2. Is the variation in resistance in the field trial consistent with patterns of intake of *E. globulus* by captive animals?
3. Are there quantitative differences between the hybrids in leaf chemical characteristics and does this reflect the observed patterns of resistance?

METHODS AND MATERIALS

Field Trial and Plant Material. Browsing of *Eucalyptus globulus* juvenile foliage on 3-yr-old trees by *Trichosurus vulpecula* was assessed in a field trial located at Weilangta, in south-east Tasmania, Australia (42° 44′ S 147° 49′ W). The trial design has been described in detail by Lopez et al. (2003). In brief, trees planted in the field trial were progeny from an 8×8 diallel crossing (80% complete excluding selfs) design where all possible crosses among eight parents were carried out. The parents were random samples of trees in native stands of *E. globulus* from both north-eastern Tasmania (termed north) and south-eastern Tasmania (termed south) races (following Dutkowski and Potts, 1999). The parental trees were growing more than 10 km apart and were considered unrelated. Four parental trees were from the north, and four were from the south. These crosses resulted in four hybrid types; intra-race crosses, north × north (NN) and south × south (SS), and two reciprocal inter-race F_1 hybrids, north × south (NS) and south × north (SN). The female parent is stated as the first letter in the cross, and the male parent is stated as the second letter in the cross. The field trial at Weilangta was planted in 1999, and a duplicate trial was planted at the same time at Geeveston, in south-east Tasmania, Australia (43° 09′ S 146° 51′ W). Trees were planted in a randomized block design with each hybrid represented as a single tree plot. There were 18 field replicates at Weilangta and 17 at Geeveston. The Geeveston field trial was fenced to prevent mammal browsing, while the Weilangta field trial was not. In

July (winter) 2002, all trees (648 in total) in the Weilangta trial were assessed for browsing damage. Each tree was observed for damage by *T. vulpecula* and given a 'browsing damage score,' which was as follows: 0 = no damage; 1 = minor browsing damage evident in the crown; 2 = major structural branch damage in the crown (due to *T. vulpecula*), with none to little browsing damage; 3 = major browsing damage in the crown; 4 = major structural branch damage and major browsing damage in the crown.

Feeding Trial. After assessing browsing damage in the Weilangta field trial, we tested the preferences of six captive *Trichosurus vulpecula* (three male and three female, 2.5–4.1 kg body mass) for juvenile foliage from the four hybrids (NN, NS, SN, SS) in a feeding trial. All animals were caught, housed, and maintained, following McArthur et al. (2000). Foliage used in the feeding trial was selected from the Geeveston field trial as it was un-browsed and hence avoided any possible effects of induced chemistry responses of the foliage. Juvenile foliage (cut branches) was selected from as many trees as possible from several replicates to ensure a full representation of each family in each hybrid sample. Cut branches from each hybrid type were combined to make up four bunches of foliage; NN, NS, SN, SS. The foliage for each trial was cut from the field at the beginning of each trial, and foliage was stored in a cool room (5°C) with cut stems in water. Animals were offered foliage as four, of the possible six, paired choice feeding trials, as the four pairs provided adequate comparisons to rank the relative preference of each hybrid type. The pairs were: Trial 1 = SN and SS; Trial 2 = NN and SS; Trial 3 = NS and SN; and Trial 4 = NN and NS. Each trial ran for 3 d and all 6 animals received the same foliage at the same time. For each trial, a random selection of cut stems were presented to each animal in their cage as two bunches (one bunch for each cross race type) in a container of water.

Sufficient foliage was presented to each animal to allow *ad libitum* feeding. On each day (at ~1500 hr), total fresh mass (g) of each bunch was measured before being placed in the container. The next morning (at ~0800 hr), the remains of each bunch were weighed (including any plant fragments found on the cage floor). Intake was estimated as the amount of foliage consumed (weight difference) from each bunch, and all intakes were expressed in terms of grams dry matter per kilogram of body mass of the consumer (g DM.kgBM^{-1}), based on the fresh to dry weight ratios calculated from the control plants. During the feeding trial, animals were offered a basal diet (see McArthur et al., 2000) at the same time as the experimental foliage. The amount of basal diet offered was 75% of that required to maintain body mass. This ensured that animals would eat the test foliage by choice, not because they were hungry, and ensured that all animals maintained body mass throughout the trial. On each day of the trial, two bunches of each hybrid fed to animals were also prepared as controls. The mean overnight change in mass of the two controls was used to adjust for changes in mass of the experimental plants. On each morning of the trial, one bunch was stripped

of the leaves, which were oven dried (35°C for 1 wk and 80°C overnight before weighing) to determine their absolute dry matter (DM). The second bunch was frozen for subsequent chemical analysis.

Chemical Analysis. For each hybrid type, the control plants from the feeding trials were combined and foliage was randomly sub-sampled 4 times for each analysis for each variable. We assayed plants for nitrogen, plant cell wall components (neutral detergent fiber, acid detergent fiber and lignin; $N = 3$ for these constituents), total essential oils and 1,8-cineole, total phenolics, condensed tannins, and six formylated phloroglucinol compounds (FPCs) (sideroxylonal A, sideroxylonal B, sideroxylonal C, macrocarpal A, macrocarpal B, and macrocarpal G; $N = 3$ for these constituents).

Primary Chemistry. Nitrogen was determined following the sulphuric acid and hydrogen peroxide method of Lowther (1980) using air-dried ground foliage, ground in a cyclone grinder through a 1-mm mesh sieve. Digested samples were colorimetrically analyzed for nitrogen (QuikChem method 10-107-06-2E, Lachat Instruments, WI, USA) on a continuous flow injection analyzer (QuikChem 800, Lachat Instruments). Standard samples of known nitrogen concentration and blank samples were included to validate the efficiency of digestion and elemental analysis. Nitrogen results are expressed as % DM.

Plant cell wall components were analyzed by sequentially fractioning dried ground foliage. Neutral detergent Fiber (NDF), acid detergent Fiber (ADF), and acid detergent lignin (lignin) were determined following the ANKOM Technology procedures (ANKOM $^{200/220}$Technology Operator's Manual, 1997). Results are expressed as % DM.

Secondary Chemistry. Essential oils (terpenes) were extracted using dichloromethane with heptadecane as an internal standard (100 mg of heptadecane was diluted in 1 l of dichloromethane) (O'Reilly-Wapstra et al., 2004). One gram of thawed foliage, cut into approximately one cm pieces, was soaked in the dichloromethane solvent for 1 hr. Extracts were analyzed by combined gas chromatography-mass spectrometry (GC-MS) on a Varian 3800 g chromatograph coupled to a Varian 1200 triple quadrupole mass spectrometer, using a 30 m × 0.25 mm Varian 'Factor Four' VF-5 ms column with a 0.25-micron film. One microliter aliquots were injected into a Varian 1177 split/splitless injector at 200°C using a split ratio of 20:1, and a GC column temperature profile of 60°C to 260°C at 12 degrees per minute. The electron energy was 70eV, and the range from m/z 35 to 350 was scanned on quadrupole 1 three times per sec. Total ion currents (TIC) were determined separately for 1,8-cineole, the sum of all oil components (referred to as total oils), and for the heptadecane internal standard. Results for 1,8-cineole and total oils were then standardized by dividing by the internal standard. A response factor for cineole relative to the heptadecane internal standard was determined from a pure standard, and the amount of 1,8-cineole was expressed as mg.g DM^{-1}. The amount of total oils was expressed as equivalents of cineole (mg.g DM^{-1}).

Total phenolics and condensed tannins were assayed using the modified prussian blue assay for total phenolics (Graham, 1992) and the acid butanol assay for condensed tannins (Porter et al., 1986). Foliage for these assays was prepared and extracted following the method outlined in Hagerman (1995). In brief, 0.6 g of air dried, ground leaf was weighed into glass tubes, and 5 ml of 70% acetone were added. Samples were sonicated at 4°C for 30 min and centrifuged for 10 min at 2800 g.min^{-1}. The supernatant was poured off and saved at 4°C. This process was repeated 3 more times for each sample. The supernatants were combined for each sample. The concentration of the total phenolics (mg.g DM^{-1}) was determined in relation to a gallic acid standard (SIGMA G-7384), and the concentration of condensed tannins (mg.g DM^{-1}) was determined in relation to a purified sorghum tannin standard.

Formylated phloroglucinol compounds were extracted following the method outlined in Wallis et al. (2003). Specific representative FPCs were determined by HPLC using a Waters Alliance 2690 HPLC with a Waters 996 UV/Vis diode array detector. The chromatogram at 280 nm was generated for peak area measurements from diode array data acquired between 230 nm and 400 nm. The column was a 250 mm × 4.6 mm Wakosil C18RS 3micron (SGE), using a flow rate of 1 ml/minute. A tertiary gradient system was used; solvent A was 0.1% trifluoroacetic acid in acetonitrile, solvent B was Milli-Q water, and solvent C was 'hexanes' (85% n-hexane, Mallinckrodt). The initial mix was 93:7 A and B, then a linear gradient to 100% A between 13 and 15 min was used. At 20 min, the solvent mix was immediately changed to 95:5 A and C, and this was held until 27 min when it was immediately changed to 90:10 A and C to help remove late eluting FPCs. At 38 min it was ramped back to starting conditions through 100% A and equilibrated for 7 min before the next sample. Peaks were identified by retention times and UV spectra in comparison to authentic standards, and representative samples were also analyzed by combined negative ion electrospray HPLC-MS to confirm that the molecular weights and tandem MS data of the peaks selected from the UV trace were also consistent with the standards (Eyles et al., 2003). Under these conditions, retention times were: sideroxylonal A 10.53 min, sideroxylonal B 15.32 min, sideroxylonal C 11.11 min, macrocarpal A 8.26 min, macrocarpal B 9.86 min, macrocarpal G 25.00 min. Results are expressed as mg.g DM^{-1} for sideroxylonal A and C and macrocarpal A and B. Results for sideroxylonal B are expressed as mg.g DM^{-1} equivalents of sideroxylonal A, and results for macrocarpal G are expressed as mg.g DM^{-1} equivalents of macrocarpal A.

Statistical Analysis. Results from the field trial were analyzed by first fitting a mixed model to the individual tree browsing data to determine if there was any significant difference in browsing score across the four hybrids and to test for replicate effects in the field trial design. Residuals were checked for normality and homoscedasticity with the general linear model procedure (PROC GLM) in SAS (SAS Institute Inc., SAS version 8), and the data were log transformed. Log transformed browse score was the dependent variable and the fixed effect

was the hybrid type. The random effects were replicate and replicate by hybrid type interaction. The model was fitted with a PROC MIXED procedure in SAS (SAS Institute Inc., SAS version 8). A *posteriori* multiple pair-wise comparisons of significant effects (least squares means) were made using the Tukey-Kramer adjustment. We performed *a priori* contrasts (contrast statement, SAS Institute Inc., SAS version 8) to test among predicted patterns of browsing damage on the parental and F_1 hybrids (Fritz et al., 1996; Hjältén et al., 2000). Figure 1 illustrates the contrasts performed and the possible expected outcomes.

Results from the feeding trial were analyzed as four separate trials: Trial 1 = SN and SS; Trial 2 = NN and SS; Trial 3 = NS and SN; and Trial 4 = NN and NS. The PROC UNIVARIATE procedure in SAS was performed for each trial to determine if the difference in intake between the two pairs was significantly different from zero (SAS Institute Inc., SAS version 8).

Results for each chemical constituent were first analyzed by fitting a one factor general linear model (PROC GLM) for each chemical variable with hybrid

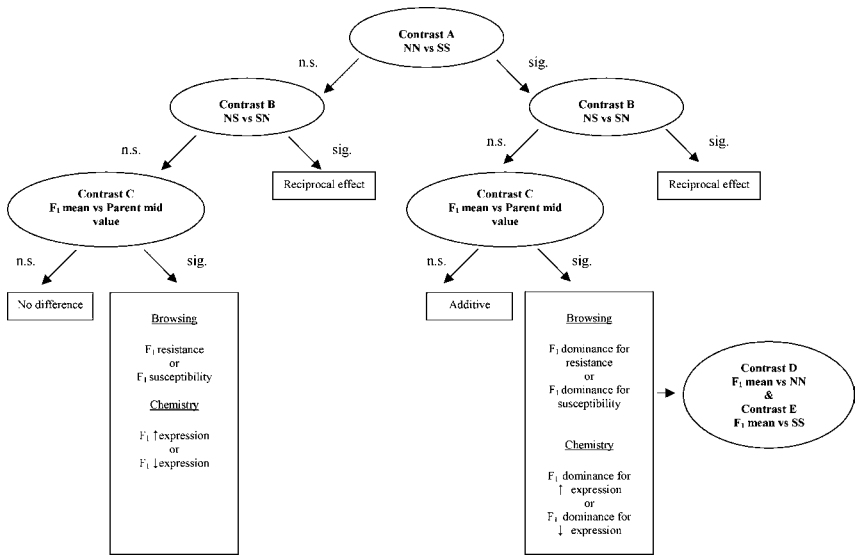

FIG. 1. Description of the contrast procedures and the potential outcomes of inheritance patterns of the browsing resistance to *T. vulpecula*, as assessed in the field trial, and of the concentration of chemical constituents of *E. globulus* foliage from the captive feeding trial. *NN* the hybrid from the northern Tasmania × northern Tasmania cross; *SS* the hybrid from the southern Tasmania × southern Tasmania cross; *NS* the hybrid from the northern Tasmania × southern Tasmania cross; *SN* the hybrid from the southern Tasmania × northern Tasmania cross. The first letter in the cross represents the maternal parent. *sig* a significant contrast; *n.s.* a non significant contrast.

type (SAS Institute Inc., SAS version 8). Residuals were checked for normality and homoscedasticity. Data for total oils and 1,8-cineole were log transformed, and data for macrocarpal A were inverse transformed (1/macrocarpal A). Following this, if there were significant effects, we performed *a priori* contrasts (PROC MIXED, SAS Institute Inc., 1990) to test among predicted patterns of concentration of chemical constituents on the parental and F_1 hybrids (Figure 1). If an effect was not significant following the general linear model analysis, no contrasts were performed.

RESULTS

Browsing damage by *T. vulpecula* on individual trees at the Weilangta field trial ranged in browsing damage score from 0 (no damage) to 4 (major structural branch damage and major browsing damage in the crown). There were no replicate or replicate hybrid effects ($P > 0.05$). There was a highly significant difference in damage score among the four F_1 hybrid types ($F_{3,51} = 37.90$, $P < 0.001$, Figure 2). The intra-race NN cross was browsed more than the other three hybrid types, while the SS cross was browsed less than the other three hybrid types. There was no significant difference between the inter-race reciprocal F_1 hybrids (NS and SN), and these were intermediate in amount of browsing damage compared to the parental intra-race crosses, supporting the hypothesis of additive inheritance on the log transformed scale (Figure 2).

Table 1 illustrates foliage intake by *T. vulpecula* of each hybrid pair and results of the univariate statistical analysis for each feeding trial. In trial 1, there was no significant difference in intake between the two hybrids SS and SN. In trial 2, foliage from the NN cross was more preferred by *T. vulpecula* than foliage from the SS cross. In trial 3, there was no difference in intake of foliage between the NS and the SN hybrids, while in trial 4 there was no difference in intake of the NN and NS hybrids, although the NN cross tended to be more preferred.

Several chemical constituents varied significantly ($P < 0.05$) among the four hybrid types (Table 2 and 3). There was a difference in the nitrogen content among the four types. The NN hybrid cross had higher nitrogen content than the SS cross. A difference between the two F_1 hybrids indicates a reciprocal cross effect for nitrogen concentration (Table 3), with each F_1 hybrid tending to be closer to its maternal parent indicating a maternal reciprocal effect. However, the range in nitrogen concentration from 1.3% to 1.4% may not be biologically significant. Results for acid detergent Fiber show significantly higher acid detergent Fiber content for the SS cross compared to the NN cross (Table 2 and 3) and F_1 dominance for decreased expression (Table 3). There was variation in total oil and 1,8-cineole concentration among hybrid types (Table 3). Contrast A shows the two intra-race hybrid crosses differed significantly in total oils and 1,8-cineole;

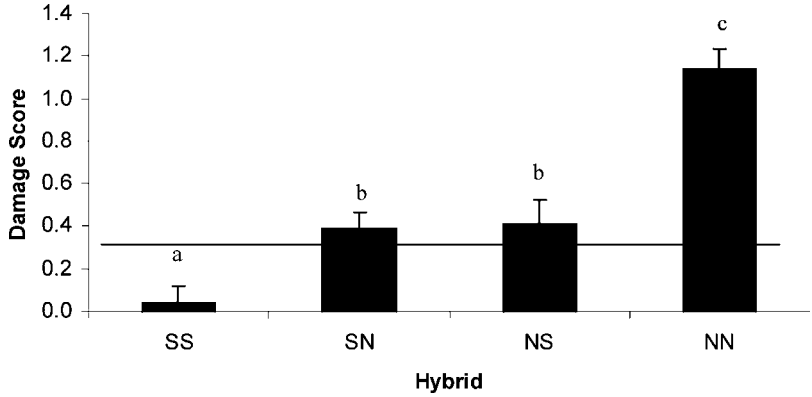

FIG. 2. Browsing damage by *T. vulpecula* for each hybrid type in the field trial. Values are least squares means with SE bars. Letters that differ indicate significant differences ($\alpha = 0.05$ after Tukey-Kramer adjustment for multiple comparisons). The line represents the back transformed mid parent value.

SS had higher concentrations of oil than NN (Table 2 and 3). There was no difference in concentration of total oils and 1,8-cineole between the two F_1 hybrids (contrast B), however, there was a significant difference between the mean F_1 hybrid concentration and the midparent value (contrast C). Significance at contrast D indicates F_1 dominance for increased expression of oil production. There was a significant difference in concentration of condensed tannins among the four hybrid types (Table 3). The SS intra-race cross had more condensed tannins than the NN cross (Table 2 and 3), and there was F_1 dominance for reduced expression (Table 3). There was no difference among the four hybrid types in concentration of sideroxylonal A, however, there were significant differences in all other FPCs (Table 3) despite the concentrations of these compounds being low (Table 2). There was F_1 dominance for decreased expression of sideroxylonal B, and F_1 dominance for increased expression of sideroxylonal C, although these patterns should be viewed with caution due to the low concentrations. A reciprocal effect was evident for all macrocarpal concentrations with the SN F_1 hybrid having higher concentrations of macrocarpals than all other hybrid types. There are no significant differences in concentration of total phenolics, neutral detergent fiber and lignin among the four hybrid types (Table 3).

DISCUSSION

In this study, we had an *a priori* expectation that the two parental hybrids would exhibit differential susceptibility to browsing by *T. vulpecula* (O'Reilly-Wapstra et al., 2002). The patterns of browsing by *T. vulpecula* on *E. globulus*,

TABLE 1. MEAN INTAKE (G DM.KGBM^{-1}) BY *T. vulpecula* OF FOLIAGE FOR EACH HYBRID TYPE IN EACH CAPTIVE FEEDING TRIAL AND RESULTS FOR THE UNIVARIATE ANALYSIS OF EACH TRIAL

Trial	Hybrid Pair	Intake (±S.E.)	*T*-value	*P*-value
1	SS	6.2 (0.59)	1.28	0.256
	SN	5.1 (1.20)		
2	NN	7.7 (1.51)	−3.81	0.012
	SS	4.6 (1.03)		
3	NS	7.7 (2.01)	−0.78	0.471
	SN	5.7 (1.36)		
4	NN	10.1 (1.66)	−2.27	0.073
	NS	6.7 (1.40)		

in previous common environment field trials planted across different geographic areas of Tasmania, have consistently shown that populations of *E. globulus* from north-eastern Tasmania are more susceptible than populations from south-east Tasmania (Volker and Orme, 1988; O'Reilly-Wapstra et al., 2002). This was supported in both the field trial and captive feeding trial results in this present study. The pure parental hybrid from the north-eastern Tasmania race (NN) was more susceptible to *T. vulpecula* than the parental hybrid from the south-eastern Tasmania race (SS). The pattern of hybrid resistance, however, was unknown. The phenotypic expression of resistance of the inter-race F_1 hybrids supports the additive pattern (Fritz, 1999) of hybrid resistance. On the log scale, the F_1 hybrid was intermediate in resistance, compared to the parental hybrids, and this pattern of browsing is consistent over two separate years of field data (Potts, unpublished data). Numerous studies in plant hybrid/insect systems have demonstrated that the patterns of hybrid resistance are highly variable (e.g., Aguilar and Boecklen, 1992; Eisenbach, 1996; Hjältén, 1997; Fritz et al., 1998). In comparison, relatively few studies have examined the resistance of hybrids to mammalian herbivores (but see Vila and D'Antonio, 1998; Dungey and Potts, 2002; Scott et al., 2002; Anderson and Paige, 2003). However, the patterns of hybrid resistance to mammalian herbivores appear just as varied as those patterns expressed to insect herbivores. Increased hybrid resistance (Vila and D'Antonio, 1998), additive inheritance of resistance (Scott et al., 2002), dominance towards susceptibility (Dungey and Potts, 2002), and no difference in hybrid resistance compared to the parental plants (Anderson and Paige, 2003) have all been demonstrated.

Confirmation of the field trial results (the differential resistance of NN and SS) in the feeding trial indicates that the difference in resistance is most likely due to foliage characteristics, such as plant secondary metabolites, as opposed to other characteristics such as tree form. Based on our previous studies (O'Reilly-Wapstra et al., 2004) and research in similar systems (Lawler et al., 2000; Wallis

TABLE 2. CONCENTRATION (LEAST SQUARES MEANS (±SE)) OF PRIMARY AND SECONDARY CHEMICAL CONSTITUENTS OF FOLIAGE OF THE FOUR HYBRID TYPES OFFERED TO *T. VULPECULA* IN THE CAPTIVE FEEDING TRIALS[a]

Constituent	Units	Hybrid			
		SS	SN	NS	NN
Nitrogen	(% DM)	1.2 (0.03)	1.3 (0.03)	1.4 (0.03)	1.4 (0.03)
NDF	(% DM)	38.6 (0.98)	36.4 (0.98)	36.3 (0.98)	37.3 (0.98)
ADF	(% DM)	25.1 (0.54)	23.0 (0.54)	21.3 (0.54)	22.5 (0.54)
Lignin	(% DM)	9.3 (0.48)	8.5 (0.48)	10.4 (0.48)	9.3 (0.48)
Total oil	($mg.gDM^{-1}$ cineole equivalents)	16.0 (1.04)	16.1 (1.04)	16.0 (1.04)	11.0 (1.04)
1,8-cineole	($mg.gDM^{-1}$)	8.0 (0.55)	7.4 (0.55)	8.5 (0.55)	5.6 (0.55)
Total phenolics	($mg.gDM^{-1}$ gallic acid equivalents)	99.3 (4.78)	84.2 (4.78)	76.5 (4.78)	85.4 (4.78)
Condensed tannins	($mg.gDM^{-1}$ sorghum equivalents)	8.3 (0.57)	2.3 (0.57)	2.4 (0.57)	3.5 (0.57)
Sideroxylonal A	($mg.gDM^{-1}$)	0.13 (0.004)	0.12 (0.004)	0.13 (0.004)	0.13 (0.004)
Sideroxylonal B	($mg.gDM^{-1}$ sideroxylonal A equivalents)	0.10 (0.002)	0.09 (0.002)	0.10 (0.002)	0.11 (0.002)
Sideroxylonal C	($mg.gDM^{-1}$)	0.07 (0.001)	0.08 (0.001)	0.07 (0.001)	0.06 (0.001)
Macrocarpal A	($mg.gDM^{-1}$)	0.48 (0.010)	0.71 (0.010)	0.49 (0.010)	0.45 (0.010)
Macrocarpal B	($mg.gDM^{-1}$)	0.35 (0.007)	0.52 (0.007)	0.36 (0.007)	0.33 (0.007)
Macrocarpal G	($mg.gDM^{-1}$ macrocarpal A equivalents)	0.76 (0.029)	1.31 (0.029)	0.78 (0.029)	0.66 (0.029)

[a] *NDF*, Neutral detergent fiber *ADF*, Acid detergent fiber.

TABLE 3. *P*-VALUES FOR THE DIFFERENCE IN BROWSING DAMAGE ACROSS THE FOUR HYBRID TYPES IN THE FIELD TRIAL, THE DIFFERENCE IN CONCENTRATION OF EACH CHEMICAL CONSTITUENT ACROSS THE FOUR HYBRID TYPES (GENERAL LINEAR MODEL ANALYSIS) AND THE CONTRAST COMPARISONS[a]

Variable	GLM	Contrast Comparison					Hypothesis supported
		A	B	C	D	E	
Browsing damage in field trial	<0.001[b]	<0.001	0.439	0.281	—[c]	—[c]	Additive[d]
Nitrogen (% DM)	<0.001	0.002	<0.001	—[c]	—[c]	—[c]	Reciprocal effect
NDF (% DM)	0.363	—[c]	—[c]	—[c]	—[c]	—[c]	No difference
ADF (% DM)	0.007	0.009	0.061	0.017	0.649	0.002	F_1 dominance for ↓ expression
Lignin (% DM)	0.127	—[c]	—[c]	—[c]	—[c]	—[c]	No difference
Total Oils	0.004	0.002	0.950	0.016	<0.001	0.980	F_1 dominance for ↑ expression[d]
1,8-cineole	0.007	0.004	0.171	0.042	0.002	0.868	F_1 dominance for ↑ expression[d]
Total phenolics	0.055	—[c]	—[c]	—[c]	—[c]	—[c]	No difference
Condensed tannins	<0.001	<0.001	0.861	<0.001	0.219	<0.001	F_1 dominance for ↓ expression
Sideroxylonal A	0.073	—[c]	—[c]	—[c]	—[c]	—[c]	No difference
Sideroxylonal B	0.006	0.016	0.072	0.007	0.002	0.266	F_1 dominance for ↓ expression
Sideroxylonal C	<0.001	<0.001	0.128	<0.001	<0.001	0.752	F_1 dominance for ↑ expression
Macrocarpal A	<0.001	0.092	<0.001	—[c]	—[c]	—[c]	Reciprocal effect
Macrocarpal B	<0.001	0.111	<0.001	—[c]	—[c]	—[c]	Reciprocal effect
Macrocarpal G	<0.001	0.036	<0.001	—[c]	—[c]	—[c]	Reciprocal effect[e]

[a] Contrast Comparison a is Between the Two Intra-Race Hybrid Crosses (Parental Crosses), Comparison B Between the Two F_1 Hybrids, Comparison C Between the Mean of the F_1 Hybrids and the Mid-Parent Value, Contrast D Between the Mean of the F_1 Hybrids and NN (the Susceptible Parent), and Contrast E Between the Mean of the F_1 Hybrids and SS (the Resistant Parent). the Final Column Refers to the Hypothesis or Inheritance Pattern that is Supported by the Hybrid Results. *NDF*, Neutral Detergent Fibre, *ADF*, Acid Detergent Fiber

[b] indicates mixed model analysis.

[c] indicates either that no contrasts were performed as there were no significant differences between hybrids in the GLM analysis, or that further contrast analysis between hybrids was redundant.

[d] indicates log transformed data.

[e] indicates inverse transformed data.

et al., 2002), we expected the formylated phloroglucinol compounds (FPCs) to follow the pattern of hybrid resistance observed in this study and to provide the mechanism of resistance in these plants. On the contrary, there was no relationship between FPC concentration and relative resistance of the four hybrid types. The most likely reason for this is that, despite some differences in concentration of FPC compounds in these leaves, the actual concentrations were very low and, therefore, perhaps biologically ineffective in acting as deterrents to *T. vulpecula* in this particular foliage. This point requires further discussion.

In comparison to the three-year-old juvenile foliage used in this study, our previous and ongoing research with *E. globulus* and preferences by *T. vulpecula* has utilized foliage from coppice re-growth (O'Reilly-Wapstra et al., 2002; O'Reilly-Wapstra et al., 2004) and young seedlings (O'Reilly-Wapstra, unpublished data). Concentrations of FPCs in these two types of foliage are higher than those measured in the present study; sideroxylonal A concentration was 0.9–8.5 mg.g DM^{-1} and 0.2–1.8 mg.g DM^{-1} for coppice and seedling foliage, respectively. It is possible that the production of higher levels of FPCs in *E. globulus* coincides with the period when the plant is most vulnerable to mammalian herbivores. *Eucalyptus* seedlings are particularly susceptible to browsing by mammals (Gilbert, 1961; Bulinski and McArthur, 1999), and we speculate that coppice re-growth is also susceptible to mammal browsers, as has been demonstrated with insect herbivores feeding on *Eucalyptus* (Abbott et al., 1993; Steinbauer et al., 1998) and with *T. vulpecula* (O'Reilly-Wapstra et al., 2002). While the concentration of FPCs in the seedling foliage (O'Reilly-Wapstra, unpublished data) are still quite low, the differences in concentration between coppice, seedling and three-year-old juvenile foliage is consistent with this idea. Additionally, although *T. vulpecula* is an arboreal herbivore and is capable of consuming foliage at all successive *E. globulus* life stages, three year old *E. globulus* trees (approximately 4 m in height) may be better capable of recovering from foliage loss. Consequently, defensive mechanisms to mammalian herbivory (FPC production) may not be necessary at this stage, and energy may be allocated to traits such as growth and reproduction. In this species, ontogenetic development of the tree results in heteroblastic leaf change from the seedling leaf phase to the juvenile phase at around 18 wks (James and Bell, 2001). Timing of juvenile to adult foliage phase change is variable (Jordan et al., 2000) but can occur at around 1.5–3 yr (James and Bell, 2001). Many studies have demonstrated developmental changes in leaf resistance to herbivores (e.g., Kearsley and Whitham, 1989; Fritz et al., 2001), including *E. globulus* resistance to insect herbivory (Steinbauer, 2002; Lawrence et al., 2003). Production of defensive chemistry to mammalian herbivory may, therefore, alter at these phase changes and become relatively unnecessary in older trees. However, further investigation of the chemical profile and timing of expression of plant secondary metabolites in *E. globulus* foliage at different life stages is necessary to elucidate this.

There were clear differences in resistance among the four hybrid types, particularly between the two parental hybrids. We suggest that the combined effect of condensed tannin and essential oil concentrations conferred this difference. Condensed tannins and essential oils were significantly higher in the SS than the NN parent. These two groups of plant secondary metabolites have been demonstrated to decrease herbivory by mammalian herbivores in many systems (Harborne, 1991). Tannins have been shown to decrease intake of *Eucalyptus rossii* and *E. consideniana* foliage by *T. vulpecula* (Marsh et al., 2003). Tannins can decrease herbivory by mammals by binding with dietary proteins (Hagerman and Butler, 1991). The maximal flow rate of salivary proline-rich proteins (available to bind with dietary tannins) in *T. vulpecula* is relatively slow compared to other marsupial herbivores (McArthur et al., 1995). This may indicate a decreased ability to cope with ingested dietary tannins. The negative effect of increasing 1,8-cineole levels in artificial diets on intake by *T. vulpecula* has also been highlighted (Wiggins et al., 2003). While the levels of 1,8-cineole required to constrain intake in that study were much higher than in *E. globulus* in the present study, clear preferences at lower concentrations were still detected in the "choice" feeding trials in the latter study. We suggest, therefore, that *T. vulpecula* still responds to foliage chemical characteristics in choice feeding trials, even when levels do not physiologically constrain intake.

We also suggest that the combined effect of tannins and essential oils in this *E. globulus* foliage explains the inheritance of resistance demonstrated in the inter-race F_1 hybrids. The SS parent had significantly higher levels of both compounds compared to the NN parent. While the F_1 hybrids deviate from the mid-parent value in each of these compounds, the direction of dominance was opposite. Condensed tannin levels in the hybrids showed dominance for decreased expression (similar to the NN parent), while essential oils showed dominance for increased expression (similar to SS parent). Consequently, the combined effects of these two groups of compounds (condensed tannin + essential oil) shows an additive pattern of expression: SS hybrid had the highest, NN hybrid had the lowest, and the two inter-race hybrids fell intermediate. This pattern is consistent with the observed patterns of additive inheritance of resistance.

In a review of the effects of hybridization on plant secondary chemistry, Orians (2000) found that hybrids typically express concentrations of compounds at intermediate levels (additive pattern) or at levels similar to one of the parents (dominance pattern). The majority of compounds in this study exhibited the dominance pattern but, interestingly, the direction of dominance varied. As discussed above, the inter-race hybrids showed dominance for decreased expression of condensed tannins, while dominance for increased expression of essential oils was also evident. This could arise, for example, if a dominant gene(s) for the reduced expression of condensed tannins (from the susceptible NN parental hybrid) and a dominant gene(s) for the increased expression of essential oils

(from the resistant SS parental hybrid) are expressed in these hybrids. Few studies have examined the pattern of inheritance of plant secondary metabolites in eucalypts, but two have reported that the inheritance patterns of essential oils in inter-specific hybrids varies. The additive pattern (Dungey et al., 2000; Farah et al., 2002) and the dominance and no difference pattern (Dungey et al., 2000) have all been demonstrated. The variable pattern of results from Dungey et al. (2000) indicate that different compounds, even from the same group (terpenes) can exhibit different inheritance patterns, as also demonstrated with the FPCs in this study.

In summary, we demonstrated that the inter-race differences in the phenotypic expression of resistance to *T. vulpecula* in *E. globulus* are inherited in an additive manner. Variation in resistance of the foliage appeared to be due to the combined effects of condensed tannins and essential oils. These compounds were both in high concentrations in the SS cross and significantly lower in the NN cross. At present, there is no evidence to suggest that past or current densities of *T. vulpecula*, and consequently browsing pressures, differ between the north-east and south-east populations of *E. globulus*. Consequently, explanations as to why these populations differ in resistance and chemical profiles remain to be elucidated. While oils and condensed tannins were inherited in a dominant manner in the inter-race F_1 hybrids, the direction of dominance was opposite, resulting in their combined concentration being inherited in an additive manner. This is consistent with the phenotypic differences in browsing. Five different patterns of hybrid inheritance of plant chemistry were demonstrated. This highlights that patterns of inheritance of resistance and plant secondary metabolites vary not only among plant species, as demonstrated in numerous systems, but also considerably within a plant species and within closely related groups of plant compounds.

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EFFECTS OF TREE PHYTOCHEMISTRY ON THE INTERACTIONS AMONG ENDOPHLOEDIC FUNGI ASSOCIATED WITH THE SOUTHERN PINE BEETLE

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Abstract—We examined the interaction between host trees and fungi associated with a tree-killing bark beetle, *Dendroctonus frontalis*. We evaluated (1) the response of four *Pinus* species to fungal invasion and (2) the effects of plant secondary metabolites on primary growth of and secondary colonization of three consistent fungal associates. Two of these fungi, *Entomocorticium* sp. *A* and *Ophiostoma ranaculosum*, are obligate mutualists with *D. frontalis*, and the third associate is a blue-staining fungus, *O. minus*, that is commonly introduced by beetles and phoretic mites. *O. minus* negatively affects beetle larvae and in high abundance can impact *D. frontalis* population dynamics. Size of lesions formed and quantity of secondary metabolites produced in response to fungal inoculations varied significantly among *Pinus* species. However, monoterpene composition within infected tissue did not significantly vary across treatments. While all eight tested metabolites negatively affected the growth rate of *O. minus*, only 4-allylanisole, *p*-cymene, and terpinene reduced the growth of the mycangial fungi. Surprisingly, growth rates of mycangial fungi increased in the presence of several secondary metabolite volatiles. *O. minus* out-competed both mycangial fungi, but the presence of secondary metabolites altered the outcome slightly. *O. ranaculosum* out-performed *E.* sp. *A* in the presence of dominant conifer monoterpenes, such as α- and β-pinene. Volatiles from the mycangial fungi, particularly *E.* sp. *A*, had a negative effect on *O. minus* growth. In general, phloem phytochemistry of particular *Pinus* species appeared to alter the relative growth and competitiveness of mutualistic and non-mutualistic fungi associated with *D. frontalis*. The outcome of interactions

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among these fungi likely has important consequences for the population dynamics of *D. frontalis*.

Key Words—*Dendroctonus*, *Ophiostoma*, *Entomocorticium*, monoterpenes, plant defenses, competition, mycelial interactions, antagonism, symbiosis, resin.

INTRODUCTION

The oleoresin system of pines is a primary mechanism of tree resistance against subcortical invasion by insects and pathogens (Paine et al., 1997; Trapp and Croteau, 2001). The physical properties of resin, particularly the quantity of resin flow, are important in deterring and excluding attacking insects such as tree-killing bark beetles (Smith, 1966; Raffa and Berryman, 1982; Christiansen and Horntvedt, 1983; Paine and Stephen, 1987; Franceschi et al., 2000). Alternatively, chemical properties of oleoresin are likely more important in disrupting the activity of insects within trees by altering the nutritional quality of subcortical tissues (Smith, 1963; Coyne and Lott, 1976; Hodges et al., 1985). Differences in the chemical composition and physical properties of oleoresin among pine species are thought to be related to differences in resistance to insects and pathogens (Hodges et al., 1979; Tisdale et al., 2003).

More specifically, differences in the oleoresin chemistry among tree species may indirectly affect tree-killing bark beetles by altering the colonization success (Christiansen, 1985; Raffa and Smalley, 1988), growth rate (Bridges, 1987; Ross et al., 1992), and competitiveness of fungi associated with beetles (Raffa, 2001). Mutualistic fungi are thought to enhance the access of beetles to phloem tissues either by helping kill the tree (pathogenic; Solheim, 1992; Paine et al., 1997), neutralizing secondary metabolites within phloem (Paine, 1984), or providing phloem nutrients directly or indirectly to progeny (Barras, 1973; Beaver, 1989). Additionally, mutualistic fungi that are antagonistic to, or resist displacement by, non-mutualistic fungi could contribute to the successful development of beetle larvae (Goldhammer et al., 1990; Klepzig and Wilkens, 1997).

Variation in primary phytochemistry (e.g., N, P, carbohydrates) and secondary phytochemistry (e.g., terpenes, phenolics) likely leads to differences in the relative success and abundance of mutualistic and non-mutualistic fungi (Raffa, 2001), which would influence beetle fitness and subsequently the initiation and speed of beetle population growth (Showalter and Filip, 1993; Trapp and Croteau, 2001). The growth of and interactions between beetle-fungal associates on substrates varying in primary and secondary phytochemistry, however, have not been thoroughly studied.

Here, we focus on three questions dealing with the effects of tree metabolites on the growth and competitiveness of fungi associated with bark beetles: 1. Do trees vary in their response to various fungi associated with bark beetles? 2. Are

growth rates of the various fungal species differentially affected by plant secondary compounds within plant tissues? 3. Are intraguild interactions among fungi influenced by tree-secondary metabolites and beetle by-products? We selected the fungal community associated with the southern pine beetles, *Dendroctonus frontalis* Zimmerman (Coleoptera: Scolytidae), as a study system because (1) *D. frontalis* colonize a broad range of *Pinus* hosts, but have differential reproductive success across pine species and sites (Hodges et al., 1979; Veysey et al., 2003), (2) the reproductive success of *D. frontalis* depends on the presence of mutualistic fungi living in phloem (i.e., endophloedic) (Barras, 1973; Goldhammer et al., 1990), and (3) the presence of non-mutualistic fungi appears to affect larval development and influence broad patterns in *D. frontalis* population dynamics (Lombardero et al., 2003; Hofstetter et al., unpublished data).

We addressed the first question by quantifying the response of four *Pinus* species to artificial inoculations of three prominent fungal associates of *D. frontalis*. We hypothesized that localized tree response (lesion size and chemical composition) to fungal infection is influenced by (1) environmental conditions and (2) tree species. To address whether the response by *Pinus* to fungal infection is an effective defensive mechanism against both fungal invasion and beetle colonization, we quantified the effects of secondary metabolites of *Pinus* on fungal colonization and growth. We used a paired-confrontation assay to determine whether secondary metabolites alter the ability of fungi to resist or suppress invasion, or colonize substrate currently occupied by another fungus. We hypothesize that the presence of particular secondary metabolites alters the competitive interaction between fungi associated with *D. frontalis*.

METHODS AND MATERIALS

Study System. *Dendroctonus frontalis* is a destructive forest pest in the southeastern United States (Price and Doggett, 1978; Coulson and Witter, 1984). This beetle is efficient at finding and exploiting potential hosts (Payne et al., 1978; Nebeker et al., 1993) and can kill large numbers of otherwise healthy, vigorous pine species (Schowalter and Filip, 1993). Successful host colonization and subsequent reproduction of *D. frontalis* depend on the presence of mutualistic fungi, *Entomocorticium* sp. A. and/or *Ophiostoma ranaculosum* (formerly *Ceratocystiopsis ranaculosus*; Jacobs and Kirisits, 2003), that are carried from tree to tree in specialized glandular structures (mycangia) within the prothorax of female beetles (Francke-Grosmann, 1967; Barras and Perry, 1972). The two mycangial fungi are only found in the presence of *D. frontalis*, and the symbiosis is apparently a product of coevolution (Harrington, 1993). In the presence of mycangial fungi, adult beetles lay more eggs, which produce larvae that grow faster, survive better, and produce larger adults (Barras, 1973; Bridges, 1983; Bridges and Perry, 1985;

Goldhammer et al., 1990). However, the two species of mycangial fungi are not equivalent; larvae that feed on *E*. sp. A grow faster and become larger than those feeding on *O*. *ranaculosum* (Goldhammer et al., 1990).

A blue staining fungus, *Ophiostoma minus*, is commonly associated with *D*. *frontalis* (Barras, 1970; Bridges et al., 1985). In southern pine forests, *O*. *minus* is primarily an ectosymbiont of *D*. *frontalis* (Nelson et al., 1934), but it may also be carried by other insects associated with *D*. *frontalis* (Hofstetter, 2004). *O*. *minus* is detrimental to developing beetle larvae (Barras, 1970; Goldhammer et al., 1990; Lombardero et al., 2003), apparently because it displaces the mycangial fungi or disrupts the interactions between larvae and mycangial fungi within phloem tissue (Ross et al., 1992; Klepzig and Wilkens, 1997). In the presence of blue-stain, adults burrow away, while larvae produce atypical, winding galleries and usually die (Barras, 1970; Lombardero et al., 2003). This is unlike beetle species that carry other species of blue staining fungi within their mycangium, in an apparent mutualism (Paine and Hanlon, 1994; Six and Paine, 1998). High levels of *O*. *minus* can contribute to the decline of local beetle populations and the collapse of regional epidemics (Hofstetter, 2004).

Fungal Cultures. We isolated mycangial fungi (*E*. sp. A, *O*. *ranaculosum*) directly from the mycangia of female *D*. *frontals* (methods similar to Barras and Perry, 1972) collected from beetle infestations in the Homochitto National Forest, Mississippi, in June 1998. We isolated *O*. *minus* from the surface of *D*. *frontalis* beetles collected from *D*. *frontalis*-infested bark, also collected in Mississippi, June 1998. We cultured five strains of each fungus on malt extract (2%) agar (MEA) and incubated them at 25°C in darkness.

1. *Do Trees Vary in Their Response to Different Fungi Associated with D*. *frontalis?* We conducted two experiments to assess the response of healthy trees to fungal inoculations. In the first, we tested the hypothesis (1a) that the localized tree response (lesion size and chemical composition) to fungal infection is influenced by soil nutrient availability. In 1993, 45 loblolly pines, 25 years of age, located within an even-aged, thinned loblolly pine plantation in Camp Beauregard near Pineville, LA (31°22′ N, 92°22′ W), were selected for fertilization treatments. Half of the trees (randomly chosen) were fertilized with di-ammonium phosphate (150 kg/ha of phosphorus and 134 kg/ha of nitrogen) during each winter from 1993 to 1998. In August 1998, we inoculated 15 fertilized and 15 unfertilized *Pinus taeda* with the three fungal species. Points of inoculation were 1 m above the ground and separated horizontally around the tree by more than 10 cm. We removed 3 phloem and bark disks (1.22 cm diam) from each tree using a sterile cork borer, inoculated the tree with one of the three fungi, and replaced and sealed the outer bark with duct tape. We randomly selected one of the five strains of each fungus for each inoculation. The inoculums consisted of a 5 mm diam agar plug of culture. After 14 d, we removed the outer bark from the inoculation site, and measured the phloem lesion area (length, width, and total area). We evaluated

fungal growth rate using an ANOVA model (JMP 3.2.1, SAS Institute Inc. 1997) that included the parameters fertilization treatment, fungal species, fertilization-fungus interaction, and tree (nested within fertilization). The parameter fungus strain (within fungal species) was not included in the model because no appreciable difference was observed in fungal growth rate among fungal strains. To correct for heteroscedasticity, we log-transformed lesion size.

In the second experiment, we tested the hypothesis (1b) that localized tree response (lesion size and chemical composition) to fungal infection is influenced by tree species, fungal species, and wounding treatments. We tested four *Pinus* species commonly attacked by *D. frontalis*. In May 1999, we inoculated (as above) 15 trees of *P. echinata* (shortleaf), *P. palustris* (longleaf), and *P. taeda* (loblolly) within one mixed-species stand in the Oakmulgee Ranger District of the Talladega National Forest, Alabama, and 15 trees of *P. virginiana* in the Bankhead National Forest, Alabama. Each tree was inoculated at separate points with each of the three fungal species and chitosan (product of fungal/beetle cell wall; methods of Klepzig and Walkinshaw, 2003) and 2% MEA control. All trees were 30–40 yr of age and 20–30 cm diam trunk (d.b.h.). We also measured phloem thickness and resin flow (methods of Lombardero et al., 2000) just before the inoculations. After 21 d, we removed the outer bark and measured the lesion area. After measurement, we removed one half of each lesion for phytochemical analysis (stored at -80°C) and the other half (stored at -5°C) for fungal isolations. We analyzed total N and C using a Carlo-Erba C:N Analyzer. We analyzed monoterpene content by placing phloem samples (1.00 g) from each lesion into HPLC grade pentane with a *p*-cymene internal standard for 24 hr then analyzed the pentane samples using a 6890 GC equipped with a 5973 MS (Hewlett-Packard Corp., Palo Alto, CA) with an HP-5MS 30 m length $\times$ 250 um ID $\times$ 0.25 um thickness column. The temperature program was 60°C for 1 min- 6°C/min to 200°C, then 15°C/min- to 250°C. Flow rate was 0.9 ml/min, and the injector temperature was 200°C. We identified compounds by their mass spectra and matched the retention time with known standards.

We evaluated lesion size (total area) and lesion phytochemistry using an ANOVA model (JMP 3.2.1) that included *Pinus* spp, fungal spp, *Pinus*-fungus interaction, and tree (nested within *Pinus*). No appreciable difference in lesion size was observed within fungal strains and, thus, was not included in the model. To correct for heteroscedasticity, we log-transformed lesion areas and square root-transformed secondary metabolite concentrations. We computed Pearson product-moment correlations among lesion size, total secondary metabolites, and tree traits with a Bonferroni correction. Individual compounds were analyzed with a MANOVA (same model as previous ANOVA, but with each individual terpene as a dependent variable). Wilk's lambda and Roy's Maximum Root statistic were used to quantify main effects for tree species, inoculation treatment and their interaction.

2. Are Growth Rates of Fungi Differentially Affected by Primary and Secondary Metabolites Within Phloem? We conducted three experiments to assess the effects of volatiles from tree secondary metabolites and fungal metabolites on fungal growth. First, we determined whether growth among fungi varies in the absence of secondary metabolites. Second, we determined whether fungal growth rates change in the presence of particular tree-secondary metabolites. Third, we determined whether volatile byproducts from neighboring fungi influenced fungal growth.

We hypothesized (2a) that differences in primary phytochemistry of trees influences the growth rate of each fungal species. We assessed the growth rates of fungi on intact phloem tissue from which volatile secondary metabolites had been removed. In August 1998, we removed a phloem disk (10 cm diam) from each of 15 fertilized and 15 unfertilized *Pinus taeda* at the Camp Beauregard site. We discarded the outer bark and autoclaved the intact phloem for 20 min at 121°C to volatilize secondary metabolites and sterilize. We divided each phloem disk into thirds and placed them on a thin layer of water agar within a Y-shaped Petri dish (Fisher Scientific Inc.). We then placed on each section a disk of MEA (5 mm diam) that had been previously colonized by *O. minus*, *O. ranaculosum*, or *E.* sp. *A*. The sealed dishes were then incubated at 20°C in the dark. Beginning three d after the inoculation, and every two d thereafter, we traced the area of phloem occupied by hyphae on the lid of the dish. Once the phloem disk was completely colonized by fungi, we removed the lids and analyzed growth (both area captured and linear growth/day) with a planimeter. Prior to inoculation, we analyzed total nitrogen and carbon of each phloem sample using a Carlo-Erba C:N analyzer. We recorded annual tree growth using dendrometer bands affixed at 2 m above ground, and measured tree height growth with a hypsometer. Lombardero et al. (2000) previously reported effects of crown size, fertilization, and drought conditions on tree growth and resin production at this site. Linear growth and area captured by fungi on phloem disks were quantitatively similar within each fungal species. For simplicity and consistency with other experiments, we used linear growth in our statistical tests. We evaluated fungal growth rate using an ANOVA model (JMP 3.2.1, SAS Institute Inc., 1997) that included fertilization treatment, fungal species, fertilization-fungi interaction, and tree (nested within fertilization). To correct for heteroscedasticity, we log-transformed fungal growth rate. We evaluated correlations among fungal growth rates and tree traits (yearly growth, height, resin flow, phytochemistry).

In the second experiment, we tested the hypothesis (2b) that particular compounds commonly found within lesion tissue reduce the growth of each fungal species. To determine the effects of individual compounds on the growth of each fungus, we placed a 0.5 cm disk of MEA colonized with actively growing hyphae of one of the three species (*O. minus*, *O. ranaculosum*, or *E.* sp. A) onto the center of a 100 × 20 mm Petri dish of 2% MEA. We tested seven volatile compounds

commonly found in oleoresin of yellow pines (Pearl, 1975; Hodges et al., 1979): α-pinene, (S,−)β-pinene, (R,+)limonene, γ-terpinene, *p*-cymene, 4-allylanisole (estragole), and myrcene (Sigma-Aldrich, Inc.). Each compound (1.0 ml) was absorbed on a sterile filter paper (55 mm diam) and placed inside the Petri dish and sealed with parafilm; this created an atmosphere that was approximately saturated with the compound. We incubated each plate upside down at 25°C in darkness. Each treatment and a control (filter paper alone) were replicated 10 times for each fungus. We traced the outer edge of fungal growth on the outside of the dish every 2 d using a map tracer. We measured growth in four directions (0°, 90°, 180°, 270°) and averaged them to give a value for each time period. We ended the assay when the fungus reached the end of the Petri dish.

In the third experiment, we tested the hypothesis (2c) that volatile compounds released by neighboring fungi negatively affect fungal growth. We measured the growth rate of *O. minus* in the vicinity of one of four fungi associated with *D. frontalis*. We placed newly inoculated malt-agar plates of *O. minus* (face down) 1 cm above a 20-d old plate of actively growing *O. ranaculosum*, *E.* sp. A, *O. minus*, *Leptographium terebrantis*, or blank malt-extract agar control. We measured the growth of the inverted *O. minus* culture every two d until the plate was completely colonized (∼10 d). We replicated each fungal combination 25 times within separate 60 cm^2 plastic Petri dishes. We analyzed the effects of volatile compounds released from fungi on growth of *O. minus* at 6 d using ANOVA (following square root transformation) followed by Tukey-Kramer HSD.

3. Are Intraguild Interactions Mediated by Secondary Metabolites? We conducted an experiment to assess the effects of secondary metabolites on interactions between fungi. We hypothesized that the presence of tree secondary metabolites alters the ability of each fungal species to resist and suppress invasion or capture territories occupied by another fungus. We quantified area of resources captured by each fungi and observed the interaction between paired fungi using a paired confrontation assay (*O. minus vs. O. ranaculosum*; *O. minus vs. E.* sp. *A*; *O. ranaculosum vs. E.* sp. *A*) on 2% MEA media in the presence of a secondary metabolite. We added an eighth treatment, uric acid, which is a large component of beetle frass and common in larval chambers. We replicated each combination and media treatment 10 times. We aseptically removed disks (5 mm diam) of colonized malt extract agar from actively growing colonies of each of two fungal species and placed the inoculum onto opposite sides of the Petri plate (100 × 20 mm). We placed the fungal disks upside down within 2 cm from the edge of the plate. We placed a filter paper (55 mm diam), saturated with a compound (1.0 ml), inside the Petri dish, or added uric acid (concentrations of 1% or 0.5% by volume) directly to media prior to poring agar into plates. We incubated the plates upside down at 25°C in darkness. We traced the outer edge of fungal growth on the outside of the dish every 2 d. At the termination of the experiment, we measured the area occupied (cm^2/2 d) and the linear growth of each fungus using a digital planimeter

for each time increment. We compared the qualitative interactions between fungi following a key by Porter (1924).

RESULTS

1. Do Trees Vary in Their Response to Different Fungi Associated with D. frontalis? Hypothesis 1a: Localized response (lesion size and chemical composition) to fungal infection is influenced by environmental conditions.-Fertilization of *P. taeda* had no effect on lesion size among fungal species ($F_{2,43} = 0.89$, $P = 0.41$). However, *O. minus* consistently produced larger lesions (mean $\pm$ std: 94.9 ± 57.9 cm^2) than both *E.* sp. *A* (2.4 ± 2.6 cm^2) and *O. ranaculosum* (5.4 ± 4.9 cm^2). Lesion sizes from *O. minus*-inoculations were positively correlated with percent nitrogen in uncolonized phloem ($r = 0.70$), lesion sizes from *O. ranaculosum* were negatively correlated with percent carbon in phloem ($r = -0.63$), and lesion sizes from *E.* sp. *A* were positively correlated with tree growth rate (change in trunk circumference) ($r = 0.55$). All other tree measurements were not correlated with lesion size ($P > 0.05$). Only *O. minus* was successfully isolated (i.e., producing a living culture) from lesion tissues. No fungi were isolated from phloem beyond the lesion area.

Hypothesis 1b: Localized response (lesion size and chemical composition) to fungal infection is influenced by tree species, fungal species, and wounding treatments.–Lesion size in response to fungal inoculations varied little among tree species (Table 1), although *O. minus* produced somewhat larger lesions in *P. taeda* and *P. virginiana* than *P. echinata* and *P. palustris* (Figure 1). The interaction between inoculation treatment and *Pinus* species had no effect on lesion size (Table 1). The only significant correlations (across trees within species) between lesion size and % nitrogen, % carbon, resin flow, or tree diameter (Table 2) was a negative relation between % C in *P. virginiana* and the lesion size from *O. minus* ($r = -0.74$).

The total amount of secondary metabolites within lesions varied among pine species and depended upon the fungal species that was inoculated (Figure 1B, Table 1). In general, *P. echinata* had higher volumes of secondary metabolites per gram of phloem, and *P. palustris* had lower volume of metabolites than the other two tree species. Inoculations of *O. minus* did not result in significant increases in total metabolites in any of the tree species relative to the other inoculation treatments (*O. minus* vs. mean of other treatments; $P > 0.05$). Six compounds were detected in moderate amounts: (in general order of most to least abundant) α-pinene, β-pinene, 4-allylanisole, myrcene, limonene, and camphene. Ratios of the compounds varied significantly among tree species (Table 1, Figure 2). For instance, *P. echinata* and *P. virginiana* had significantly lower concentrations of β-pinene and 4-allylanisole than *P. taeda* and *P. palustris* across all inoculation treatments ($P < 0.05$). Also, limonene concentration in *P. virginiana* was high

TABLE 1. ANOVA TABLE FOR LESION SIZE, TOTAL MONOTERPENES AND MANOVA FOR EACH MONOTERPENE QUANTITY (g/g phloem) IN THE FOUR *Pinus* SPECIES FOR EACH INOCULATION TREATMENT

Source	df	lesion size	total Terp.	α-pinene	β-pinene	myrcene	limonene	4-allylanisole	camphene
Pinus spp	3	0.76	2.91*	3.62*	3.58*	4.48**	5.53**	17.4**	1.04
Inculation treatment	4	80.4**	5.54**	21.4**	16.7**	12.83**	3.11*	12.2**	14.4**
Pinus spp × Inoculation	12	0.90	1.51	0.84	1.05	0.61	0.81	1.32	0.62
Tree {*Pinus* spp}	16	1.65	1.25	2.51**	2.79**	3.11*	6.27*	3.89**	1.53

Note. Wilks' lambda from MANOVA: *Pinus* spp: 0.092**, Treatment: 0.338**, *Pinus* × Treatment: 0.209**, Tree {*Pinus* spp}: 0.018**. *Roy's Maximum Root from* MANOVA: *Pinus* spp. 2.55**, Treatment: 1.62**, *Pinus* × Treatment: 1.44**, Tree {*Pinus* spp}: 2.78**
MSEs, from left to right (with $df = 94$): 0.707,11.19, 247.5, 77.34, 0.322, 3.86, 4.62, 0.039

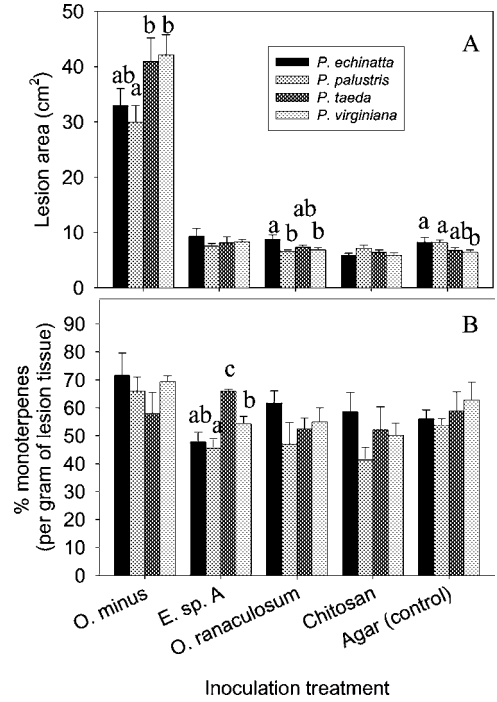

FIG. 1. (A) Lesion size (cm^2) and (B) monoterpene levels (including 4-allylanisole) in 4 *Pinus* species after 14 days in response to inoculation of *Ophiostoma minus*, *Entomocorticium* sp. *A*, *O. ranaculosus*, chitosan, and agar control.

relative to the other three *Pinus* species. Monoterpenes of low concentration (i.e., myrcene, limonene, and camphene) were not detected in some of the inoculation treatments within *Pinus* species (particularly inoculations other than *O. minus* in *P. echinata* and *P. palustris*).

2. Are Growth Rates of Fungi Differentially Affected by Plant Phytochemistry? Hypothesis 2a: Primary phytochemistry influences the growth rate of each fungus. Mean fungal growth on isolated phloem tissue varied across fungal species ($F_{2,43} = 291.1$, $P < 0.001$) with *O. minus* growing about twice as fast as *E.* sp. *A* or *O. ranaculosum* (mean $\pm$ SE $= 2.24 \pm 0.08$, 1.12 ± 0.03, and 0.97 ± 0.03 mm/d, respectively). Fertilization of *P. taeda* had no effect on fungal growth rates (for each fungus: $F_{1,14} < 3.1$, $P > 0.11$), nor did it interact with fungal species ($F = 0.52$, $P = 0.60$). Nitrogen content of phloem was similar between fertilized and unfertilized trees ($0.46 + 0.08$ vs. $0.43 + 0.08$; $F_{1,13} = 0.68$, $P = 0.42$). The growth of *O. minus* was negatively correlated with percent nitrogen in phloem and positively correlated with tree radial growth rate, but growth

TABLE 2. MEASURED TRAITS IN FOUR *Pinus* SPECIES (MEANS ± SD, $N = 15$)

	P. taeda[a]	*P. taeda*[b]	*P. echinata*[b]	*P. palustris*[b]	*P. virginian*[b]
Phloem % nitrogen	0.45 ± 0.08	0.21 ± 0.03	0.22 ± 0.03	0.21 ± 0.04	0.25 ± 0.02
Phloem % carbon	40.7 ± 4.7	50.3 ± 0.7	51.0 ± 1.8	49.0 ± 0.5	46.7 ± 1.1
Trunk diameter (cm)	24 ± 1	30 ± 4	27 ± 3	33 ± 4	28 ± 4
Constitutive resin (g/d)	0.7 ± 0.6	1.4 ± 0.7	1.8 ± 1.8	2.1 ± 1.8	0.2 ± 0.2
Tree height (m)	19.7 ± 1.2				
Tree growth rate[c]	8.9 ± 2.1				

[a] Located at Camp Beauregard, LA in 1998. N and C samples from Camp Beauregard were analyzed separately from Talladega samples.
[b] Located in Talladega National Forest, AL in 1999.
[c] Annual changes in tree circumference (cm) at breast height.

rates of mycangial fungi were not correlated with any measured traits of trees (Table 2). Average fungal growth rates on intact phloem were 17, 24, and 50% less than on 2% malt-extract agar (*O. ranaculosum*, *O. minus*, and *E.* sp A, respectively; $P < 0.01$ for all).

Hypothesis 2b: Volatile compounds commonly found within lesion tissue reduce the growth of fungal species. Fungal growth rates on malt-extract agar were highly sensitive to volatiles of tree-secondary metabolites (Figure 3). Growth of *O. minus* was significantly reduced in the presence of all compounds tested. *Entomocorticium* sp. *A* grew faster in the presence of myrcene, but grew slower in the presence of all other compounds compared to 2% malt agar alone. *Ophiostoma ranaculosum* grew faster in the presence of limonene, myrcene, and α- and β-pinene than on malt agar alone. All fungi grew slowest in the presence of volatiles from 4-allylanisole followed by *p*-cymene.

Hypothesis 2c: Volatile compounds produced by neighboring fungi reduce fungal growth. Growth rate of *O. minus* was generally reduced by the volatiles of other actively growing fungi (Figure 4). Aerial contact with actively growing cultures of *O. minus*, *E.* sp. *A*, and *O. ranaculosum* all reduced the growth of *O. minus* relative to the control; in the middle of the trial, when growth rates were most rapid, these effects were all significant ($P < 0.05$). Mycangial fungi (particularly *E.* sp. *A*) had the greatest negative affect on *O. minus* growth. Only *L. terebrantis* did not reduce *O. minus* growth rate relative to the blank control.

3. Are Intraguild Interactions Mediated by Secondary Metabolites? Hypothesis 3: The presence of tree secondary metabolites alters the ability of each fungal species to resist and suppress invasion or capture territories occupied by another fungus. The ability of fungi to resist or suppress growth by neighboring fungi was altered by the presence of particular secondary metabolites (Table 3). For instance, interactions between *O. minus* and *O. ranaculosum* were altered in the presence of limonene and high uric acid concentrations. All volatiles except *p*-cymene and α-pinene altered the interaction between *O. minus* and *E.* sp. *A* relative to

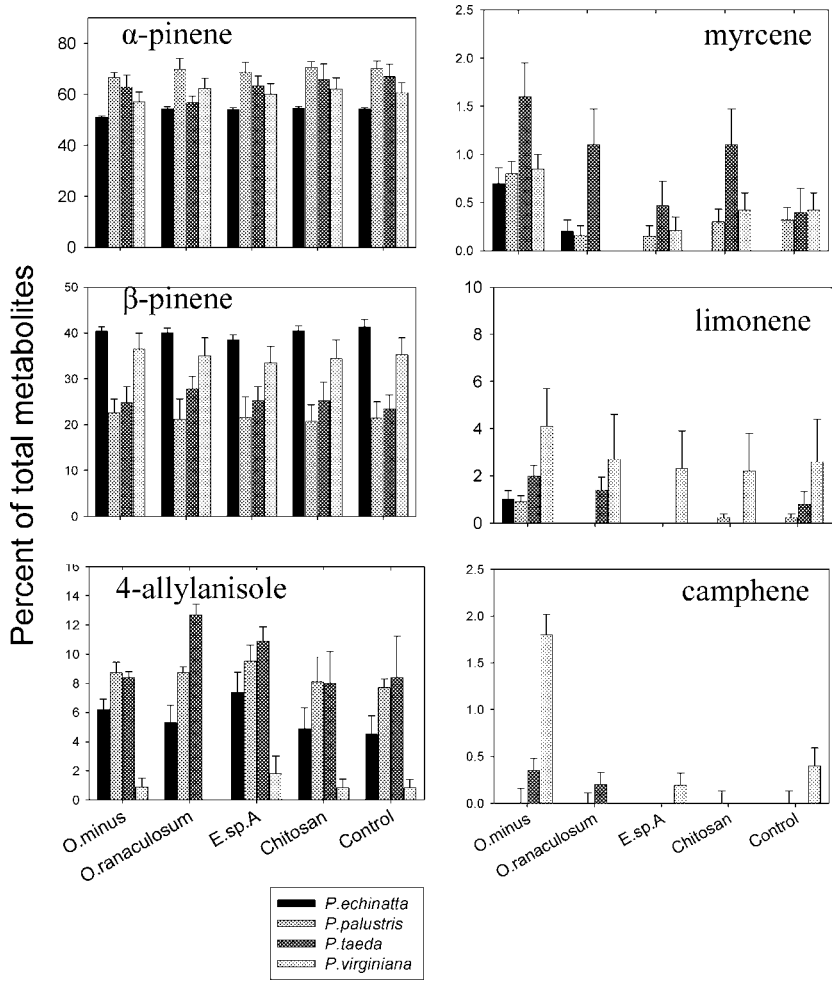

FIG. 2. Secondary metabolite concentrations in lesion tissues within *P. taeda*, *P. echinata*, *P. palustris*, and *P. virginiana* in response to an agar control, chitosan, *Entomocorticium* sp. *A*, *O. ranaculosum*, or *O. minus*.

the interaction observed between the two fungi on malt-extract agar alone. *Entomocorticium* sp. *A* and *O. minus* showed no signs of competitive interactions (no altered or slowed growth) in the presence of β-pinene. In the absence of secondary metabolites, *O. ranaculosum* captured about 46% of the area in competition with *E.* sp. *A* (control in Figure 5). However, *O. ranaculosum* captured significantly more resources in the presence of α-pinene, β-pinene, and 4-allylanisole, and

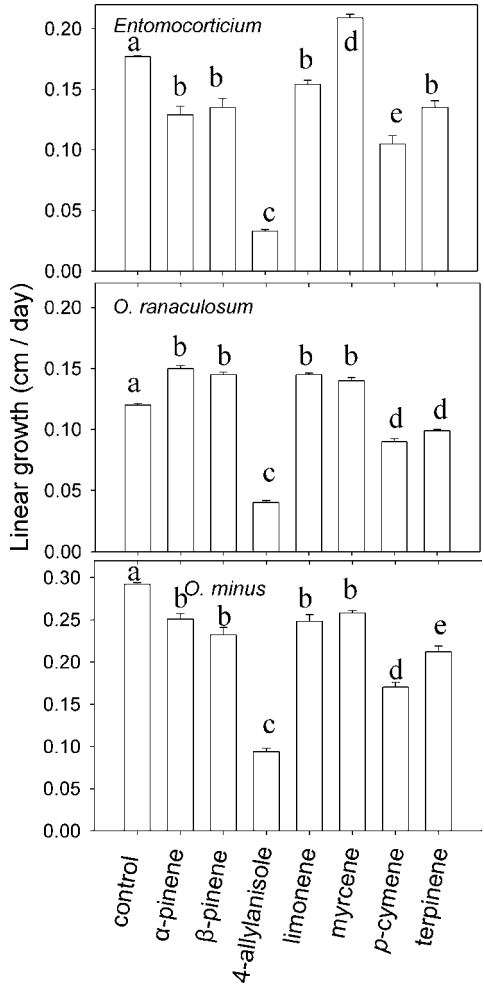

FIG. 3. Mean linear growth rate (cm/day) of each fungus on 2% malt-extract agar in the absence (control) or presence of volatiles from a particular monoterpene. Error bars indicate standard deviation and letters indicate significant differences across treatments ($P < 0.05$).

significantly less in the presence of myrcene, *p*-cymene and terpinene (Figure 5). *O. minus* always captured more area than either mycangial fungi (60–85% of the area; Figure 6). The amount of area captured by *O. minus* vs. mycangial fungi was highest in the presence of 4-allylanisole and similar in the other treatments. In the absence of secondary metabolites, but in the presence of *O. minus*, *E.* sp. *A* colonized more phloem area than *O. ranaculosum* (control in Figure 6).

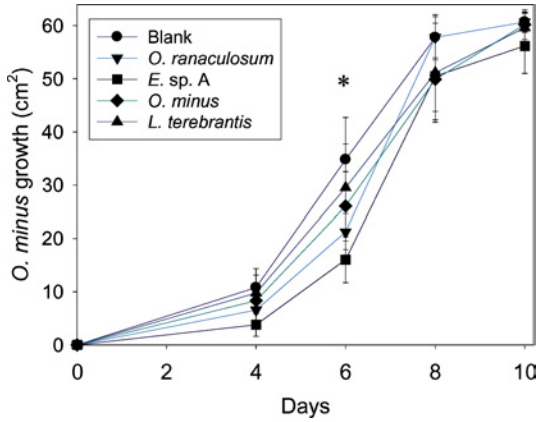

FIG. 4. Area growth (cm^2) of *O. minus* per day in presence of volatiles from other fungi associated with *D. frontalis*-infested trees, including other strains of *O. minus*. *Leptographium terebrantis* is usually found in the roots and does not usually colonize tissues inhabited by *O. minus*.

However, the relative performance of the two mycangial fungi (while in competition with *O. minus*) was altered by secondary metabolites; e.g., *O. ranaculosum* outperformed *E.* sp. *A* in assays with *O. minus* in the presence of α- or β-pinene volatiles (Figure 6).

DISCUSSION

1. Do Trees Vary in Their Response to Different Fungi Associated with D. frontalis? Secondary metabolites within lesion tissue vary depending on the infecting agent and pine species (Figure 2, Table 1). However, no clear pattern between chemical content and lesion size in phloem could be detected (Figure 1). Tree growth rate, d.b.h., % nitrogen or carbohydrates, and resin flow were correlated with lesion size in several tree species but there was no consistent pattern across all tree species. However, given that lesion size was not correlated with fungal growth rate on removed intact phloem from the same *P. taeda*, secondary metabolites (removed from intact phloem by autoclave) likely affect fungal growth ($\approx$lesion size) within trees. Greater induced response to *O. minus* relative to the other mycangial fungi *indicates* that (1) these tree species are highly sensitive (production of an elicitor within the tree; Delorme and Lieutier, 1990) to *O. minus* infection, (2) *O. minus* is pathogenic and thus triggers a greater response compared to that of the mycangial fungi, or (3) *O. minus* is capable of growing faster within resinous lesion tissues than the mycangial fungi. Particular secondary metabolites

TABLE 3. QUALITATIVE RESULTS FROM COMPETITION WITHIN PAIRED COMBINATIONS OF *E*. sp. A, *O. ranaculosum*, AND *O. minus* IN THE PRESENCE OF A MONOTERPENE VOLATILE OR URIC ACID ADDED TO GROWTH SUBSTRATE

Treatment	Pair combination		
	Entomocorticium vs. O. ranaculosum	*O. minus vs. O. ranaculosum*	*O. minus vs. Entomocorticium*
Control	1	2	3
p-Cymene	1	2	3
4-allylanisole	1	2	2,3
Myrcene	1,2[b]	2	2,3
Limonene	1[c]	1,2	2,3
α-Pinene	1,2[a]	2	3
β-Pinene	1	2	1,2
Terpinene	2[b]	2	2,3
0.5% Uric acid	1	2	2,3
1% Uric acid	1	2,3	2,3

Note. Following Porter (1924): "1" designates mutually intermingling growth; "2"-one fungus dominates, the other fungus shows little growth into other fungus; "3"-mutual slight inhibition (slowed growth) of both fungi than overgrowth by one of the fungi. Unless stated otherwise, *O. minus* dominated (grew over) the other fungus while the other fungus stopped growing. No two fungi where mutually inhibited at a distance greater than 2 mm. Two numbers per cell (e.g., 2, 3) indicate that replicates varied within the treatment.

[a] *O. ranaculosum* grew into area previously captured by *E*. sp. A.

[b] *E*. sp. *A* grew into area previously captured by *O. ranaculosum*.

[c] When fungi interacted, *E*. sp. *A* grew under *O. ranaculosum* (which grew only on the surface).

within induced resins may inhibit or slow growth of beetle-mutualistic fungi, or a reduction in available nutrients within lesion tissues might have a greater negative impact on beetle-mutualistic fungi than *O. minus*.

2. Are Growth Rates of Fungi Differentially Affected by Secondary Metabolites? Volatiles of secondary metabolites differentially affected the growth of fungus cultures (Figure 3). In the case of *O. minus*, all secondary metabolites reduced growth. Even with reduced growth rates, *O. minus* still grew faster than the mycangial fungi. Surprisingly, several secondary metabolites increased growth rates of the mycangial fungi relative to the growth rates in the absence of metabolites. Enhanced growth in the presence of a secondary metabolite might result from stimulated (increased) directional growth toward or away from the compound source (Rice, 1970), oxygenation of the compound (Knobloch et al., 1989), a specific response of the fungus to the optical rotation (chirality), number of double bonds (Cobb et al., 1968), or use as a carbon source (Paine and Hanlon, 1994). The two mycangial fungi responded differently to each compound, suggesting that each mycangial fungi might perform differently within different host trees. The major volatile constituents of induced phloem (and oleoresin) might not always enhance

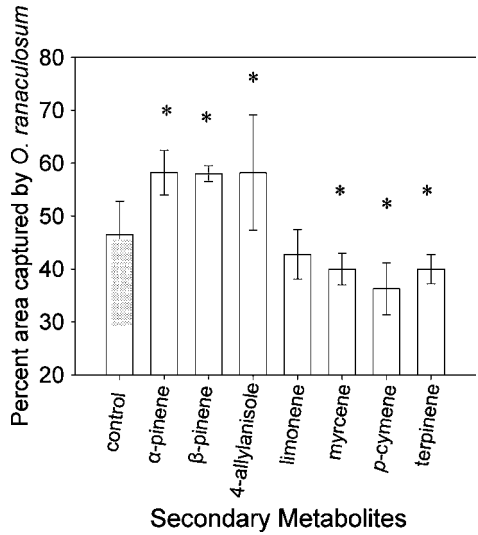

FIG. 5. Percent area captured after 30 days by *O. ranaculosum* compared to *E.* sp. *A* in the absence (control) or presence of a monoterpene (volatiles) in a paired-confrontation assay. Error bars indicate standard deviations and asterisks represent significant difference from control ($^{*}P < 0.05$, $^{**}P < 0.01$).

the resistance of trees to beetle invasion. Each fungus may also respond differently to various concentrations of each metabolite (Paine and Hanlon, 1994). However, small concentrations of secondary metabolites, like 4-allylanisole and terpinene can have strong negative effects on the mycangial fungi (Figure 3) and reduce the attraction of *D. frontalis* to host trees (Strom et al., 1994).

3. Are Intraguild Interactions Mediated by Secondary Metabolites? The relative growth and ability of the mycangial fungi *O. ranaculosum* and *E.* sp *A* to capture resources in the presence of *O. minus* was altered by secondary metabolites (Figure 6, Table 2). Thus, differences in compound concentrations or lack of particular compounds (e.g., no 4-allylanisole observed in *P. virginiana*) and the abundance of *O. minus* within trees may influence the relative frequencies of mycangial fungi within beetle infestations. In our competition studies, *E.* sp. *A* was more effective at reducing *O. minus* growth into colonized areas on artificial media than *O. ranaculosum* (Figure 6; Klepzig and Wilkens, 1997). However, secondary metabolites altered the relative ability of each mycangial fungus to compete with *O. minus*. For instance, *O. ranaculosum* was a relatively better competitor with *O. minus* than *E.* sp. *A* in the presence of α- or β-pinene. Goldhammer et al. (1989) found that high levels of uric acid in growth media reduced *O. ranaculosum* and *O. minus* growth, but increased the growth rate of *E.* sp. *A*. Uric acid

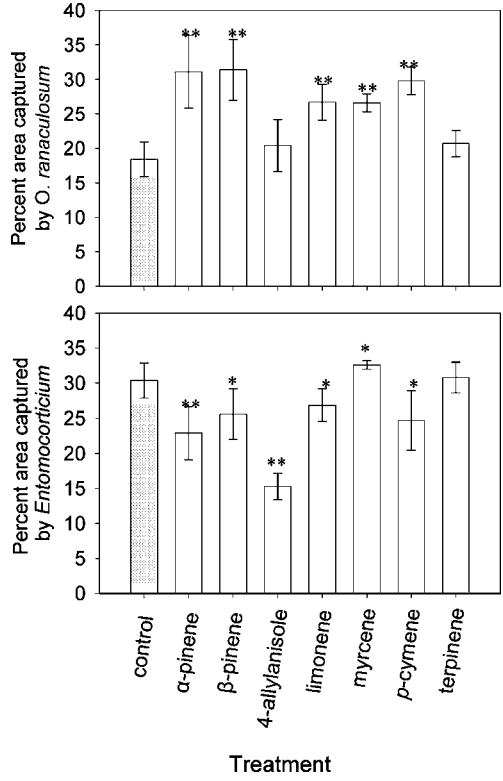

FIG. 6. Percent area captured after 30 days by *O. ranaculosum* or *E.* sp. *A* in paired assay with *O. minus* in the absence (control) or presence of a secondary metabolite (volatile). Error bars indicate standard deviations. Asterisks indicate significant difference from control ($^{*}P < 0.05$; $^{**}P < 0.01$).

also reduced *O. minus*' ability to capture resources previously captured by *E.* sp *A*. Uric acid levels likely increase in larval chambers as beetles develop, and, thus, become more important for competing fungi during the late larval or pupal stage. Phloem chemistry could affect the nature of interactions between fungi by altering the production, diffusion, or volatilization of fungal-produced compounds (Boddy, 2000).

Tree chemistry and interactions among fungi may select for the presence of multiple mutualistic fungi in beetle species that associate with mycangial fungi. Sampling of *D. frontalis* populations, ranging from one colonized tree to greater than 100 trees, have revealed the continual presence of both mycangial fungus (Bridges, 1983; Coppedge et al., 1995; Hofstetter, 2004). In fact, most bark beetle

species that have mycangial fungi appear to have two co-existing species of fungal mutualists (Six, 2002). Alternatively, the presence of mites (e.g., *Tarsonemus* guild) may promote the presence of a second mycangial fungus (e.g., "exploiter" species, Stanton, 2003) (Hofstetter et al., unpublished data).

Effect of Tree-Fungal Interactions on Beetle Populations

Differences in tolerance (represented by growth rate or competition) of the fungi to various compounds have ecological and economic significance. *O. minus* reduces survival of *D. frontalis* larvae (Lombardero et al., 2003) and reduces the growth rate and longevity of *D. frontalis* infestations (Hofstetter et al. unpublished data). Increased levels of *O. minus* within trees also reduce the economic value of wood as a result of blue staining in the xylem (Kreber and Byrne, 1994). All monoterpenes reduced the growth of *O. minus* and, thus, provide some defense against *O. minus* infection. High production of 4-allylanisole or terpinene, in particular, may reduce the relative abundance of blue-stain coverage within trees. Interestingly, increased growth and competitiveness of *O. minus* may minimize the destructive impacts of *D. frontalis* in pine plantations and forests by reducing beetle abundance (Lombardero et al., 2003; Hofstetter, 2004).

Differences in the frequencies of the two-mycangial fungi may affect beetle size, development rate, and reproduction within beetle populations (Goldhammer et al., 1990; Coppedge et al., 1995) and likely influences beetle population growth rate (Bridges, 1983; Ayres et al., 2000). Our results suggest that pine species composition within forests could affect the frequencies of each fungus within an infestation. For example, high levels of particular secondary metabolites within a stand of pines (e.g., shortleaf vs. longleaf) may favor the growth of *O. ranaculosum* relative to *E.* sp. *A*. Seasonal variation in secondary metabolites could generate seasonal variation in the relative abundance of mycangial fungi.

The frequency of each mycangial fungus within local beetle populations may change as beetle populations transition from low to outbreak densities. In endemic (low density) populations, *D. frontalis* usually colonizes trees that already have reduced defenses (e.g., lightning struck tree) that may favor the growth of *O. minus* and *E.* sp *A* relative to *O. ranaculosum* (Figures 3–5). Surveys of endemic populations (between September–December) by Bridges (1983) revealed that *E.* sp. *A* was more abundant than *O. ranaculosum* in 7 out of 8 *D. frontalis* populations. Alternatively, *O. ranaculosum* may become more prevalent in epidemic populations as a result of increased colonization of healthy (resinous) trees. Increased attack rates on trees may also favor *O. minus* growth (relative to mycangial fungi) if high attack rates result in lower secondary metabolites within host trees. Surveys of ten infestations during an epidemic in Alabama revealed that the relative proportions of *E.* sp. *A* and *O. ranaculosum* cycle throughout the year. Temporal variation in mycangial fungi may result from seasonal changes in beetle

attack rate, fungal growth rate, or interspecific interactions (Hofstetter, 2004). In any case, the relative frequency of each fungus in combination with the tree-host selection behavior of *D. frontalis* likely influences local population dynamics of *D. frontalis*.

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POLYAMINE PROFILES OF HEALTHY AND PARASITE-INFECTED *Vaccinium myrtillus* PLANTS UNDER NITROGEN ENRICHMENT

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Abstract—Addition of nitrogen (N) to the field layer of boreal forests has been shown to increase the occurrence of the parasitic fungus *Valdensia heterodoxa* on *Vaccinium myrtillus* plants. We investigated whether N addition to soil alters the levels of polyamines in *V. myrtillus* shoots, and discuss here whether such changes could promote the spread of the parasitic fungus on *V. myrtillus*. Using HPLC, we analyzed the concentrations of free and conjugated polyamines in healthy and naturally *V. heterodoxa*-infected *V. myrtillus* plants, which had received a moderate or high dose of N fertilizer, or no additional N. Fertilization with N increased the concentrations of free diamines (putrescine and diaminopropane), but had no significant effect on conjugated amines. Thus, N-induced changes in the constitutive levels of soluble conjugated amines do not seem to explain the increased parasite susceptibility of *V. myrtillus* under N enrichment. Generally, the concentrations of free diamines and insoluble conjugated putrescine were higher in diseased than in healthy shoots, suggesting parasite-induced accumulation of diamines. Free spermine seemed to accumulate in unfertilized, diseased plants, but in fertilized plants this induction was dampened, suggesting that N-induced alterations in spermine metabolism may promote the spread of parasites on *V. myrtillus* under N-enrichment.

Key Words—diaminopropane, putrescine, spermidine, spermine, nitrogen fertilization, boreal forest, plant-pathogen interactions.

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INTRODUCTION

Increased rates of nitrogen (N) deposition, originating from heavy traffic, intensive agriculture, and industry, have been implicated as major anthropogenic factors affecting the structure and functioning of diverse ecosystems (Vitousek et al., 1997; Bobbink et al., 1998; Lee, 1998). N amendment may lead to increased N uptake by plants (Tomassen et al., 2003), for which N is an essential macronutrient and often available in growth-limiting amounts (Näsholm et al., 1998; Turkington et al., 1998), and to increased cellular content of N (Minocha et al., 2000 and references therein). Increased levels of N in plant tissues may change their chemical composition, and thus affect the nutritive value of the plants for consumers, i.e., microbes and herbivores. Hence, N amendment may have profound ecological consequences at different trophic levels of an ecosystem (Vitousek et al., 1997; Bobbink et al., 1998; Lee, 1998). The ecological effects of increasing N deposition may be especially pronounced in areas where background levels are historically low and the plants have physiologically adapted to the low availability of soil N (Bobbink et al., 1998). Such areas include boreal forest ecosystems.

Characteristic species in the field-layer of N-poor boreal forests are ericaceous dwarf shrubs, e.g., *Calluna* and *Vaccinium* species. Recent research indicates that N fertilization of the field layer in boreal forests leads to a transition from *Vaccinium myrtillus* L.- dominated to grass- (especially *Deschampsia flexuosa* (L.) Trin.) dominated vegetation (Nordin et al., 1998; Strengbom et al., 2002, 2003). In parallel, the incidence of a parasitic fungus, *Valdensia heterodoxa* Peyronel (Ascomycota, Sclerotiniaceae; Vogelgsang and Shamoun, 2002) on *V. myrtillus* leaves increases, leading to severe necrosis and premature shedding of *V. myrtillus* leaves (Strengbom, 2002; Strengbom et al., 2002, 2003). Thus, N amendment seems to predispose *V. myrtillus* to parasite infection. Earlier studies elucidated changes that occur in secondary phenolic metabolism (Witzell and Shevtsova, 2004) and in the primary N metabolism (amino acids) of N-fertilized *V. myrtillus* plants (Nordin et al., 1998; Strengbom, 2002). Nevertheless, the metabolic basis of the N-induced susceptibility of *V. myrtillus* to *V. heterodoxa* is still not completely understood.

Nitrogen amendment is likely to influence the activity of several metabolic pathways in plants, leading to altered rates of production and accumulation of several plant chemicals that may be important in plant-parasite interactions. For instance, the levels of small aliphatic amines, polyamines, often increase in plants as a consequence of N application (Taulavuori et al., 1999; Minocha et al., 2000). This may result from N-induced increases in concentrations of the precursor amino acids (Weidner et al., 1996; Tomassen et al., 2003), such as ornithine and N-rich arginine, which can be decarboxylated to the diamine putrescine (PUT). Transfer of aminopropyl groups from decarboxylated S-adenosyl methionine (SAM) to PUT yields the triamine spermidine (SPD) and the tetramine spermine (SPM).

In addition to these ubiquitous polyamines, some plants also contain several less common amines, such as diaminopropane (DAP), which is formed by polyamine oxidation via the activity of polyamine oxidase (PAO) (Bouchereau et al., 1999; Bagni and Tassoni, 2001). Polyamines are involved in a myriad of physiological and developmental processes (e.g., cell proliferation and differentiation, flowering, and senescence) and are necessary for normal growth of plants (Bouchereau et al., 1999; Igarashi and Kashiwagi, 2000). The metabolism of polyamines is highly responsive to external stimuli, apparently reflecting their importance in the regulation of plant metabolism under environmental challenges (Bouchereau et al., 1999).

Plant polyamines occur as free bases, but since they are positively charged, they can readily bind to negatively charged molecules, such as proteins (forming insoluble conjugates; ISC). The insoluble fraction also includes di- or trisubstituted hydroxycinnamic acid conjugates, through which polyamines can link to cell wall polymers, such as hemicellulose and lignin (Bagni and Tassoni, 2001, and references therein; Walters et al., 2001). Polyamines can also conjugate with small molecules (forming soluble conjugates; SC), mainly phenolic acids, via amide bonds (Martin-Tanguy, 1997; Bouchereau et al., 1999; Walters, 2000). Changes in polyamine metabolism have been recorded in several plant-pathogen interactions (Walters, 2000). Much of the research on polyamines in plant-pathogen interactions has focused on SC forms (Walters, 2000, 2003a,b). Some of the phenolic conjugation partners of amines possess defensive or anti-microbial activity (Nicholson and Hammerschmidt, 1992; Bennett and Wallsgrove, 1994; Dixon and Paiva, 1995), and it has been proposed that conjugation with polyamines may reduce the toxicity of phenolics (Legaz et al., 1998; Bouchereau et al., 1999). On the other hand, increased concentrations of SC polyamines have been detected in some incompatible plant-pathogen interactions, and found to correlate positively with pathogen resistance (Walters, 2003a, and references therein). Recent studies have emphasized that in addition to conjugated polyamines, free polyamines also influence the outcome of some plant-pathogen interactions (Yamakawa et al., 1998; Walters, 2003a, and references therein).

The goals of our study were to investigate whether N fertilization changes the levels of free, SC, and ISC polyamines in boreal *V. myrtillus* plants, and to relate the data acquired to existing information (Strengbom, 2002; Strengbom et al., 2003, 2003) concerning N effects on the parasite resistance of *V. myrtillus*. We analyzed the concentrations of free, SC, and ISC PUT, SPD, and SPM in healthy and *V. heterodoxa*-infected annual shoots of *V. myrtillus* plants, grown in the herbaceous layer of a Norway spruce dominated boreal forest. The plants received no additional N (unfertilized control), a moderate, or a high dose of N fertilizer. We also compared the levels of some phenolic acids that were analyzed after hydrolysis of the polyamine extracts. We were especially interested in finding out whether putative N-induced changes in the pool of SC polyamines could be

linked to the increased susceptibility of *V. myrtillus* to parasite infection under N-enrichment.

METHODS AND MATERIALS

Study Site and Sample Collection. The plant material was collected from an experimental forest at the Svartberget research station, located in northern Sweden, 60 km NW of Umeå (64°14′N, 19°46′E). Norway spruce (*Picea abies* (L.) H. Karst) dominates the tree layer of the forest, and characteristic plant species of the field layer are *V. myrtillus*, *V. vitis-idaea* L., *Linnaea borealis* L., and *D. flexuosa*. In the study area, the background level of N deposition is about 3.5 kg N ha^{-1} y^{-1} (see Strengbom, 2002). The experiment was set up in 1996 as a randomized complete block design. Within each of five blocks (150 × 150 m), three 31.6 × 31.6 m plots were randomly assigned to N fertilization treatments, of 0 (unfertilized control), 12.5 (moderate N level), and 50 (high N level) kg ha^{-1} y^{-1}. The N doses used were ecologically realistic for northern and central European conditions (cf. Dise et al., 1998; Tietema et al., 1998). The incidence of *V. heterodoxa* leaf spot disease on the experimental plots was monitored during 1996–2000, and the data show that the disease is more prevalent on fertilized than on unfertilized plots (Strengbom, 2002; Strengbom et al., 2002).

In the beginning of June 2001, current annual shoots bearing symptoms of *V. heterodoxa* infection, i.e., brownish, necrotic spots (diseased shoots) and visibly healthy shoots (healthy shoots) were collected from unfertilized and fertilized plots (six replicate shoots per permutation, where the pooled material of three shoots made up one replicate). In order to control the physiological age of the shoots, only shoots without any senescence symptoms were collected. Moreover, all shoots were approximately the same size. We standardized the minimum distance between pairs of healthy and diseased shoot to approximately 2 m, to avoid large variations due to possible microclimatic differences within the plots. Since the structure and size of *V. myrtillus* clones in this area are not known, some healthy and diseased shoots may have originated from the same ramet. However, we considered the possible systemic inductions in polyamines as random variation, which could not be controlled. The plant material was transported from the forest on dry ice and stored at −20°C until analyzed.

Analysis of Polyamines and Phenolic Acids. About 0.2 g of frozen plant material was ground in liquid N, extracted in 5% $HClO_4$, and centrifuged at 37 000 g. The pellet was resuspended in the original volume of $HClO_4$. This suspension and the supernatant were hydrolyzed overnight at 110°C in 6N HCl in order to release polyamines from their conjugated forms. Polyamines in the crude and hydrolyzed supernatant (for free and SC polyamines, respectively) and the hydrolyzed pellet (ISC polyamines) were analyzed as dansylchloride derivatives as described earlier (Sarjala and Kaunisto, 1993).

Phenolic acids were released from crude $HClO_4$ extracts by hydrolysis as described by de Armas et al. (1999). The phenolic fractions from crude extracts before and after hydrolysis were analyzed using a Hitachi LaChrom HPLC system equipped with a PDA-detector. The compounds were separated on a Chromolith^{TM} RP-18e (Merck KGaA, Darmstadt, Germany) column (100 × 4.6 mm i.d.). The mobile phase consisted of methanol (B) and H_2O (pH adjusted to 3 with H_3O_4P; A). Elution gradient was: 12–13% B (0–2 min), 13–23% B (2–8 min); 23–35% B (8–12 min); 35–70% B (12–24 min). The flow rate was 2 ml/min and the column temperature 30°C. On the basis of their UV-spectra (200–400 nm), three of the most abundant peaks in the HPLC-chromatogram of the hydrolyzed samples were tentatively identified as *p*-hydroxybenzoic acid (λ_{max}224.7), protocatechuic acid (λ_{max}208.9, 256.7, 291.0), and a benzoic acid derivative (λ_{max}227.5). Peak areas (measured at 270 nm) per unit sample mass before and after hydrolysis were calculated, and the results are expressed as percentages of the respective compounds released by hydrolysis.

Statistics. The effects of fertilization, disease, and their interaction on the levels of polyamines were tested with 2-way ANOVA. Data were log (x + 1) or arcsin transformed prior to analysis when necessary. Student's *t*-tests were used to test the differences between means of healthy and diseased shoots within a single N fertilization level. Data related to phenolic compounds were analyzed using a Kruskall-Wallis test. All statistical tests were carried out using SPSS version 12.0.1 for Windows (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Effects of N on Polyamines. PUT, DAP, SPD, and SPM were the major polyamines detected in *V. myrtillus*, SPD being the most abundant in unfertilized, healthy shoots. N addition resulted in elevated concentrations of free PUT ($F_{2,30} = 361.32$, $P = 0.003$; Figure 1). In addition, DAP levels tended to be higher in the shoots of fertilized plants, especially in diseased shoots (Figure 1). However, we did not find statistically significant N effects on DAP, free SPD, or free SPM. Addition of N did not significantly affect the levels of conjugated (SC or ISC) polyamines. Neither SC nor ISC forms of DAP were detected in *V. myrtillus* shoots.

Infection-associated Changes in Polyamines. The diseased *V. myrtillus* shoots had higher concentrations of free PUT ($F_{1,30} = 1930.80$, $P = 0.001$) and DAP ($F_{1,30} = 29.51$, $P = 0.032$; Figure 1) than healthy shoots. In most cases, the levels of free SPD and SPM also tended to be elevated in the infected shoots, but these differences were not significant. In unfertilized plants, however, the level of free SPM was higher in diseased plants (*t*-test for equality of means, $t = 2.45$, $d.f. = 5$, $P = 0.028$; Figure 1). This difference was reduced by N addition (Figure 1).

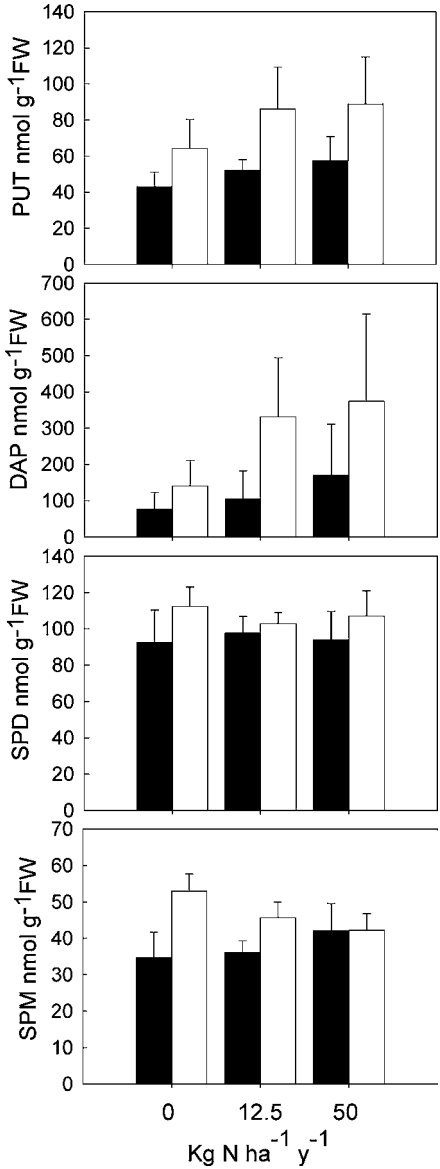

FIG. 1. Concentrations of free putrescine (PUT), diaminopropane (DAP), spermidine (SPD), and spermine (SPM) in healthy (closed bars) and diseased (*V. heterodoxa*-infected; open bars) shoots of *V. myrtillus* plants grown under three nitrogen treatments (0, 12.5 and 50 kg N ha^{-1} y^{-1}). (Vertical bars represent S.E.).

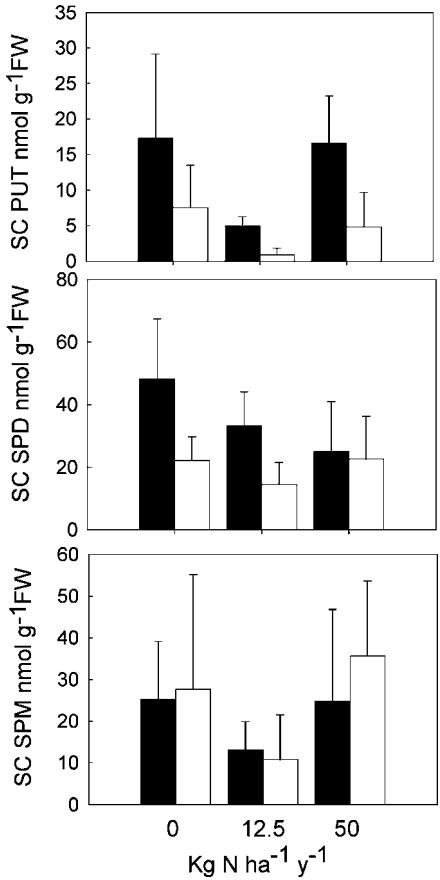

FIG. 2. Concentrations of soluble conjugated (SC) putrescine (PUT), spermidine (SPD), and spermine (SPM) in healthy (closed bars) and diseased (*V. heterodoxa*-infected; open bars) shoots of *V. myrtillus* plants grown under three nitrogen treatments (0, 12.5 and 50 kg N ha^{-1} y^{-1}). (Vertical bars represent S.E.).

The diseased shoots tended to contain less SC PUT, and in both the control and moderate N-treatments also less SC SPD in their leaves (Figure 2). However, we found no significant differences in levels of SC polyamines between healthy and diseased shoots.

The ISC PUT concentration was higher in diseased shoots than in healthy shoots ($F_{1,30} = 72.65$, $P = 0.013$; Figure 2). In unfertilized plants, the concentration of ISC SPM was higher in diseased plants than in healthy plants (t-test for equality of means, $t = 7.50$, $d.f. = 5$, $P < 0.001$; Figure 3).

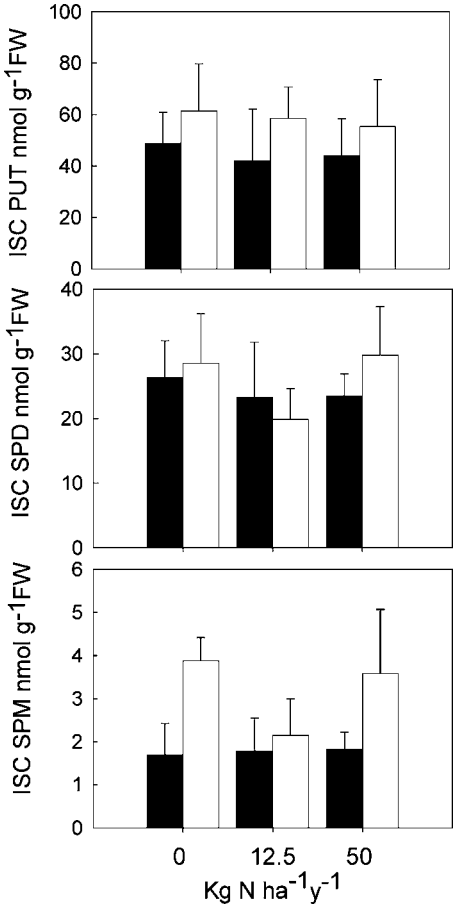

FIG. 3. Concentrations of insoluble conjugated (ISC) putrescine (PUT), spermidine (SPD) and spermine (SPM) in healthy (closed bars) and diseased (*V. heterodoxa*-infected; open bars) shoots of *V. myrtillus* plants grown under three nitrogen treatments (0, 12.5 and 50 kg N ha^{-1} y^{-1}). (Vertical bars represent S.E.).

Effects of N on the Relative Proportions of Free and Conjugated Polyamines in Healthy and Diseased Shoots. To display visually the effects of N fertilization on the relative proportions of specific polyamines within the free and conjugated pools in healthy and diseased shoots, results for PUT, SPD, and SPM are presented as percentages of their total sums (Figure 4). The total pool of PUT was dominated by the free and ISC forms, whereas SPD and SPM more frequently occurred as SCs. In healthy plants, less than 5% of the total SPM pool was present as the ISC form, whereas about 10% of the total SPD and about 40% of the total PUT pool

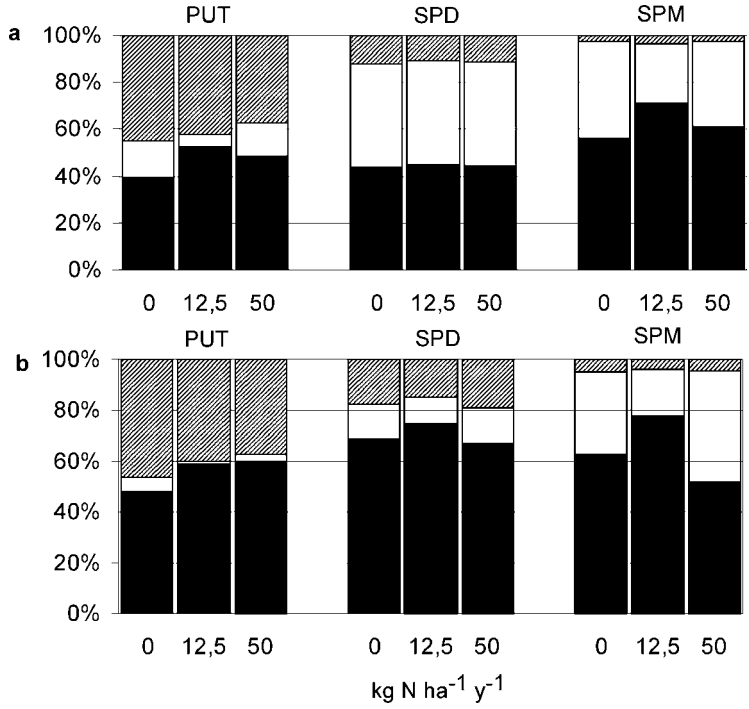

FIG. 4. Relative abundance of free (closed bars), soluble conjugated (open bars) and insoluble conjugated (hatched bars) forms of putrescine (PUT), spermidine (SPD), and spermine (SPM) in healthy (a) and diseased (*V. heterodoxa*-infected b) shoots of *V. myrtillus* plants grown under three nitrogen treatments (0, 12.5 and 50 kg N ha^{-1} y^{-1}).

was found as IS conjugates (Figure 4a). Nitrogen addition had no significant effect on the relative abundance of the different polyamine forms. Infection reduced the relative abundance of SC PUT at all N levels ($F_{1,30} = 4.409$, $P = 0.044$), but did not significantly change the relative abundance of different forms of other polyamines (Figure 4).

Hydrolyzable Phenolics in PCA-extracts. A significantly smaller proportion of p-hydroxybenzoic acid was released by hydrolysis from the extracts of fertilized plants than from unfertilized plants (N effect: $\chi^2 = 6.27$, $P = 0.043$), suggesting that relatively little conjugation of this compound with polyamines occurs under N-amendment. For the protocatechuic acid, a similar trend ($\chi^2 = 5.81$, $P = 0.055$) was observed, which seemed to be due to decreased conjugation in the moderate N-treatment (Figure 5). Parasite infection had no significant effect on the abundance of any of the analyzed hydrolyzable phenolic acids in PCA-extracts.

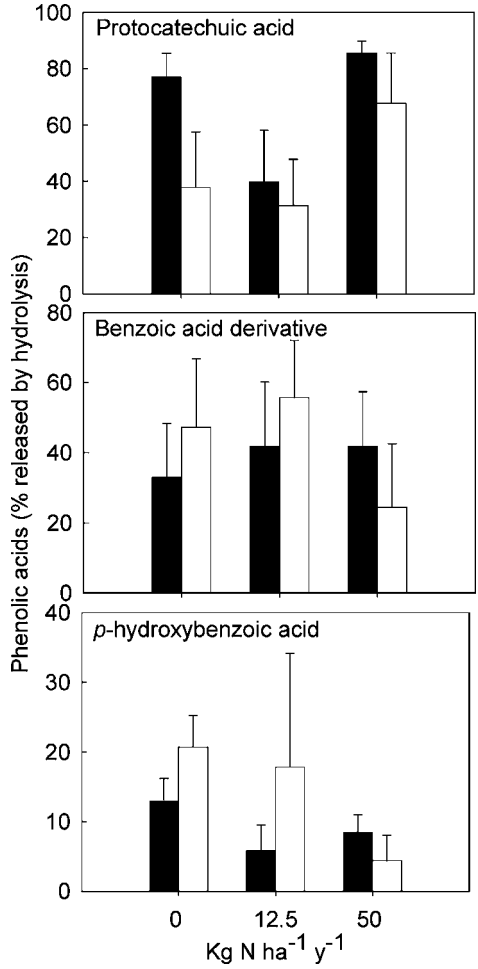

FIG. 5. Percentage of three phenolic acids released by hydrolysis from crude $HClO_4$ extracts of healthy (closed bars) and diseased (*V. heterodoxa*-infected; open bars) shoots of *V. myrtillus* plants grown under three nitrogen treatments (0, 12.5, and 50 kg N ha^{-1} y^{-1}). (Vertical bars represent S.E.).

DISCUSSION

The N-induced elevation we found in the concentration of free PUT is consistent with earlier reports concerning N effects on polyamines (Altman and Levin, 1993; Minocha et al., 2000). Accumulation of free PUT in fertilized plants may be due, at least in part, to stimulated biosynthesis, which could be fueled by

N-induced increases in the availability of amino acid precursors, particularly arginine (cf. Tomassen et al., 2003). Arginine dominates the amino acid profiles of *V. myrtillus* leaves during the late growth season, and its concentration increases in response to N additions (Ohlsson et al., 1995).

SC polyamines have frequently been associated with the expression of plant resistance to pathogen attacks (Legaz et al., 1998; Walters, 2000, 2003a). Therefore, we hypothesized that changes may occur in the constitutive levels of SC polyamines in N-fertilized *V. myrtillus* plants, and that these changes could be causally linked to the plants' increased susceptibility to parasitic fungi. We anticipated that N amendment could either increase or decrease the concentrations of SC polyamines. For instance, if N amendment increases the synthesis of polyamines, then higher proportions might be converted to conjugates as storage forms (Altman and Levin, 1993; Martin-Tanguy, 1997). If conjugation inhibits the antimicrobial actions of phenolics (Legaz et al., 1998), or those of the free polyamines (cf. Walters et al., 2001), increased conjugation could weaken the resistance of *V. myrtillus* to parasitic fungi.

On the other hand, the results from earlier studies indicate that N amendment may reduce the synthesis or accumulation of some phenolic acids in *V. myrtillus* leaves (Witzell and Shevtsova, 2004; for N-effect on phenolics, see also Haukioja et al., 1998). This, we postulated, could reduce the availability of phenolic conjugation partners for polyamines and, thus, SC levels. If SC polyamines have defensive activity in the studied plant-parasite association, N-induced reductions in phenolic acid-polyamine conjugates might explain the observed increase in susceptibility to pathogens. Indeed, the results from the phenolic analysis imply that N-amendment, at least at a moderate level, reduces the conjugation of some phenolics with polyamines in *V. myrtillus* shoots. Nevertheless, the results of the polyamine analyses show that although there was an apparent trend towards lower SC polyamine levels in plants that received a moderate N dose, neither the absolute concentrations of SC (or ISC) polyamines, nor their relative proportions, were significantly influenced by N amendment. Thus, our results suggest that N-induced effects on SC polyamines do not explain the increased susceptibility of *V. myrtillus* to parasites under N enrichment reported by Strengbom (2002) and Strengbom et al. (2002).

The increase in the absolute concentration of free diamines (PUT, DAP) in *V. myrtillus* shoots was associated with infection by *V. heterodoxa*. Since our results show that the increase of PUT in *V. myrtillus* shoots was not dependent on the N treatment, it appears to have been a general stress response to infection. Parasite-induced elevation of free DAP may be due to stimulated oxidation of SPM and SPD in diseased shoots (cf. Drolet et al., 1986; Bors et al., 1989; Bouchereau et al., 1999; Bagni and Tassoni, 2001; Walters, 2003b). The fact that free DAP increases in diseased *V. myrtillus* plants seemed to be strengthened by N-addition could be due to enhanced synthesis or activity of PAO under N enrichment. Elevated levels

of ISC PUT and SPM in infected plants could be linked to increased synthesis and accumulation of stress or defense-related proteins (cf. Terras et al., 1995; Yamakawa et al., 1998).

A significant increase in free SPM was associated with *V. heterodoxa* infection in unfertilized *V. myrtillus* plants, and a weaker increase in N-fertilized plants. In addition to its involvement in the induction of pathogenesis-related (PR) proteins (Yamakawa et al., 1998; Hiraga et al., 2000), spermine metabolism in diseased plants has been associated with enhanced oxidative metabolism, activation of cysteine proteases with aspartate specificity (caspases) that are active in the induction of programmed cell death, maintenance of membrane integrity, and retardation of senescence (Bouchereau et al., 1999; Walters, 2003b and references therein). Such activities of SPM could be important in interactions between plants and parasites such as *V. heterodoxa* that cause extensive necrosis and premature senescence of leaves. Given the increased susceptibility of *V. myrtillus* to *V. heterodoxa* in N-fertilized plants (Strengbom, 2002; Strengbom et al., 2002), the mitigating effect of N on the infection-associated accumulation of free SPM could be one of a number of metabolic changes promoting the spread of parasite infection under N-enrichment. However, an intriguing finding is that the level of macromolecule-bound (ISC) SPM was elevated by infection not only in unfertilized plants, but also in plants receiving the high N dose.

Throughout the studied range of N-regimes, the relative abundance of SC PUT was reduced in *V. myrtillus* shoots that were infected by *V. heterodoxa*, whereas the abundance of free PUT tended to increase in these (diseased) shoots. A similar trend was observed for SPD. This suggests that some of the SC polyamines may have been metabolized to yield free polyamines in infected *V. myrtillus* plants (cf. Cochlan and Walters, 1990). Thus, the free polyamines or their phenolic conjugation partners may be more active separately than as conjugates in this particular plant-pathogen association. The results from the phenolic analysis, which suggested that infection had no significant effect on the association of certain phenolic acids with polyamines, further support the view that SC polyamines do not play a central role in the resistance of *V. myrtillus* against *V. heterodoxa*.

In summary, we found that significant alterations in the absolute concentrations of polyamines in *V. myrtillus* leaves, as well as in the relative abundance of free and conjugated forms of individual polyamines, were associated with parasite infection. However, the *V. myrtillus* plants maintained stable levels of polyamines within the studied range of N-regimes, and we found no evidence suggesting that N-induced changes in SC polyamines could explain the earlier reports of increased susceptibility of *V. myrtillus* to *V. heterodoxa* under N-enrichment (Strengbom, 2002; Strengbom et al., 2002, 2003). Nevertheless, our results imply that infection-associated accumulation of free SPM could be involved in restricting the spread of this fungus on *V. myrtillus* plants in their natural, N-poor habitats. Therefore, SPM metabolism seems to be worthy of more detailed investigations

in future studies concerning N-induced effects on the pathogen resistance of *V. myrtillus* (cf. Walters, 2003a,b).

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THE EFFECTS OF HUMIC SUBSTANCES ON PINUS CALLUS ARE REVERSED BY 2,4-DICHLOROPHENOXYACETIC ACID

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Abstract—The reversal of humic matter-induced inhibition of callus growth and metabolism by 2,4-dichlorophenoxyacetic acid (2,4-D) was studied in *Pinus laricio*. Two forest humic fractions (relative molecular mass (M_r) > 3500), derived from soil under *Fagus sylvatica* (Fs) and *Abies alba* (Aa) plantation, were used. *Pinus laricio* callus was grown for a subculture period (4 weeks) on Basal Murashige and Skoog (MS) medium plus forest humic matters (Fs or Aa), at a concentration of 1 mg C/l, and then was transferred, for an additional four weeks, to a MS medium culture without humic matter, but with different hormones: indole-3-acetic acid (IAA, 2 mg/1) or 2,4-dichlorophenoxyacetic acid (2,4-D, 0.5 mg/1) and/or 6-benzylaminopurine (BAP, 0.25 mg/1). Growth of calluse, glucose, fructose, and sucrose contents, and activities of soluble and bound invertases, glucokinase, phosphoglucose isomerase, aldolase, and pyruvate kinase were monitored. The results show a negative effect of humic fractions on callus growth, due to decreased utilization of glucose and fructose, and decreased activities of glycolytic enzymes. The effects are reversible. Substitution of humic fractions with 2,4-D+BAP or 2,4-D is followed by an increase of glycolytic enzyme activities and, consequently, by the utilization of glucose and fructose that induces a restart of growth. In contrast, the inhibitory effects of humic fractions persist when they are substituted with BAP alone, indicating that only the auxin 2,4-D is capable of reversing the negative effects. A possible competitive action on the auxin-binding site between 2,4-D and the chemical structures in the forest humic fractions is suggested.

Key Words—Humic substances, invertase, glucokinase, phosphoglucose isomerase, aldolase, pyruvate kinase, *Pinus laricio* callus, 2,4-dichlorophenoxyacetic acid.

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INTRODUCTION

Soil organic matter is one of the most useful indicators of soil fertility. A considerable part of organic soil compounds is represented by humic substances. These molecules are characterized by large differences in molecular size, solubility, and chemical composition that are related to changes in climate, vegetation, parent material, and to numerous complex biological, chemical, and physical soil processes. There is much evidence to suggest that humic substances can exert beneficial effects on soil structure, increasing soil aggregation (Soane, 1990), water-holding capacity (De Jong et al., 1983), and cation-exchange capacity (Stevenson, 1994). Moreover, they can influence plant metabolism in regulating plant biochemical and physiological processes (Vaughan and Malcolm, 1985), irrespective of their stimulatory or inhibitory roles (Nardi et al., 1999). Numerous studies have investigated the biological activities of forest humic matter, indicating that vegetation cover is related to their biological activity (Pizzeghello et al., 2001), and also that forest species are affected in different ways by their humic substances (Nardi et al., 2000). In order to consider the biological activity of humic matter with respect to vegetation, our previous studies (Muscolo et al., 1999a, 2001a, 2002) were carried out by testing two forest humic substances derived from soil under *Fagus sylvatica*, L. (Fs) and *Abies alba*, Miller (Aa) plantation on *Pinus laricio* callus and *Pinus laricio* seeds. The results showed that both the humic substances utilized inhibited *Pinus laricio* seed germination, thus affecting the ascorbate system, the amylase activity, and the amino acid metabolism; these observations could explain the difficulty of the natural regeneration of *Pinus laricio* seeds in forest soils. The growth of *Pinus laricio* callus was inhibited by both humic fractions that blocked the utilization of hexoses, the only source of carbon skeleton for callus in culture (Muscolo et al., 1999b, 2001b).

This knowledge on the mechanism of the action of humic substances may be important in understanding the role of humus in the forest ecosystem, where the presence of a lot of active substances (i.e., organic acids, hormones) could interfere with its biological activity.

In the light of this, using an *in vitro* approach to avoid environmental interferences, we wanted to verify if the inhibitory effect induced by humic substances could be reversed by hormones. Thus, *Pinus laricio* callus was grown on Murashige and Skoog (MS) Basal medium supplemented with humic matter (Fs or Aa) and then was transferred into a culture without a humic fraction but with different hormones (IAA or 2,4-D and /or BAP).

Sucrose, fructose, and glucose, and the main enzymes involved in carbohydrate metabolism, were monitored.

METHODS AND MATERIALS

Humus Extraction. Humic matter was extracted from two experimental sites located in the North-East of Italy near the town of Cortina d'Ampezzo (latitude 46° 30′ 29″ N, longitude 12° 08′ 43″ E), each representative of one principal type of vegetation, which have been designated as "Aa" (*Abies alba*, Miller) and "Fs" (*Fagus sylvatica*, L.). The soils were classified as Calcic Luvisols (LVkm) and Humic Cambisols (CMu), according to the FAO-UNESCO system criteria (FAO-UNESCO, 1990). Humic substances were extracted by using 0.1 N KOH in a soil: solution ratio of 1:10 (w/v) at 50°C for 16 hr under a N_2 atmosphere. The extract was desalted by dialysis and ion (H^+) exchange chromatography on Amberlite IR-120 H^+ resin (Stevenson, 1994), then acidified to pH 2.1 with glacial acetic acid. After dialysis against distilled water in Spectrapore 3 tubings (3500 mol. wt cut-off), the retained high relative molecular mass and dialysed low relative molecular mass fractions were further purified by vacuum distillation and ion exchange chromatography (Nardi et al., 1991). In this study, only humic fractions of $M_r > 3500$ were used. Both humic fractions were adjusted to pH 6.0 and used at a final concentration of 1 mg C/l, according to the previous tests showing that in the range of 0.1–5.0 mg C/l, the concentration of humic matter that most interfered with *Pinus laricio* callus was 1 mg C/l. In addition, 1 mg C/l is the concentration of humic substances commonly present in forest soils (Cheng et al., 1996).

Chemical Characterization and Isotopic Determination. Quantitative ^{13}C-NMR spectra were obtained by suspending ca. 300 mg of humic extract in 0.5 ml of 2 M NaOH + 1.5 ml D_2O (Deuterium oxide) for one day at room temperature under N_2. The suspension was filtered through acid-washed glass wool, which was washed with 1 ml of D_2O, and the filtrates were combined. ^{13}C-NMR spectra were recorded at 125.7 MHz on a Bruker AMX-500 spectrometer, using inverse gated decoupling (IGD) experiments for quantitative intensity distribution. Peak areas were obtained by integration. The chemical shift was expressed in ppm on a scale relative to external 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt, at 0 ppm (Piccolo and Conte, 1998). The NMR spectra were divided in five regions: 0–48 ppm (Aliphatic C), 48–105 ppm (Peptidic and Carbohydratic C), 105–145 ppm (Aromatic C), 145–165 ppm (Phenolic C), and 165–190 ppm (Carboxylic C). These assignments were based, in part, on values given in Candler et al. (1997).

Callus Growth. Callus induction was attempted, *in vitro*, from needles of *Pinus laricio*. The initiation medium was composed of MS (Murashige and Skoog, 1962) Basal medium supplemented with Bacto agar (0.8% w/v), sucrose (3% w/v), and growth regulators (2,4-D 0.5 mg/1 and BAP 0.25 mg/1) adjusted to pH 5.7. Callus (1.0 g) was subcultured for 4 wk on MS Basal medium with Fs or Aa humic

fractions (filtered sterilized) but without 2,4-D or BAP (subculture period I). After this period, the callus was transferred to a MS Basal medium culture without humic matter but with BAP (0.25 mg/1) or 2,4-D (0.5 mg/1) or 2,4-D+BAP (0.5 + 0.25 mg/1) or IAA (2mg/1), for a consecutive subculture period of 4 wk (subculture period II). Callus continuously grown on MS basal medium without hormones or humic fractions was used as control. All treatments were carried out at 25°C in the dark. At the end of the treatment, the calluses were weighed and used for enzymatic analysis and soluble sugars determination.

Soluble Sugars Determination. Calluses were extracted ×3 with boiling 80% ethanol (v/v). Homogenates were centrifuged at 12000 *g*, and the ethanolic extract was evaporated under vacuum (Rotavapor RE 111; Büchi, Switzerland), resolubilized in distilled water, and subjected to enzymatic assay. Glucose, fructose, and sucrose were determined by using respectively, hexokinase, glucose-6-phosphate dehydrogenase, and phosphoglucose isomerase, and after enzymatic inversion to D-glucose and D-fructose by the enzyme β-fructosidase (Boehring test combination 716 260) (Bergmeyer and Bernt, 1974). Absorbance was detected at 340 nm (UV-2100 spectrophotometer; Shimadzu, Japan).

Protein Content and Enzyme Activities. Crude homogenates were prepared by grinding the calluses in a prechilled mortar and pestle, with 0.1 M HEPES-NaOH buffer (pH 7.7), containing 10 mM $MgCl_2$, 0.4 mM Na-EDTA, 100 mM Na-ascorbate, 1% (w/v) polyvinylpyrrolidone (PVP), 1% (w/v) bovine serum albumin (BSA), and 5 mM glutathione reduced form (GSH). Extracts were centrifuged at 20000 *g* for 20 min at 4°C. Soluble proteins were quantified with the method of Bradford (1976), using bovine serum albumin as a standard.

The supernatant resulting from this extraction was designated the soluble acid invertase (SAI, EC 3.2.1.26) free fraction. The pellet was suspended in a homogenizing buffer containing 1 M NaCl and left 12 hr at 4°C. The supernatant from this extraction was referred to as bound acid invertase fraction (BAI, EC 3.2.1.26). Invertase activity was measured by using 1% sucrose as the substrate, and the assay was performed at 30 °C in an acetate buffer at pH 4.6 for 1 hr (Borkowska and Szczerba, 1991). The resulting reducing sugars were estimated colorimetrically at 560 nm, by arseno-molybdate method (Nelson, 1944).

Pyruvate kinase (PK, EC 2.7.1. 40): to 450 μl 0.1 M triethanolamine (TEA) adjusted with NaOH to pH 7.75 were added 50 μl 3 mM β-NADH-Na_2-salt in 0.1 M TEA pH 7.75, 50 μl 0.15 M $MgSO_4$-$6H_2O$ and 0.15 M KCl in 0.1 M TEA (pH 7.75), 50 μl L-lactic dehydrogenase diluted to 225 U ml^{-1} with TEA (pH 7.75), and 50 μl extract. The reaction was started after a lag time of 10 min at 30 °C by adding 50 μl 0.225 M 2-phosphoenolpyruvate-Na-H_2O in 0.1 M TEA (pH 7.75) (Nowotny et al., 1998).

Phosphoglucose isomerase (PGI, EC 5.3.1.9): to 530 μl 0.2 M Tris adjusted with 0.1M HCl to pH 9.0 were added 75 μl 20 mM β-NADH-Na_2-salt in distilled water, 75 μl 80 mM fructose-6-phosphate-Na_2 in 0.2 M Tris, pH 9.0, and 20 μl

glucose 6-phosphate dehydrogenase (from yeast) diluted to 30 U ml^{-1} with 0.2 M Tris, pH 9.0. The reaction was started by adding 50 μl extract after a lag time of 20 min at 30 °C (Nowotny et al., 1998).

Glucokinase (GK, EC 2.7.1. 11) activity was measured by coupling glucose phosphate production with NAD reduction by glucose-6-phosphate dehydrogenase and monitoring at A_{340}. The assay contained 50 mM Bicine-NaOH (pH 8.5), 5 mM $MgCl_2$, 1 mM ATP, 1 mM glucose, 1 mM NAD, 1 IU/ml glucose 6-phosphate dehydrogenase, and 50 μl extract. The assay was initiated with glucose (Doehlert et al., 1988).

Aldolase (ALD, EC 4.1.2.13) was assayed spectrophotometrically at A_{340}. The assay contained 50 mM HEPES-NaOH (pH 7.2), 5 mM $MgCl_2$, 1 mM fructose-1,6-bis phosphate, 0.2 mM NADH, 1 IU/ml glycerol-3-phosphodehydrogenase, 1 IU/ml triose phosphate isomerase, and 50 μl extract (Doehlert et al., 1988).

Statistical Analysis. The reported data represent mean values of five replicates. Comparisons between the means were made using the Student-Newman-Keuls test (Sokel and Rohlf, 1969).

RESULTS AND DISCUSSION

Currently, NMR spectroscopy is considered an essential tool in the analysis of humic matter; it is employed to obtain structural information of carbon nuclei in humic matter and on relative amounts of each carbon fraction (Wilson et al., 1987; Preston, 1996). Quantitative ^{13}C-NMR spectra show different features between the two humic substances analyzed (Table 1). Fs humic fraction, derived from soil under *Fagus sylvatica* plantation, contained a greater amount of phenolic and carboxylic C groups than Aa humic fraction, suggesting a possible enrichment of lignin aromatic structures in Fs. In contrast, Aa humic fraction, derived from soil under *Abies alba*, had a higher content of aliphatic, peptidic, and carbohydratic C-groups obtained from polysaccharide and lipid decomposition (Table 1).

TABLE 1. DISTRIBUTION OF C INTENSITY IN DIFFERENT REGIONS (PPM) OF ^{13}C-NMR SPECTRA OF FOREST (FS AND AA) HUMIC FRACTIONS

	Relative intensity (total area %)[a]				
Humic fraction	0–48 aliphatic C	48–105 peptidic and carbohydratic C	105–145 aromatic C	145–165 phenolic C	165–190 carboxylic C
Aa	17.3 ± 0.3	39.8 ± 0.4	21.9 ± 03	9.3 ± 0.2	11.7 ± 0.2
Fs	16.0 ± 0.2	33.9 ± 0.2	21.9 ± 0.4	10.5 ± 0.2	17.7 ± 0.3

[a] Values are mean ± SE ($N = 5$).

TABLE 2. EFFECTS OF DIFFERENT TREATMENTS ON *Pinus laricio* CALLUS GROWTH

Treatments		Fresh weight (mg)[a]	
Subculture period I	Subculture period II	Subculture period I	Subculture period II
Control	Control	2.4 ± 0.10 d	3.0 ± 0.09 f
2,4-D+BAP	2,4-D+BAP	5.8 ± 0.13 a	10.9 ± 0.13 a
BAP	BAP	3.0 ± 0.09 c	4.0 ± 0.09 de
2,4-D	2,4-D	4.2 ± 0.11 b	8.4 ± 0.12 b
IAA	IAA	2.6 ± 0.20 cd	4.2 ± 0.11 d
Aa	Aa	1.4 ± 0.08 e	0.9 ± 0.04 i (dead)
Aa	2,4-D+BAP	1.4 ± 0.09 e	5.3 ± 0.20 c
Aa	BAP	1.4 ± 0.09 e	1.3 ± 0.09 h
Aa	2,4-D	1.4 ± 0.08 e	4.7 ± 0.19 cd
Aa	IAA	1.4 ± 0.09 e	3.0 ± 0.09 f
Fs	Fs	1.0 ± 0.08 f	0.5 ± 0.03 i (dead)
Fs	2,4-D+BAP	1.0 ± 0.09 f	4.3 ± 0.10 d
Fs	BAP	1.0 ± 0.09 f	1.1 ± 0.06 h
Fs	2,4-D	1.0 ± 0.07 f	3.7 ± 0.15 e
Fs	IAA	1.0 ± 0.08 f	2.3 ± 0.09 g

Note. Subculture I and subculture II represent two consecutive culture treatments of 4 wk duration each of the same callus.
Control = callus on MS medium; 2,4-D = 2,4-dichlorophenoxyacetic acid; BAP = 6 benzylaminopurine; IAA = indole-3-acetic acid; Fs = humic fraction from *Fagus sylvatica* plantation; Aa = humic fraction from *Abies alba* plantation.
[a] Values are mean ± SE ($N = 5$). Mean ± SE in the same column, followed by the same letter are not statistically different at $P = 0.05$.

These findings provide evidence that the composition of humic substances may depend on origin and type of organic matter as previously reported by Tan (1998).

Callus incubated on basal medium without growth regulators or humic fractions had a slow growth during subculture periods I and II. A similar behavior was observed when IAA or BAP supplements alone were used. The treatment 2,4-D+BAP produced the largest mean callus weight. Aa or Fs humic fractions significantly decreased callus growth causing, at the end of subculture period II, the callus death (Table 2). Callus grown for four wk with Fs and then transferred, for a consecutive subculture period, to a basal MS medium with 2,4-D+BAP or 2,4-D alone, restarted growth. A significant restart of growth was also observed in callus treated initially with Aa and then with 2,4-D+BAP or 2,4-D alone. A slight restart was noticed when the calluses were cultured initially with Fs or Aa and then subcultered with IAA present (Table 2). Callus incubated initially on MS medium with Fs or Aa humic fractions and then transferred, for a subsequent four wk, to

a medium with BAP only, did not restart growth, showing values similar to those observed in callus grown continuously in the presence of humic fractions.

These results were confirmed by visible changes in the calluses; callus grown for the whole period with only humic fractions was brown, while that grown for 4 wk in the presence of humic fractions and for an additional 4 wk with 2,4-D+BAP or 2,4-D had a few small green areas on it, confirming the restored callus growth. In contrast, callus grown for 4 wk with humic fractions (Aa or Fs) and then for 4 weeks with BAP or IAA, appeared similar to that grown with humic fractions alone.

Heterotrophic cell cultures are dependent on an exogenous carbon source for metabolism, and, generally, this carbon source is the growth-limiting factor. A wide variety of carbon sources (sucrose, glucose, fructose) are used to support the growth of cell culture (Botha and O'Kennedy, 1998). In most cases, plant cell cultures are grown in a medium containing sucrose as the only carbohydrate source. Sucrose catabolism is necessary to provide carbon for respiration process; in most investigated species, it is rapidly hydrolyzed to glucose and fructose before use (Hawker, 1985). Sucrose may be metabolized either by sucrose synthase or invertase, although the contribution of each enzyme can vary with respect to tissue type, function, and age (Sung et al., 1989). Sucrose synthase is crucial to sucrose utilization in fruit development (Wang et al., 1993), and invertase is particularly active in growing tissue (Borkowska and Szczerba, 1991). In callus treated for the entire period (subculture period I and II) with Fs or Aa humic fractions, glucose and fructose levels, produced from sucrose, were higher compared to those detected in control and in callus treated with hormones.

In callus cultured for four wk on medium culture with Fs or Aa and then transferred to a culture medium with 2,4-D+BAP or 2,4-D alone, glucose and fructose values were lower compared to those observed in callus treated with humic fractions alone (Table 3). In contrast, in callus grown in presence of humic fractions for four wk and then with BAP or IAA, glucose and fructose values were similar to those in callus treated for the whole period with humic fractions Aa or Fs alone (Table 3). Moreover, as shown in Table 3, the decrease of sucrose content in callus tissue cultured with different treatments confirmed the cleavage of sucrose into two hexoses.

These data are supported by values of soluble and bound invertase activities. The results reported in Table 4 show that (during subculture period I) both humic fractions, and in particular the Fs, have increased activities in comparison to control and hormone treatments. At the end of subculture period II, humic substances caused callus death, so it was impossible to detect the enzymatic activities. In callus treated for four wk with Fs or Aa and then transferred to a Basal MS medium with 2,4-D+BAP or 2,4-D alone, it was possible to observe a decrease in levels of both invertases. These data indicate that the Fs or Aa humic fractions that inhibited growth and caused an accumulation of hexoses, resulted from an apparent increase of invertase activities.

TABLE 3. GLUCOSE, FRUCTOSE, AND SUCROSE CONTENT (MG ML^{-1}) IN *Pinus laricio* CALLUS GROWN WITH DIFFERENT TREATMENTS

Treatments		Glucose		Fructose		Sucrose	
Subculture period I	Subculture period II	Subculture period I	Subculture period II	Subculture period I	Subculture period II	Subculture period I	Subculture period II
Control	Control	3.80 c*	3.80 e	10.20 cd	10.20 d	0.93 c	0.93 c
2,4-D+BAP	2,4-D+BAP	1.45 e	1.45 g	2.93 f	2.95 h	0.33 e	0.33 e
BAP	BAP	3.80 c	3.80 e	10.60 c	10.60 d	1.97 a	1.97 a
2,4-D	2,4-D	1.95 d	1.90 f	3.47 e	3.45 h	0.82 d	0.82 c
IAA	IAA	3.60 c	3.60 e	9.90 d	10.0 d	0.91 c	0.91 c
Aa	Aa	9.97 b	9.97 b	13.30 b	13.30 b	0.22 f	0.22 f
Aa	2,4-D+BAP	9.97 b	3.87 e	13.30 b	5.70 g	0.22 f	0.19 f
Aa	BAP	9.97 b	10.71 b	13.30 b	13.90 b	0.22 f	1.14 b
Aa	2,4-D	9.97 b	3.90 e	13.30 b	6.50 f	0.22 f	0.98 c
Aa	IAA	9.97 b	9.91 b	13.30 b	11.60 c	0.22 f	0.82 c
Fs	Fs	11.33 a	11.33 a	14.50 a	14.50 a	1.10 b	1.10 bc
Fs	2,4-D+BAP	11.33 a	4.11 d	14.50 a	6.53 f	1.10 b	0.33
Fs	BAP	11.33 a	11.05 a	14.50 a	14.95 a	1.10 b	0.65 d
Fs	2,4-D	11.33 a	5.43 c	14.50 a	7.40 e	1.10 b	1.31 b
Fs	IAA	11.33 a	10.08 b	14.50 a	11.65 c	1.10 b	0.75 d

Note. Subculture I and subculture II represent two consecutive culture treatments of 4 wk duration each of the same callus. Control = callus on MS medium; 2,4-D = 2,4-dichlorophenoxyacetic acid; BAP = 6 benzylaminopurine; IAA = indole-3-acetic acid; Fs = humic fraction from *Fagus sylvatica* plantation; Aa = humic fraction from *Abies alba* plantation.
*Values in the same column, followed by the same letter are not statistically different at $P = 0.05$.

TABLE 4. SOLUBLE (SAI) AND BOUND (BAI) INVERTASE ACTIVITIES (MG GLUCOSE GFW^{-1} H^{-1}) IN *Pinus laricio* CALLUS GROWN WITH DIFFERENT TREATMENTS

Treatments		SAI		BAI	
Subculture period I	Subculture period II	Subculture period I	Subculture period II	Subculture period I	Subculture period II
Control	Control	0.30 de*	0.30 h	0.25 e	0.25 f
2,4-D+BAP	2,4-D+BAP	0.25 e	0.25 h	0.51 c	0.51 d
BAP	BAP	0.35 de	0.35 h	0.39 d	0.39 e
2,4-D	2,4-D	0.44 d	0.44 g	0.06 f	0.06 g
IAA	IAA	1.70 c	1.70 e	5.10 a	5.10 a
Aa	Aa	2.42 b	N.D.	0.30 e	N.D.
Aa	2,4-D+BAP	2.42 b	0.79 f	0.30 e	0.50 d
Aa	BAP	2.42 b	1.90 d	0.30 e	0.98 b
Aa	2,4-D	2.42 b	0.90 f	0.30 e	0.41 e
Aa	IAA	2.42 b	1.80 de	0.30 e	0.88 b
Fs	Fs	10.80 a	N.D.	1.40 b	N.D.
Fs	2,4-D+BAP	10.80 a	2.44 c	1.40 b	0.47 de
Fs	BAP	10.80 a	9.44 a	1.40 b	0.95 b
Fs	2,4-D	10.80 a	5.59 b	1.40 b	0.62 c
Fs	IAA	10.80 a	9.14 a	1.40 b	0.75 c

Note. Subculture I and subculture II represent two consecutive culture treatments of 4 wk duration each of the same callus.
Control = callus on MS medium; 2,4-D = 2,4-dichlorophenoxyacetic acid; BAP = 6 benzylaminopurine; IAA = indole-3-acetic acid; Fs = humic fraction from *Fagus sylvatica* plantation; Aa = humic fraction from *Abies alba* plantation.
*Values in the same column, followed by the same letter are not statistically different at $P = 0.05$. N.D. = not detectable.

Glucokinase, the enzyme that converted the products of sucrose cleavage to hexoses phosphates, was inhibited by humic fractions compared to the control and other treatments; this activity showed, at the end of the subculture period I, values of about 0.60 nmoles NADPH mg FW^{-1} min^{-1} (Table 5). The enzyme most affected by both humic fractions was PGI, which converts glucose-6-phosphate to fructose-6-phosphate (Appeldoorn et al., 1997). Callus grown with Fs or Aa showed a decrease in PGI activity compared to levels in the control treatment. In callus treated for four wk with Fs or Aa humic fractions and then with 2,4-D+BAP or 2,4-D alone, the levels of PGI activity increased remarkably, reaching values of about 35 nmoles NADPH mg FW^{-1} min^{-1}. In callus grown first with humic fractions and then with BAP or IAA, PGI activity was low (about 4.00 nmoles NADPH mg FW^{-1} min^{-1}) (Table 5).

In the presence of humic fractions, the values for the activity of aldolase, the enzyme that converts hexoses phosphates to triose phosphates (Ashihara et al., 1988), were lower than control and hormone treatments. In callus grown with humic fractions for four wk, and then transferred for an additional four wk to a

TABLE 5. EFFECTS OF DIFFERENT TREATMENTS ON GLUCOKINASE (GK) AND PHOSPHOGLUCOSE ISOMERASE (PGI) ACTIVITIES (NMOLES NADPH MG FW^{-1} MIN^{-1}) IN *Pinus laricio* CALLUS

Treatments		GK		PGI	
Subculture period I	Subculture period II	Subculture period I	Subculture period II	Subculture period I	Subculture period II
Control	Control	0.80 b*	0.80 b	30.00 c	30.00 d
2,4-D+BAP	2,4-D+BAP	0.93 a	0.93 a	43.00 b	43.00 b
BAP	BAP	0.82 b	0.82 b	46.10 a	46.10 a
2,4-D	2,4-D	0.85 b	0.85 ab	42.30 b	42.30 b
IAA	IAA	0.83 b	0.83 b	30.00 c	30.00 d
Aa	Aa	0.61 c	N.D.	1.70 d	N.D.
Aa	2,4-D+BAP	0.61 c	0.90 a	1.70 d	35.10 c
Aa	BAP	0.61 c	0.86 a	1.70 d	4.10 e
Aa	2,4-D	0.61 c	0.92 a	1.70 d	35.95 c
Aa	IAA	0.61 c	0.85 ab	1.70 d	4.00 e
Fs	Fs	0.60 c	N.D.	1.04 e	N.D.
Fs	2,4-D+BAP	0.60 c	0.90 a	1.04 e	35.37 c
Fs	BAP	0.60 c	0.82 b	1.04 e	4.33 e
Fs	2,4-D	0.60 c	0.85 ab	1.04 e	35.27 c
Fs	IAA	0.60 c	0.85 ab	1.04 e	4.43 e

Note. Subculture I and subculture II represent two consecutive culture treatments of 4 weeks duration each of the same callus.
Control = callus on MS medium; 2,4-D = 2,4-dichlorophenoxyacetic acid; BAP = 6 benzylaminopurine; IAA = indole-3-acetic acid; Fs = humic fraction from *Fagus sylvatica* plantation; Aa = humic fraction from *Abies alba* plantation.
*Values in the same column, followed by the same letter are not statistically different at $P = 0.05$.
N.D. = not detectable.

MS medium with BAP or IAA, a slight increase of ALD activity was observed (about 3.4 nmoles NADPH mg FW^{-1} min^{-1}); the highest levels of ALD activity were reached when the callus was transferred from humic fractions to 2,4-D+BAP or 2,4-D medium.

The same behavior was observed for PK activity, an important regulatory enzyme of the glycolytic pathway that catalyzes the conversion of phosphoenolpyruvate to pyruvate (Ambashi and Kayastha, 2002). Callus treated for the whole culture period with humic fractions and that grown first with humic fractions and after with BAP or IAA, both had a lower PK activity compared to other treatments (Table 6).

The results show that the callus fractions that utilized Fs or Aa were negatively affected; and the callus growth inhibition was due to the inhibition of glycolytic enzymes involved in carbohydrate catabolism. The block of the glycolytic pathway may induce an inadequate invertase activity response, suggesting that not only the levels of glucose and fructose, but also the amount of the end products of glycolysis

TABLE 6. ALDOLASE (ALD) AND PYRUVATE KINASE (PK) ACTIVITIES (NMOLES NADPH MG FW^{-1} MIN^{-1}) IN *Pinus laricio* CALLUS GROWN WITH DIFFERENT TREATMENTS

Treatments		ALD		PK	
Subculture period I	Subculture period II	Subculture period I	Subculture period II	Subculture period I	Subculture period II
Control	Control	4.30 b*	4.30 c	6.70 b	6.70 c
2,4-D +BAP	2,4-D +BAP	6.90 a	6.90 a	8.30 a	8.30 b
BAP	BAP	6.80 a	6.80 a	6.50 b	6.50 c
2,4-D	2,4-D	7.00 a	7.00 a	6.70 b	6.70 c
IAA	IAA	4.25 b	4.25 c	6.30 b	6.30 c
Aa	Aa	2.00 c	N.D.	2.30 c	N.D.
Aa	2,4-D +BAP	2.00 c	6.20 b	2.30 c	9.76 a
Aa	BAP	2.00 c	3.40 d	2.30 c	3.97 d
Aa	2,4-D	2.00 c	6.42 ab	2.30 c	6.31 c
Aa	IAA	2.00 c	3.31 d	2.30 c	3.77 d
Fs	Fs	1.70 d	N.D.	1.20 d	N.D.
Fs	2,4-D +BAP	1.70 d	6.08 b	1.20 d	8.03 b
Fs	BAP	1.70 d	3.37 d	1.20 d	3.55 d
Fs	2,4-D	1.70 d	6.05 b	1.20 d	7.00 c
Fs	IAA	1.70 d	3.47 d	1.20 d	3.35 d

Note. Subculture I and subculture II represent two consecutive culture treatments of 4 weeks duration each, of the same callus.
Control = callus on MS medium; 2,4-D = 2,4-dichlorophenoxyacetic acid; BAP = 6 benzylaminopurine; IAA = indole-3-acetic acid; Fs = humic fraction from *Fagus sylvatica* plantation; Aa = humic fraction from *Abies alba* plantation.
*Values in the same column, followed by the same letter are not statistically different at $P = 0.05$. N.D. = not detectable.

may stimulate or inhibit this enzyme. Moreover, these data show that the effect caused by these substances is reversible. After four wk, the substitution of humic fractions with 2,4-D+BAP or 2,4-D was followed by an increase of glycolytic enzyme activities and consequently by the increased utilization of glucose and fructose, that induced a restart of growth. The inhibitory effects on callus growth and metabolism were slightly reversed when these substances were substituted with IAA, but it persisted when these substances were substituted with BAP, suggesting that only the auxin 2,4-D is able to reverse these negative effects significantly.

This may be explained by the fact that 2,4-D and IAA have different roles on callus induction in various species (Tao et al., 2002); we have found that, in *Pinus laricio*, 2,4-D is better than IAA in inducing regenerative callus. As reported by Shafer et al. (1985), for carrot culture, auxin IAA does not induce regenerative callus, probably because it is partially degraded within the cells so that it is more difficult to reach the concentration necessary to induce callogenesis. This may

be true also for *Pinus laricio* and explain why IAA, possessing less callogenic activity, is not able to reverse the inhibitory effects of humic matter.

These results indicate competition between the humic fractions Fs and Aa and auxin 2,4-D, suggesting that this effect is due to interference with functional groups, in particular the carboxylic group, that, as suggested by Rubery (1981), is needed for auxin-binding receptors.

The data also show a different inhibitory effect of the two humic fractions. The Fs fraction mostly affects PGI activity, utilization of glucose and fructose, and, consequently, callus growth. The Fs humic fraction has a greater amont of phenolic and carboxylic-C groups that, as suggested by Mato et al. (1972) and Pflug and Ziechman (1981), could also play a role in determining humic substances activity.

Humic matter, thus, shows a complex biological activity depending on its origin and concentration, and, consequently, it can affect plant metabolic parameters in different ways.

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PLANT GROWTH INHIBITION BY *cis*-CINNAMOYL GLUCOSIDES AND *cis*-CINNAMIC ACID

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Abstract—*Spiraea thunbergii* Sieb. contains 1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (CG) and 6-*O*-(4′-hydroxy-2′-methylene-butyroyl)-1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (BCG) as major plant growth inhibiting constituents. In the present study, we determined the inhibitory activity of CG and BCG on root elongation of germinated seedlings of lettuce (*Lactuca sativa*), pigweed (*Amaranthus retroflexus*), red clover (*Trifolium pratense*), timothy (*Phleum pratense*), and bok choy (*Brassica rapa* var *chinensis*) in comparison with that of two well-known growth inhibitors, 2,4-dichlorophenoxyacetic acid (2,4-D) and (+)-2-*cis*-4-*trans*-abscisic acid (*cis*-ABA), as well as two related chemicals of CG and BCG, *cis*-cinnamic acid (*cis*-CA) and *trans*-cinnamic acid (*trans*-CA). The EC_{50} values for CG and BCG on lettuce were roughly one-half to one-quarter of the value for *cis*-ABA. *cis*-Cinnamic acid, which is a component of CG and BCG, possessed almost the same inhibitory activity of CG and BCG, suggesting that the essential chemical structure responsible for the inhibitory activity of CG and BCG is *cis*-CA. The *cis*-stereochemistry of the methylene moiety is apparently needed for high inhibitory activity, as *trans*-CA had an EC_{50} value roughly 100 times that of CG, BCG, and *cis*-CA. Growth inhibition by CG, BCG, and *cis*-CA was influenced by the nature of the soil in the growing medium: alluvial soil preserved the bioactivity, whereas volcanic ash and calcareous soils inhibited bioactivity. These findings indicate a potential role of *cis*-CA and its glucosides as allelochemicals for use as plant growth regulators in agricultural fields.

Key Words—*Spiraea thunbergii* Sieb, Rosaceae, Thunberg Spirea, allelopathy, plant growth inhibitors, *cis*-cinnamic acid, *cis*-cinnamoyl glucosides.

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INTRODUCTION

Environmental safety is a major concern in many countries. The use of natural products would be a helpful way to reduce the risk from synthetic agrochemicals because, in general, the ecosystem can decompose and recycle natural products. Compounds that regulate plant growth have been sought in plants and microorganisms, and plants contain growth-regulating compounds that control their own growth (phytohormones) or that of other plants (allelochemicals).

In recent decades, research aimed at identifying natural chemicals that might serve as commercial herbicides or as prototypes for the development of synthetic analogs has intensified. The most widely used herbicide in the world, 2,4-dichlorophenoxyacetic acid (2,4-D; Figure 1), is an auxin analog. Some

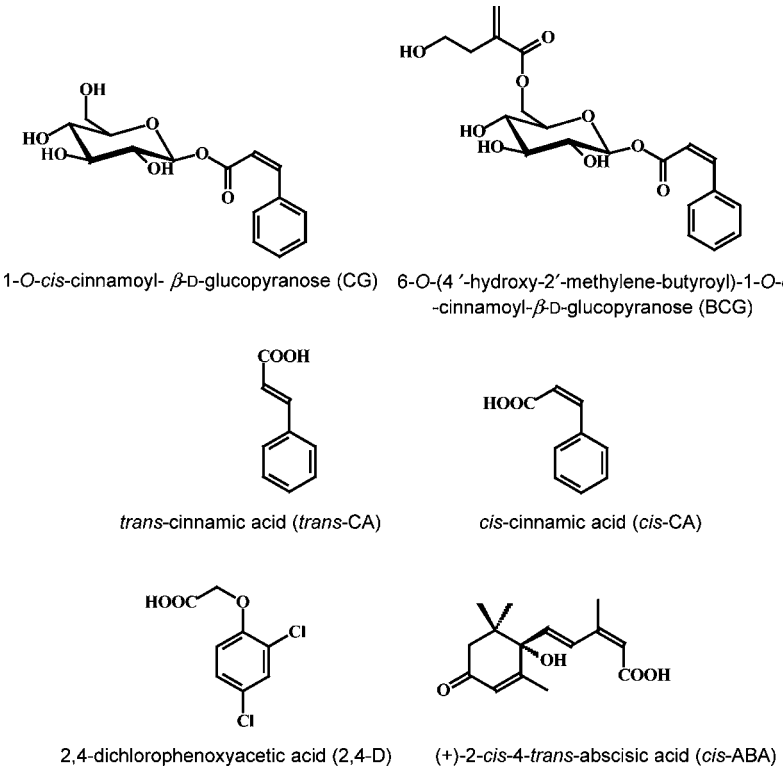

FIG. 1. Chemical structures of 1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (CG), 6-*O*-(4′-hydroxy-2′-methylene-butyroyl)-1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (BCG), *trans*-cinnamic acid (*trans*-CA), *cis*-cannamic acid (*cis*-CA), 2,4-dichlorophenoxyacetic acid (2,4-D), and (+)-2-*cis*-4-*trans*-abscisic acid (*cis*-ABA).

phytohormones, such as (+)-2-*cis*-4-*trans*-abscisic acid (*cis*-ABA; Figure 1), gibberellins, and brassinosteroids, are used as commercial plant growth regulators. The commercial herbicide phosphino-thricylalanylalanin (bialaphos) has also been found among the fermentation products of *Streptomyces hygroscopicus*.

Morita et al. (2001) clarified the growth-inhibition activity of leachates from the leaves of 56 species of woody plants grown in Japan, and found that three Rosaceae plants (*Spiraea thunbergii* Sieb., *S. cantoniensis*, and *S. pruniflora*) had the highest inhibitory activity when assessed with lettuce roots. Hiradate et al. (2004) isolated the inhibitory compounds from *S. thunbergii* using the concept of "*total activity*," which means biological activity per unit weight of the organism containing the bioactive compound, and elucidated the chemical structures from spectroscopic evidence. The compounds were novel *cis*-cinnamoyl glucosides: 1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (CG; Figure 1) and 6-*O*-(4′-hydroxy-2′-methylene-butyroyl)-1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (BCG; Figure 1).

In the present study, we clarified the inhibitory activity (*specific activity*, biological activity per unit weight of the compound) of CG and BCG on the root elongation of five plant species by comparing the two compounds with two well-known plant growth inhibitors (2,4-D and *cis*-ABA). The inhibitory activity of CG and BCG was also compared with that of *cis*-cinnamic acid (*cis*-CA; Figure 1) and *trans*-cinnamic acid (*trans*-CA; Figure 1) to reveal the portion of the chemical structure that is essential for the inhibitory activity of CG and BCG. The activities of CG, BCG, *cis*-CA, and *trans*-CA were also tested in various soil types (alluvial soil, volcanic ash soil, and calcareous soil) to assess the effects of soils on the inhibitory activities.

METHODS AND MATERIALS

Chemicals. 2,4-D (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and *cis*-ABA (Toray Industries, Inc., Tokyo, Japan) were used as reference compounds for the bioassay. Two natural plant growth inhibitors, CG and BCG, were isolated and purified from the leaves of *S. thunbergii* according to the method described by Hiradate et al. (2004). To prepare *cis*-CA, we transformed *trans*-CA (Wako Pure Chemical Industries, Ltd.) into an isomeric mixture of *cis*- and *trans*-CA by irradiating it with an ultraviolet lamp (254 nm) overnight. Pure *cis*-CA was isolated by separating the isomeric mixture using preparative HPLC as described by Sun et al. (2002). To determine the concentrations of CG, BCG, and *cis*-CA, we dissolved 60 μg of *trans*-CA in 0.60 ml of CD_3OD-d_4 in an NMR tube (5-mm i.d.). A solution of each of the purified compounds (hydrated) was also prepared by dissolving an unweighed quantity of the compounds in 0.60 ml of CD_3OD-d_4 in the NMR tube (concentration unknown). Hydrogen-1 (^1H) NMR signals of these samples were recorded quantitatively (acquisition time: 5.46 sec) using an NMR spectrometer (^1H: 600 MHz, JNM α-600, JEOL, Tokyo). We compared the

integrated peak area of the methylene ^{1}H of *trans*-CA with that of the methylene ^{1}H of the cinnamic acid moiety of each compound, and the concentration of each compound was calculated thereby.

Phytotoxic Activities in the Absence of Soil. A filter paper (27 mm diam, Type 1, Toyo Roshi Kaisha, Ltd., Tokyo) was placed in a glass petri dish (27 mm diam). Test compounds were dissolved in water at various concentrations and a 0.7-ml portion of each test solution was added to the filter paper in the petri dishes of each treatment. Five to six pre-germinated (20°C in the dark) seedlings of lettuce (*Lactuca sativa* cv. Great Lakes 366), pigweed (*Amaranthus retroflexus*), red clover (*Trifolium pratense*), timothy (*Phleum pratense*), and bok choy (*Brassica rapa* var. *chinensis*) were used as a single replicate for each treatment, with different species placed on different filter papers to avoid any potential allelopathic effects. Seedlings were incubated for 48 hr at 20°C in the dark, and the inhibitory activity of each test solution on root elongation was detected by measuring the length of each root and comparing it with that of the untreated control (with only distilled water provided). The effective concentration required to induce half of the maximum inhibition (EC_{50}) and its 95% confidence interval were calculated by using the probit method from SPSS for Windows ver. 11.0.1J statistical software (SPSS Japan Inc., Tokyo).

Phytotoxic Activities in the Presence of Soil. An Alluvial soil (Aquept, Soil Survey Stuff, 1999; Anthrosol, FAO, 1998) and a volcanic ash soil (Melanudand, Soil Survey Stuff, 1999; Silandic Andosol, FAO, 1998) were collected from the A_p horizons of paddy and upland agricultural fields in Tsukuba, Japan. A calcareous soil (Hapludalf, Soil Survey Stuff, 1999; Chromic Luvisol, FAO, 1998) was collected from the A_p horizon of an upland agricultural field in Yomitan, Okinawa, Japan. The soils were air-dried and finely sieved (<0.5 mm), and a 500-mg portion (oven-dry basis) of each soil was placed in each well (15.6-mm diam, 17-mm height, 1.9-cm^2 growing area) of a 24-well plate (Nunclon, NalgeNunc, Denmark). We then added 1 ml of a solution containing 10^{-8} to 10^{-2} M of plant growth inhibitor and 0.75% of agar (gelling temperature, 30 to 31°C; Nakalai Tesque, Inc., Kyoto, Japan) at 40°C to each soil-containing well and mixed the components. After incubating the mixture at 30°C for 24 hr in the dark, we placed four lettuce seeds on the gelled agar-soil mixture in the well. The lettuce seeds were grown for 72 hr at 20°C in the dark, and the inhibitory activity was detected by measuring root elongation and comparing it with that of the control (with no plant growth inhibitor, but in the presence of the corresponding soil). This experiment was replicated three times.

RESULTS AND DISCUSSION

Figure 2 shows the inhibitory activities of CG, BCG, 2,4-D, *cis*-ABA, *cis*-CA, and *trans*-CA on the root growth of lettuce seedlings in the absence of soil. In this

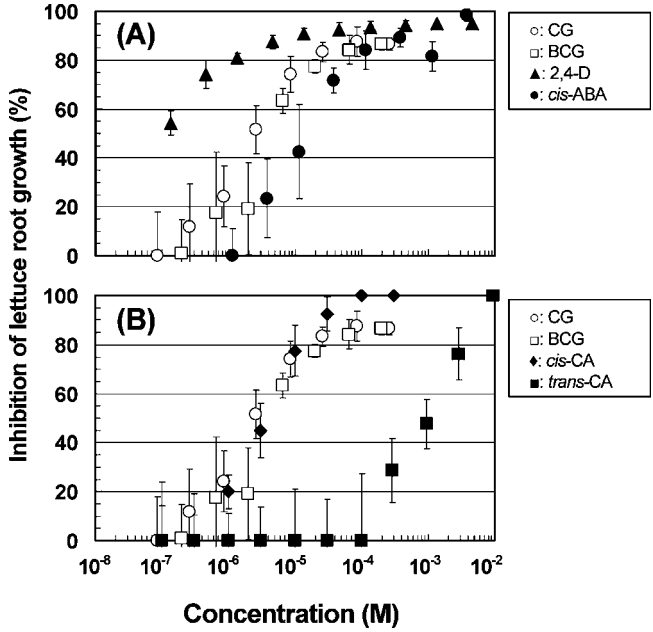

FIG. 2. Inhibitory activities of *cis*-cinnamoyl glucosides (○; CG, □; BCG) in comparison with those of the reference compounds (A: ▲; 2,4-D, ●; *cis*-ABA) and cinnamic acids (B: ♦; *cis*-CA, ■; *trans*-CA) in the absence of soil. Inhibition of root elongation was detected by comparing the root lengths with those of untreated controls after incubation for 48 hr at 20°C in the dark. Bars indicate standard deviations ($N = 5$–6).

experiment, the growth inhibition in the presence of CG, BCG, 2,4-D, *cis*-ABA, and *cis*-CA was attributable to the effects of the compounds themselves, and not to effects of pH or salts, because single treatment with 3×10^{-4} M HCl or 10^{-4} M $CaCl_2$ alone did not affect the growth of lettuce roots (data not shown). As shown in Figure 2, CG, BCG, 2,4-D, *cis*-ABA, and *cis*-CA inhibited the root growth of lettuce seedlings at concentrations below 10^{-4} M.

cis-ABA is one of the most active natural growth inhibitors. Koshimizu et al. (1966) reported that the effective concentration of *cis*-ABA required to induce half the maximum inhibitory action (EC_{50}) on the leaf sheath of rice seedlings was around the 10^{-6} M level. Structural modification of this compound has been attempted to strengthen its specific activity and to make it resistant against biodegradation into inactive forms (Nakano et al., 1995; Todoroki et al., 1997; Arai et al., 1999). In this study, the EC_{50} of *cis*-ABA for root elongation of lettuce seedlings was observed at the 10^{-5} M level (14.9 μM, Table 1).

TABLE 1. INHIBITORY ACTIVITIES OF REFERENCE COMPOUNDS (2,4-D AND *cis*-ABA), CINNAMOYL GLUOSIDES (CG AND BCG), AND CINNAMIC ACIDS (*cis*-CA AND *trans*-CA) ON ROOT GROWTH OF LETTUCE (*Lactuca sativa* CV. GREAT LAKES 366) SEEDLINGS IN FILTER PAPER AND AGAR BIOASSAY SYSTEMS IN THE ABSENCE OF SOIL, AS INDICATED BY THE EFFECTIVE CONCENTRATION AT 50% INHIBITION (EC_{50}) AND 95% CONFIDENCE INTERVAL VALUES (μM) DETERMINED BY PROBIT ANALYSIS

	On filter paper	On agar
2,4-D	0.34 (0.07–1.21)[a]	0.24 (0.19–0.30)
cis-ABA	14.94 (6.39–32.81)	—[b]
CG	3.98 (1.66–9.05)	2.61 (1.12–6.02)
BCG	6.88 (3.39–14.21)	8.68 (2.97–22.76)
cis-CA	3.67 (3.07–4.38)	1.62 (0.17–4.39)
trans-CA	770.92 (549.47–1120.47)	883.40 (379.72–2252.94)

[a] Data in parentheses indicate the 95% confidence interval.
[b] No data available.

The inhibitory activities of CG and BCG on root elongation of lettuce seedlings both occurred at the 10^{-6} M level (Figure 2, Table 1). The EC_{50} values for CG and BCG on lettuce were roughly one-half to one-quarter of the value for *cis*-ABA, meaning that CG and BCG had considerably stronger inhibitory activity than *cis*-ABA. Although the inhibitory activities of CG and BCG were almost one tenth that of 2,4-D, a synthetic commercial herbicide (Figure 2A, Table 1), their inhibitory potential is still among the highest yet reported for natural products.

To establish the portion of the chemical structure that is essential for the high inhibitory activity of CG and BCG, we compared their inhibitory activity with that of *cis*- and *trans*-CA. CG, BCG, and *cis*-CA had comparable levels of inhibitory activity (Figure 2B, Table 1). This suggests that *cis*-CA represents the chemical moiety that is essential for the high inhibitory activity of CG and BCG. We obtained additional confirmation that the stereochemistry of the methylene moiety of CA should be *cis* to provide high inhibitory activity by observing that the *trans*-isomer (*trans*-CA) showed an activity that was two orders of magnitude weaker than that of CG, BCG, and *cis*-CA (Figure 2B, Table 1).

Inhibitory activities of 2,4-D, *cis*-ABA, CG, BCG, *cis*-CA, and *trans*-CA on root elongation of pigweed, red clover, timothy, and bok choy were also established (Figure 3). In all plant species that we tested, the inhibitory activities of CG, BCG, and *cis*-CA were equally high, whereas that of *trans*-CA was significantly weaker. This result supports the suggestion that the portion of the chemical structure that is essential for high inhibitory activity is *cis*-CA and that the stereochemistry of the methylene moiety of CA needs to be *cis* to provide high inhibitory activity. This result is consistent with the observation that the *trans*-isomers of CG and BCG

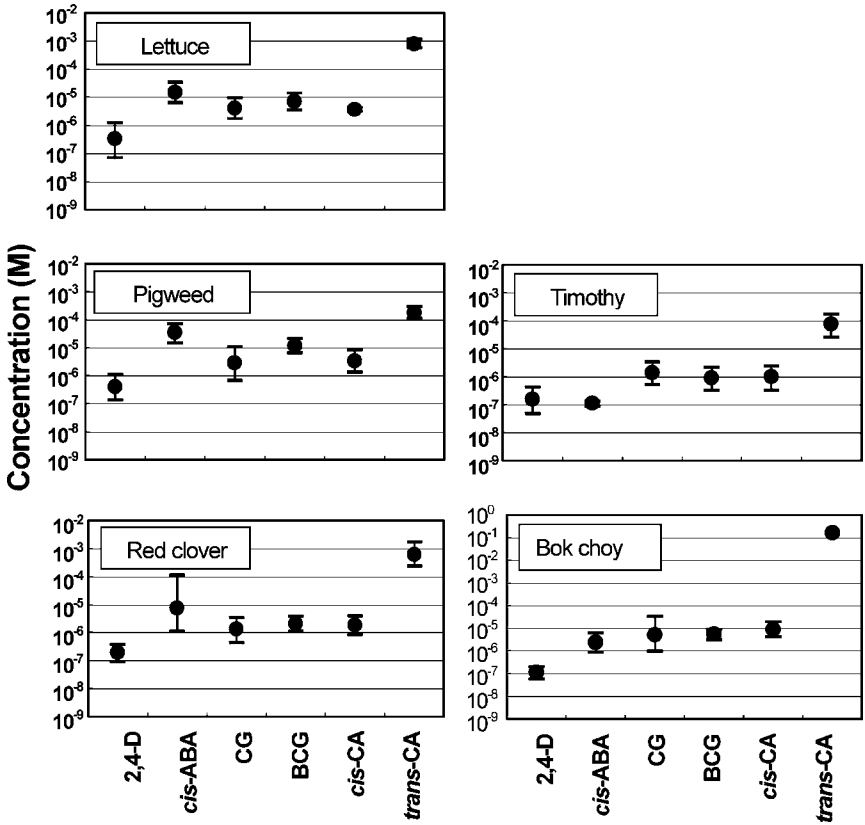

FIG. 3. Inhibitory activities of reference compounds (2,4-D and *cis*-ABA), *cis*-cinnamoyl glucosides (CG and BCG), and cinnamic acids (*cis*-CA and *trans*-CA) on root elongation of germinated seedlings of lettuce (*Lactuca sativa* cv. Great Lakes 366), pigweed (*Amaranthus retroflexus*), red clover (*Trifolium pratense*), timothy (*Phleum pratense*), and bok choy (*Brassica rapa* var. *chinensis*). Inhibition of root growth was detected by comparing root lengths with those of untreated control seedlings after incubation for 48 hr at 20°C in the dark. Data were subjected to probit analyses, and estimated EC_{50} values (•) and 95% confidence intervals (bars) are shown.

produce almost no inhibition of root elongation in lettuce seedlings (Hiradate et al., 2004).

trans-Cinnamic acid is a common phenylpropanoid synthesized from phenylalanine by L-phenylalanine ammonia-lyase (PAL). Bonner and Galston (1944) showed that *trans*-CA is secreted from the roots of guayule (*Parthenium argentatum*). Since then, the allelopathic potential of *trans*-CA has been frequently

reported (e.g., Baziramakenga et al., 1994; Chon et al., 2003). Since *trans*-CA can be converted into *cis*-CA by sunlight and by the presence of an electron-transfer facilitator, it is possible that the conversion of *trans*-CA into *cis*-CA is involved in the allelopathic phenomenon. This transformation might explain the reported synergism between *trans*-CA and polygodial (Fujita and Kubo, 2003) and ABA (Li et al., 1993).

The presence of natural *cis*-CA in plants has recently been reported in *Brassica parachinensis* (Yin et al., 2003). Its biosynthesis, however, is not well understood, but possible pathways have been proposed: (1) sunlight-mediated conversion from *trans*-CA, (2) spontaneous conversion from *trans*-CA in the presence of an electron-transfer facilitator, (3) isomerase-mediated conversion from *trans*-CA, and (4) direct enzymatic biosynthesis from L-phenylalanine (Yin et al., 2003). Further study is necessary.

The inhibitory activity of CG, BCG, and *cis*-CA was higher than that of *cis*-ABA for lettuce, pigweed, and red clover, but the reverse was observed for timothy and bok choy (Figure 3). This suggests that the mechanisms of growth inhibition might differ between *cis*-ABA and *cis*-CA derivatives. In any case, the inhibitory activity of CG, BCG, and *cis*-CA was generally comparable with that of *cis*-ABA.

The effects of soil on the inhibitory activities of these compounds is shown in Figure 4. In this bioassay system, we added agar to the soils to permit stable growth of the lettuce. This step was necessary because the addition of agar had been shown to decrease the coefficient of variation (CV) for the root growth of lettuce in our preliminary research (data not shown). The inhibitory activities of all compounds in the absence of soil (Figure 4, •) were almost identical to those in the filter paper bioassay system (Table 1, Figure 2), indicating that the adsorption of these compounds on the agar was negligible.

The inhibitory activity of CG, BCG, and *cis*-CA decreased in the presence of volcanic ash and calcareous soils (Figure 4). This was clear in comparisons made at 10^{-5} and at 3×10^{-5} M for each treatment. The alluvial soil had minimal effects on growth inhibition. In volcanic ash and calcareous soils, the amount of active surface hydroxyls, which adsorb and inactivate carboxylic acids by ligand exchange reactions, is larger than in alluvial soil (Hiradate and Uchida, 2004). Therefore, an alluvial soil would be more advantageous for the bioactivity of CG, BCG, and *cis*-CA than would volcanic ash or calcareous soils. It is possible that CG and BCG released from *S. thunbergii* work as effective allelochemicals in soil environments.

The inhibitory activity of CG, BCG, and *cis*-CA was lower than that of the synthetic commercial herbicide 2,4-D. The latter has been listed as a possible endocrine disrupter, along with its possible metabolite 2,4-dichlorophenol (Crosby, 1998). Both are frequently found in soils and in ground water (Hall et al., 1993;

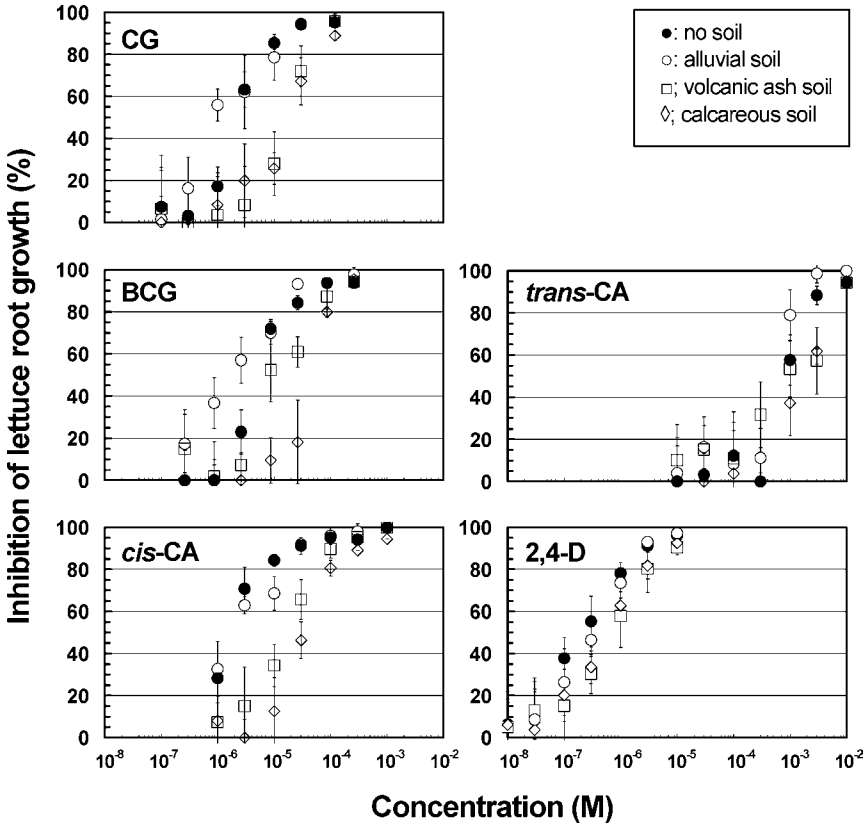

FIG. 4. Inhibitory activities of *cis*-cinnamoyl glucosides (CG and BCG), cinnamic acids (*cis*-CA and *trans*-CA), and the reference compound (2,4-D) on root elongation of lettuce (*Lactuca sativa* cv. Great Lakes 366) in agar media in the absence (●) or presence of soil (○; alluvial soil, □; volcanic ash soil, ◇; calcareous soil). Inhibition of root growth was detected by comparing the root lengths with those of untreated controls for each soil after incubating for 72 hr at 20°C in the dark. Bars indicate standard deviation ($N = 12$).

Wood and Anthony, 1997; Balinova and Mondesky, 1999; Pierzynski et al., 2000). Synthetic products such as 2,4-D often undergo little decomposition in soil environments because the enzymatic and other systems required to degrade them may not be present in nature. In contrast, both *cis*-CA and its glucosides are natural products that could be utilized by various indigenous soil organisms, because cinnamic acid is a common secondary metabolite in most organisms. It is, thus, likely that *cis*-CA and its glycosides are worth considering as plant growth regulators,

as they are inexpensive to synthesize and possess a low risk of environmental toxicity.

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(*Z,Z*)-6,9-HENEICOSADIEN-11-ONE: MAJOR SEX PHEROMONE COMPONENT OF PAINTED APPLE MOTH, *Teia anartoides*

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Abstract—(*Z,Z*)-6,9-Heneicosadien-11-one (*Z6Z9*-11-one-21Hy) was identified as the major sex pheromone component of the painted apple moth (PAM), *Teia anartoides* (Lepidoptera: Lymantriidae), on the basis of (1) comparative gas chromatographic-electroantennographic detection (GC-EAD) analyses, GC-mass spectrometry (MS), high-performance liquid chromatography (HPLC)-MS, and HPLC-UV/visible spectroscopy of pheromone gland extracts and authentic standards; (2) GC-EAD analyses of effluvia of calling females; and (3) wind tunnel and field trapping experiments with a synthetic standard. In field experiments in Australia, synthetic *Z6Z9*-11-one-21Hy as a single component attracted male moths. Wind tunnel experiments suggested that a 4-component blend consisting of *Z6Z9*-11-one-21Hy, (6*Z*,9*R*,10*S*)-*cis*-9,10-epoxy-heneicosene (*Z*6-9*R*10*S*-epo-21Hy), (*E,E*)-7,9-heneicosadien-6,11-dione (*E7E9*-6,11-dione-21Hy), and 6-hydroxy-(*E,E*)-7,9-heneicosadien-11-one (*E7E9*-6-ol-11-one-21Hy) (all present in pheromone gland extracts) might induce more males to orient toward, approach, and contact the source than did

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Z6Z9-11-one-21Hy as a single component. Additional experiments are needed to determine conclusively whether or not Z6-9*R10S*-epo-21Hy, *E7E9*-6,11-dione-21Hy, and *E7E9*-6-ol-11-one-21Hy might be minor sex pheromone components of PAM. Moreover, attractiveness of synthetic pheromone and virgin PAM females needs to be compared to determine whether synthetic pheromone could replace PAM females as trap baits in the program to monitor eradication of exotic PAM in New Zealand.

Key Words—Lymantriidae, *Teia anartoides*, painted apple moth, sex pheromone, (*Z,Z*)-6,9-heneicosadien-11-one, (*Z,E*)-6,8-heneicosadien-11-one, (6*Z*,9*R*,10*S*)-*cis*-9,10-epoxy-heneicosene, (*E,E*)-7,9-heneicosadien-6,11-dione; 6-hydroxy-(*E,E*)-7,9-heneicosadiene-11-one.

INTRODUCTION

The painted apple moth (PAM), *Teia anartoides* Walker (Lepidoptera: Lymantriidae), is endemic to southeastern Australia (Common, 1990; Edwards, 1996). Caterpillars cause minor damage to a wide range of trees and shrubs (Edwards, 1996), especially Acacias (*Acacia* spp.), and many other native and introduced plants, including cultivated crops and forest trees such as *Pinus radiata* (Pinaceae) (Common, 1990).

In 1999, PAM caterpillars were discovered in Auckland, New Zealand (Ridley, 1999). Realizing the threat of this exotic moth to New Zealand's native plants, agricultural, horticultural, and silvicultural industries as well as international trade, New Zealand's government initiated a program to eradicate PAM. Progress in this ongoing program is monitored by traps baited with laboratory-reared virgin PAM females. Continuous trap captures of PAM males indicate the persistence of residual PAM populations. Synthetic female pheromone for use as trap bait would facilitate monitoring of the eradication program, and prompted attempts to identify the PAM pheromone.

A number of compounds have been reported as possible sex pheromone components of PAM, including (*Z*)-6-heneicosen-11-one (Suckling et al., 2002; Muto and Mori, 2003), (*Z,E*)-6,8-heneicosadien-11-one (Jury et al., 2003; Muto and Mori, 2003), (6*Z*,9*R*,10*S*)- and (6*Z*,9*S*,10*R*)-*cis*-9,10-epoxy-heneicosene, as well as (6*Z*,9*R*,10*S*)- and (6*Z*,9*S*,10*R*)-*cis*-9,10-epoxy-eicosene (Muto and Mori, 2003). None of these studies report bioassay data that demonstrate attraction of PAM males to either one or all of these components. Here, we report chemical analyses, and wind tunnel and field experiments, demonstrating that (*Z,Z*)-6,9-heneicosadien-11-one (*Z6Z*9-11-one-21Hy) is the major sex pheromone component of female PAM.

METHODS AND MATERIALS

Experimental Insects. PAM caterpillars were reared on gypsy moth diet (Bell et al., 1981) (photoperiod: 14L:10D; temperature: 24°C; relative humidity:

55–60%) at the USDA Quarantine Facility in Newark, DE, USA, and at Forestry Research, Rotorua, New Zealand. Male and female pupae were sent by courier to Simon Fraser University (SFU), and were kept in SFU's Global Forest Quarantine Facility.

Collection of Pheromones. Abdominal tips with pheromone glands of calling, 2- to 3-d-old females were removed and extracted in HPLC-grade hexane. To obtain effluvia of calling females, 30 females were placed in a Pyrex glass chamber maintained at a photoperiod of 14L:10D and a temperature of 25°C. A water aspirator was used to draw humidified, charcoal-filtered air at 80 ml/min through the chamber and through a glass column (6 × 30 mm) filled with Porapak Q (50–80 mesh, Waters Associated Inc. Milford, MA, USA). To minimize isomerization of volatiles, the Porapak Q trap was kept at 5°C. After 48 hr, absorbed volatiles were desorbed with 2 ml of redistilled pentane.

Analyses of Pheromone and General Instrumentation. Aliquots of 0.1 female equivalent (FE) of pheromone gland extract, or 30 female-hour equivalents (FHE = all volatiles released by 30 PAM females during 1 hr of aeration) of Porapak Q extracts were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) (Arn et al., 1975) with a Hewlett-Packard (HP) 5890A gas chromatograph (GC) equipped with a column (30 m × 0.25 or 0.32 mm ID) coated with either DB-5, DB-210, or DB-23 (J&W Scientific, Folsom, CA, USA; further details in Gries et al., 2002). High-performance liquid chromatography (HPLC) of pheromone gland extract (100–200 FE) employed a Waters LC 626 chromatograph equipped with a Waters 486 variable wavelength UV/visible detector set to 210 nm, HP Chemstation software (Rev.A.07.01), and a reverse phase Nova-Pak C18 column (60Å, 4 μm; 3.9 × 300 mm) eluted with acetonitrile (1 ml/min).

HPLC fractions of pheromone gland extract containing the candidate chiral pheromone components *cis*-9,10-epoxy-heneicosene and (*Z,Z*)-6,9-heneicosadien-11-ol (as determined by GC-mass spectrometry) were extracted with pentane, concentrated to 2 μl, and analyzed by gas chromatography on a custom-made chiral stationary phase column [1:1 mixture of hepakis-(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin and OV-1701 (König et al., 1992; Pietruszka et al., 1992); splitless injection; temperature of injection port and FID: 240°C; temperature program: 120°C (0 min), then 5°C per min to 160°C]. *cis*-9,10-Epoxy-(*Z*)-6-eicosene, and 6-hydroxy-(*E,E*)-7,9-heneicosadien-11-one (*E7E9*-6-ol-11-one-21Hy) occurred at low quantities in pheromone gland extracts, and no attempts were made to determine their absolute configuration.

GC-MS analyses of pheromone extracts and of synthetic standards employed a Varian Saturn 2000 Ion Trap fitted with the above-referenced DB-5 column.

HPLC-MS analyses were carried out with an Agilent 1100 LC/MSD (G1946D) equipped with a binary pump, autosampler, thermostatted column compartment (set at 10°C), diode array detector (acquiring UV/Visible spectra from

190–600 nm), and an Agilent SB-C18 column (2.1 × 75.0 mm with 3.5 μm packing) fitted with a guard column (2.1 × 12 mm) consisting of XDB-C8 (5 μm particle size). The injection volume was 0.1 μl for both synthetic standards and pheromone extract. The mobile phase consisted of an isocratic solvent system, with 10% of solvent A [H_2O (95%): MeOH (5%)] and 90% of solvent B [MeOH (100%)] at a flow rate of 0.25 ml/min. MSD scan range and time, respectively, were 100–400 amu and 2.82 sec, with the fragmentator at 200 volts. The N_2 nebulizer pressure was 20 psi, and the flow rate of the drying gas 10 l/min at a temperature of 350°C. The capillary voltage was set at 3.5 kV. Samples were processed by atmospheric pressure electrospray (APES), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photo ionization (APPI).

Nuclear magnetic resonance (NMR) spectroscopy of synthetic compounds was conducted on a Varian AS500 spectrometer at 499.77 MHz for ^{1}H NMR and 125.68 MHz for ^{13}C spectra. ^{1}H chemical shifts are reported as parts per million [ppm, relative to TMS (0.00 ppm)]. Infrared (IR) spectroscopy of synthetic compounds was conducted on a Bomem MB Series FTIR spectrometer. Elemental analyses of synthetic chemicals were performed with a Carlo Erba Model 1106 elemental analyzer.

Source of Synthetic Standards. (*Z,Z*)-6,9-Heneicosadiene (*Z6Z9*-21Hy), (9*R*, 10*S*)-*cis*-9,10-epoxy-(*Z*)-6-eicosene (*Z*6-9*R*10*S*-epo-20Hy), (9*S*,10*R*)-*cis*-9,10-epoxy-(*Z*)-6-eicosene (*Z*6-9*S*10*R*-epo-20Hy), (9*R*,10*S*)-*cis*-9,10-epoxy-(*Z*)-6-heneicosene (*Z*6-9*R*10*S*-epo-21Hy), and (9*S*,10*R*)-*cis*-9,10-epoxy-(*Z*)-6-heneicosene (*Z*6-9*S*10*R*-epo-21Hy) were donations from Edward W. Underhill (National Research Council of Canada, Plant Biotechnology Institute, Saskatoon, Sask. Canada). (*Z*)-6-Heneicosen-11-one (*Z*6-11-one-21Hy) was purchased (Bedoukian, Danbury, CT, USA). (*Z,E*)-6,8-Heneicosadien-11-one (*Z6E*8-11-one-21Hy) and (*Z,E*)-6,9-heneicosadien-11-one (*Z6E*9-11-one-21Hy) were available from previous work (Gries et al., 1997). (*Z,Z*)-6,9-Eicosadien-11-one (*Z6Z*9-11-one-20Hy) and (*Z,Z*)-6,9-heneicosadien-11-one (*Z6Z9*-11-one-21Hy) were produced by oxidation of corresponding alcohols available in our laboratory (Gries et al., 2003). Racemic (*Z,Z*)-6,9-heneicosadien-11-ol (*Z6Z*9-11-ol-21Hy), and enantiomers thereof, were available from previous work (Gries et al., 2003).

Syntheses. We describe syntheses and report spectroscopic data for (*E,E*)-7,9-heneicosadien-6,11-dione (*E7E9*-6,11-dione-21Hy) and 6-hydroxy-(*E,E*)-7,9-heneicosadien-11-one (*E7E9*-6-ol-11-one-21Hy), two previously unknown components in pheromone gland extracts of insects. Chemicals obtained from commercial sources were used without further purification unless otherwise indicated. All moisture- and air-sensitive reactions were conducted under argon. Column chromatography refers to flash chromatography using silica gel 60 (230–400 mesh, E. Merck, Darmstadt, Germany) (Still et al., 1978).

*6-Hydroxy-(*E,E*)-7,9-heneicosadien-11-one (7) and (*E,E*)-7,9-Heneicosadien-6,11-dione (8) (Figure 1)*. Silylation of 1-octyne-3-ol (**1**) (Aldrich Chem.

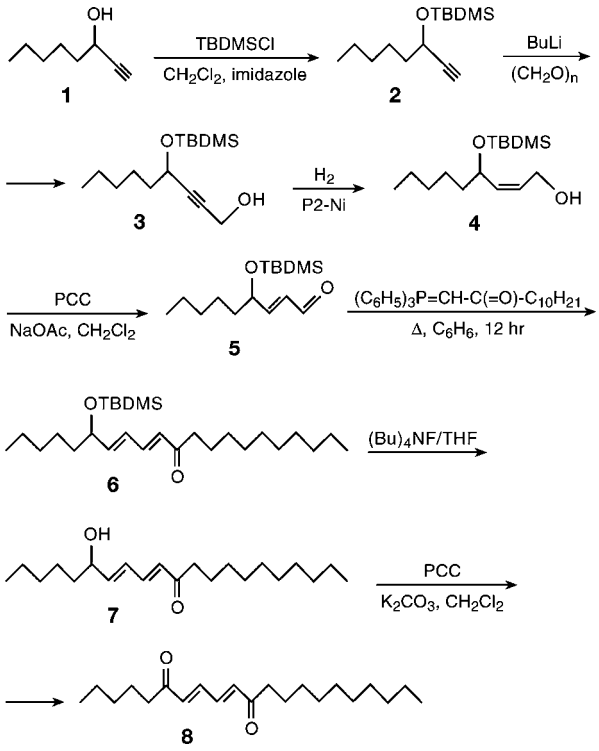

FIG. 1. Scheme for the syntheses of (*E*,*E*)-7,9-heneicosadien-6,11-dione and 6-hydroxy-(*E*,*E*)-7,9-heneicosadien-11-one.

Co) and subsequent formylation of silyl ether **2** (99% yield) gave alcohol **3** (94% yield) (Marshall and Zou, 2000). Hydrogenation of **3** (2.75 g, 0.01 mol) with P2-Nickel catalyst (Brown and Ahuja, 1973) afforded alcohol **4** (2.6 g, 96% yield) of which 50% (1.3 g, 4.7 mmol) was oxidized by pyridinium chlorochromate (PCC) (1.60 g) in dichloromethane in the presence of sodium acetate (0.60 g, 7.3 mmol). The reaction mixture was filtered through silica (15 g) with hexane-ether (19:1) as eluent, and solvents were removed *in vacuo*. Without further purification, crude aldehyde **5** (Hayashi, 1990) was refluxed 12 hr in benzene with the stabilized ylid 1-triphenylphosphoranylidene-2-dodecanone. This ylid was obtained (90% yield) by deprotonation (butyllithium) and alkylation (*n*-nonyl iodide) of 1-triphenylphosphoranylidene-2-propanone (Avocado Research Chem. Ltd., Lancashire, UK) (Taylor and Wolf, 1972; Black et al., 1996). Following reflux, benzene was removed *in vacuo*, and unreacted ylid and triphenylphosphine oxide were removed by filtering the resulting mixture through silica (15 g) with hexane-ether (9:1) as eluent. Without further separation, filtrates containing

silylated keto-alcohol **6** were concentrated and treated with a THF solution of Bu_4NF for 2 hr. The reaction mixture containing the desired keto-alcohol **7** (formation of only one geometric isomer observed) was diluted with water and extracted with ether, the ether layer was washed with water and brine, then dried ($MgSO_4$), concentrated, and purified by flash chromatography (50 g silica) with hexane/ether (3:1) and (1:1) as consecutive eluents. Yield of pure keto-alcohol **7** was 0.30 g (20% based on alcohol **4**, overall yield 17%). M.p. 43°C. Anal. calcd. for $C_{21}H_{38}O_2$ (%): C, 78.28; H, 11.88, found: C, 78.26, H, 12.20. IR (KBr): 3650, 3630, 2955, 2918, 2850, 1686, 1605, 1088, 996 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.90–0.98 (m, 6H), 1.21–1.35 (m, 20H), 1.54–1.64 (m, 4H), 2.55 (t, 2H, $J = 7.6$ Hz), 4.25 (1H, m), 6.13-6.23 (m, 2H), 6.37 (dd, 1H, $J = 15.0$, 11.0 Hz), 7.15 (dd, 1H, $J = 15.5$, 11.0 Hz). ^{13}C NMR ($CDCl_3$) δ: 14.01, 14.11, 22.56, 22.67, 24.35, 24.94, 29.31 (2), 29.43, 29.48, 29.56, 31.68, 31.88, 37.05, 40.74, 72.09, 127.89, 129.81, 141.49, 145.62, 200.95.

Oxidation of **7** (0.15 g, 0.47 mmol) with PCC (0.15 g, 0.70 mmol) in the presence of potassium carbonate (0.050 g, 0.036 mmol) for 2 hr in dry CH_2Cl_2 afforded diketone **8** (0.14 g, 93% yield based on keto-alcohol **7**). M.p. 76°C. Anal. calcd. for $C_{21}H_{36}O_2$ (%): C, 78.70; H, 11.32, found: C, 79.08, H, 11.12. IR (KBr): 3650, 2955, 2916, 2850, 1678, 1587, 1407, 1216, 1096, 1010 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.86-0.92 (m, 6H), 1.28–1.36 (m, 18H), 1.59–1.67 (m, 4H), 2.60 (t, 4H, $J = 7.5$ Hz), 6.49 (dd, 2H, $J = 11.5$, 2.8 Hz), 7.18 (dd, 2H, $J = 11.7$, 2.8 Hz). ^{13}C NMR ($CDCl_3$) δ: 13.91, 14.11, 22.45, 22.67, 23.72, 24.04, 29.22, 29.30, 29.40, 29.46, 29.55, 31.38, 31.88, 41.31, 41.35, 135.96, 135.97, 138.79, 138.80, 200.10, 200.11.

Wind Tunnel and Field Experiments. Wind tunnel experiments employed a wind tunnel (160 cm long, 68 cm wide, 70 cm tall) and protocol as developed by Miller and Roelofs (1978). The air speed was 30 cm/sec, and the temperature was 22°C (±2°C). Experiments bioassayed the responses of individually tested 1- to 3-d-old males to filter paper (Whatman International Ltd., Maidstone, England) impregnated with test chemicals in HPLC-grade hexane or acetonitrile. All test chemicals were >95% chemically pure. Six criteria (Table 1) were used to assess the attractiveness of lures. Experiment 1 tested *Z*6*Z*9-11-one-21Hy singly and in combination with either *Z*6-9*R*10*S*-epo-21Hy (a), *E*7*E*9-6,11-dione-21Hy plus *E*7*E*9-6-ol-11-one-21Hy (b), or (a) plus (b). Experiment 2 tested *Z*6*Z*9-11-one-21Hy singly and in binary, ternary, and quaternary combinations with *Z*6-9*R*10*S*-epo-21Hy, *E*7*E*9-6,11-dione-21Hy, and *E*7*E*9-6-ol-11-one-21Hy (Table 1).

Field experiments were conducted in Campbelltown, N.S.W., Australia, in the residential area of Wedderburn (S 34 deg. 09 min., E 150 deg. 49 min) generally in forest types classified as "dry schlerophyll" (Sydney, Hawkesbury sandstone). The area was dominated by trees of *Eucalyptus* spp. with some *Acacia* spp., and stocked with numerous shrubs in formerly disturbed or open field habitats. Delta-type traps made from 2-l milk cartons (Gray et al., 1984) were coated with

TABLE 1. WIND-TUNNEL BIOASSAYS WITH MALES OF PAINTED APPLE MOTH, *Teia anartoides*, RESPONDING TO FILTER PAPERS IMPREGNATED WITH CANDIDATE PHEROMONE COMPONENTS

Exp.	Treatment[a]	Males responding to assessment criteria[b] (N = male moths tested)						
			A	F	O	H	Ap	L
1	*Z*6*Z*9-11-one	($N = 12$)	6	6	5	0	0	0
	*Z*6*Z*9-11-one + *Z*6-9*R*10*S*-epo	($N = 12$)	9	9	3	1	1	1
	*Z*6*Z*9-11-one + *E*7*E*9-6,11-dione + *E*7*E*9-6-ol-11-one	($N = 12$)	8	7	0	0	0	0
	*Z*6*Z*9-11-one + *Z*6-9*R*10*S*-epo + *E*7*E*9-6,11-dione + *E*7*E*9-6-ol-11-one	($N = 13$)	13	13	13	6	4	4
2	*Z*6*Z*9-11-one	($N = 13$)	8	6	1	0	0	0
	*Z*6*Z*9-11-one + *Z*6-9*R*10*S*-epo	($N = 13$)	6	6	5	4	3	1
	*Z*6*Z*9-11-one + *E*7*E*9-6,11-dione + *E*7*E*9-6-ol-11-one	($N = 13$)	2	3	0	0	0	0
	*Z*6*Z*9-11-one + *Z*6-9*R*10*S*-epo + *E*7*E*9-6,11-dione	($N = 13$)	12	9	8	1	1	1
	*Z*6*Z*9-11-one + *Z*6-9*R*10*S*-epo + *E*7*E*9-6-ol-11-one	($N = 13$)	9	8	6	3	2	1
	*Z*6*Z*9-11-one + *Z*6-9*R*10*S*-epo + *E*7*E*9-6,11-dione + *E*7*E*9-6-ol-11-one	($N = 13$)	11	11	9	5	5	4

[a]Compound abbreviations as follows: *Z*6*Z*9-11-one = (*Z*,*Z*)-6,9-heneicosadien-11-one (57 ng = 1 female equivalent of pheromone gland extract); *Z*6-9*R*10*S*-epo = (*Z*6,9*R*,10*S*)-*cis*-9,10-epoxy-heneicosene (7 ng); *E*7*E*9-6,11-dione = (*E*,*E*)-7,9-heneicosadien-6,11-dione (3.5 ng); *E*7*E*9-6-ol-11-one = 6-hydroxy-(*E*,*E*)-7,9-heneicosadien-11-one.

[b]A = Activation; F = flight; O = 4 orientation towards pheromone lure; H = halfway flight toward lure; Ap = approaching lure; L = landing on lure.

Tanglefoot (The Tanglefoot Company, Grand Rapids, MI, USA) and suspended from trees at a height of 2 m and spacings of 20–25 m in complete randomized blocks, which were separated by 0.1 to 3 km. Traps were baited with a piece of dental cotton roll (10 × 15 mm) (Richmond Dental, Charlotte, NC, USA), which was impregnated with test chemicals just prior (0–2 hr) to the onset (~10:00 am) of experiments. To prevent rearrangement of (labile) test chemicals during storage, they were placed in vials, diluted in solvent (hexane, pentane/ether, or acetonitrile), kept on dry ice, and allowed to warm up to ambient temperature only during the 15–30-min preparation of test lures.

Field experiment 1 tested the major candidate pheromone component *Z*6*Z*9-11-one-21Hy (5 μg) singly and in quaternary combination with secondary candidate pheromone components *Z*6-9*R*10*S*-epo-21Hy, *E*7*E*9-6,11-dione-21Hy, and *E*7*E*9-6-ol-11-one-21Hy at ratios of 5:5:5:5 or 5:0.5:0.5:0.5. Taking into account that the 4-component blend (5: 0.5: 0.5: 0.5) appeared most attractive, experiment 2 tested *Z*6*Z*9-11-one-21Hy (5 μg) singly and in combination with either one or all three of *Z*6-9*R*10*S*-epo-21Hy, *E*7*E*9-6,11-dione-21Hy, and *E*7*E*9-6-ol-11-one-21Hy at 10:1 ratios. Considering that *Z*6-9*R*10*S*-epo-21Hy appeared to enhance attractiveness of *Z*6*Z*9-11-one-21Hy in experiment 2, experiments 3 and 4 explored whether attractiveness of this 2-component blend could be enhanced by additional components. Experiment 3 tested *Z*6*Z*9-11-one-21Hy (5 μg) plus *Z*6-9*R*10*S*-epo-21Hy (0.5 μg) in combination with either *E*7*E*9-6,11-dione-21Hy (0.5 μg), *E*7*E*9-6-ol-11-one-21Hy (0.5 μg), or both, whereas experiment 4 tested *Z*6*Z*9-11-one-21Hy (5 μg) plus *Z*6-9*R*10*S*-epo-21Hy (0.5 μg) in combination with either *Z*6*Z*9-11-one-20Hy (0.5 μg), *Z*6-11-one-21Hy (0.5 μg), *Z*6*E*8-11-one-21Hy (0.5 μg), *Z*6*E*9-11-one-21Hy (0.5 μg), *Z*6*Z*9-11-ol-21Hy (0.5 μg) or *Z*6-9*R*10*S*-epo-20Hy (0.5 μg). Experiment 5 tested 3 components singly: *Z*6*Z*9-11-one (5 or 0.5 μg), the corresponding rearrangement product *Z*6*E*8-11-one-21Hy (5 μg), and *Z*6-9*R*10*S*-epo-21Hy (5 μg). In all experiments, lures were replaced after two days without re-randomizing treatment positions.

Trap catch data were analyzed by nonparametric analysis of variance (Friedman's test) followed by comparison of means by Scheffé test (Zar, 1984; SAS/STAT, 1988). In all analyses, $\alpha = 0.05$.

RESULTS

Chemical Analyses. GC-EAD analyses of PAM pheromone gland extract revealed many components that consistently elicited antennal responses from male PAM antennae (Figure 2). By comparing mass spectra and retention indices (Van den Dool and Kratz, 1963) of PAM-produced compounds with those of our reference library of moth pheromones (RG & GG, unpublished), and by comparative GC, GC-MS, and GC-EAD analyses of PAM-produced components and authentic standards on three GC columns (DB-5, DB-23, DB-210), we determined

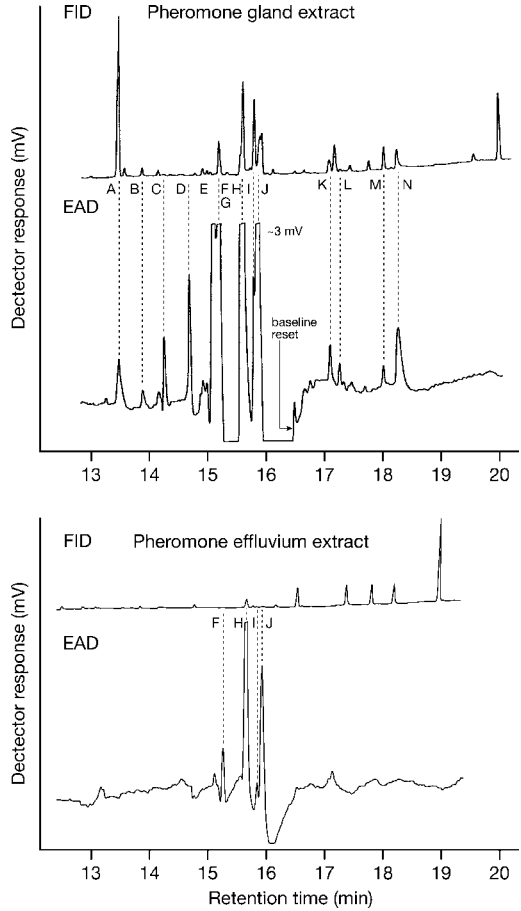

FIG. 2. Flame ionization detector (FID) and electroantennographic detector (EAD: male *Teia anartoides* antenna) responses to 0.1 female equivalent (FE) of pheromone gland extract of female *T. anartoides* (*top*), or to 30 female-hour equivalents (FHE = all volatiles released by 30 females during 1 hr of aeration) of Porapak Q extract (*bottom*). Chromatography: DB-5 column; splitless injection; temperature program: 100°C/1 min, 10°C per min to 280°C. **A** = (*Z*,*Z*)-6,9-heneicosadiene (Z6Z9-21Hy); **B** = heneicosatriene (with unknown double bond positions); **C** = (Z6)-*cis*-9,10-epoxy-eicosene (Z6-9,10-epo-20Hy); **D** = (*Z*,*Z*)-6,9-eicosadiene-11-one (Z6Z9-11-one-20Hy); **E** = (*Z*,*Z*)-6,9-heneicosadien-11-ol (Z6Z9-11-ol-21Hy); **F** = (Z6)-*cis*-9,10-epoxy-heneicosene (Z6-9,10-epo-21Hy); **G** = (*Z*)-6-heneicosen-11-one (*Z*6-11-one-21Hy); **H** = (*Z*,*E*)-6,8-heneicosadien-11-one (Z6E8-11-one-21Hy); **I** = (*Z*,*E*)-6,9-heneicosadien-11-one (*Z*6*E*9-11-one-21Hy); **J** = (*Z*,*Z*)-6,9-heneicosadien-11-one (*Z*6*Z*6-11-one-21Hy); **K** and **L** = unknown rearrangement products of *Z*6*Z*9-11-one-21Hy; **M** = (*E*,*E*)-7,9-heneicosadien-6,11-dione (*E*7*E*9-6,11-dione-21Hy); **N** = 6-hydroxy-(*E*,*E*)-7,9-heneicosadien-11-one (*E*7*E*9-6-ol-11-one-21Hy).

that components **A-J** were Z6Z9-21Hy (**A**), a heneicosatriene with undetermined double bonds (**B**), *Z*6-9,10-epo-20Hy (**C**), *Z6Z*9-11-one-20Hy (**D**), *Z6Z*9-11-ol-21Hy (**E**), *Z*6-9,10-epo-21Hy (**F**), *Z*6-11-one-21Hy (**G**), *Z6E*8-11-one-21Hy (**H**), *Z6E*9-11-one-21Hy (**I**), and *Z6Z*9-11-one-21Hy (**J**). Although labile *Z6Z*9-11-one-21Hy rearranges to *Z6E*8-11-one-21Hy during GC analyses (Gries et al., 1997; Wimalaratne, 1998), trace amounts of *Z6Z*9-11-one-21Hy remained detectable by PAM antennae (Figure 2), suggesting that pheromone extracts may contain larger quantities of this labile ketodiene than are evident from Figure 2.

To determine unequivocally the presence and ratio of the three ketodienes (*Z6Z*9-11-one-21Hy, *Z6E*8-11-one-21Hy, *Z6E*9-11-one-21Hy) in pheromone gland extracts, extracts were analyzed by HPLC-MS, a procedure that minimized isomerization of labile ketodienes. Comparative HPLC-MS analyses of synthetic *Z6Z*9-11-one-21Hy and of PAM pheromone extract revealed that the most abundant (~57 ng per FE) candidate pheromone component in PAM pheromone extracts eluted at the same time as synthetic *Z6Z*9-11-one-21Hy (Figure 3). Identical HPLC retention times (Figure 3), mass spectra (Figure 3), and UV spectra (Figure 4) of this component and of synthetic *Z6Z*9-11-one-21Hy confirmed that *Z6Z*9-11-one-21Hy was present in relatively large quantities in PAM pheromone extracts. *Z6E*8-11-one-21Hy and *Z6E*9-11-one-21Hy were also present but at lower quantities (Figure 3).

The absolute configurations of candidate pheromone components *Z6Z*9-11-ol-21Hy (**E** in Figure 2) and *Z*6-9,10-epo-21Hy (**F** in Figure 2) were determined by HPLC fractionation of PAM extract (20 FE), followed by GC analyses of concentrated (2 μl) HPLC fractions and authentic standards on a chiral stationary phase GC column. These analyses revealed that PAM females produce both enantiomers (1:1) of *Z6Z*9-11-ol-21Hy (data not shown), and the (9*R*,10*S*)-enantiomer of *Z*6-9,10-epo-21Hy (Figure 5).

Mass spectra and molecular weights of late-eluting components **M** and **N** (Figure 6) suggested that they were heneicosadienes with two oxygens. Considering that PAM females produce epoxides and ketones (Figure 2), we hypothesized that compound **M** (MW = 320) was an epoxy-ketone or diketone, and compound **N** (MW = 322) an epoxy- or keto-alcohol. Chromatographic "tailing" of **N** (Figure 2) suggested the presence of a hydroxy-group in **N**. Hydrogenation of the **M**- and **N**-containing HPLC fraction, and GC-MS analyses of the hydrogenation products, produced a new mass spectrum with fragmentation ion *m/z* 169 indicative of a carbonyl group on C11. To test the hypothesis that **M** and **N** were biosynthetically related to *Z6Z*9-11-one-21Hy or *Z6E*8-11-one-21Hy, and to confirm the presence of a second oxygen in **M** and **N**, we epoxidized and hydrogenated synthetic *Z6E*8-11-one-21Hy. One of the resulting compounds had the same retention time and mass spectral characteristics as the **M** hydrogenation product. Bearing in mind that epoxidation and hydrogenation of *Z6E*8-11-one-21Hy could afford both epoxy-ketones and diketone by-products (Malinovskii, 1965), we further hypothesized

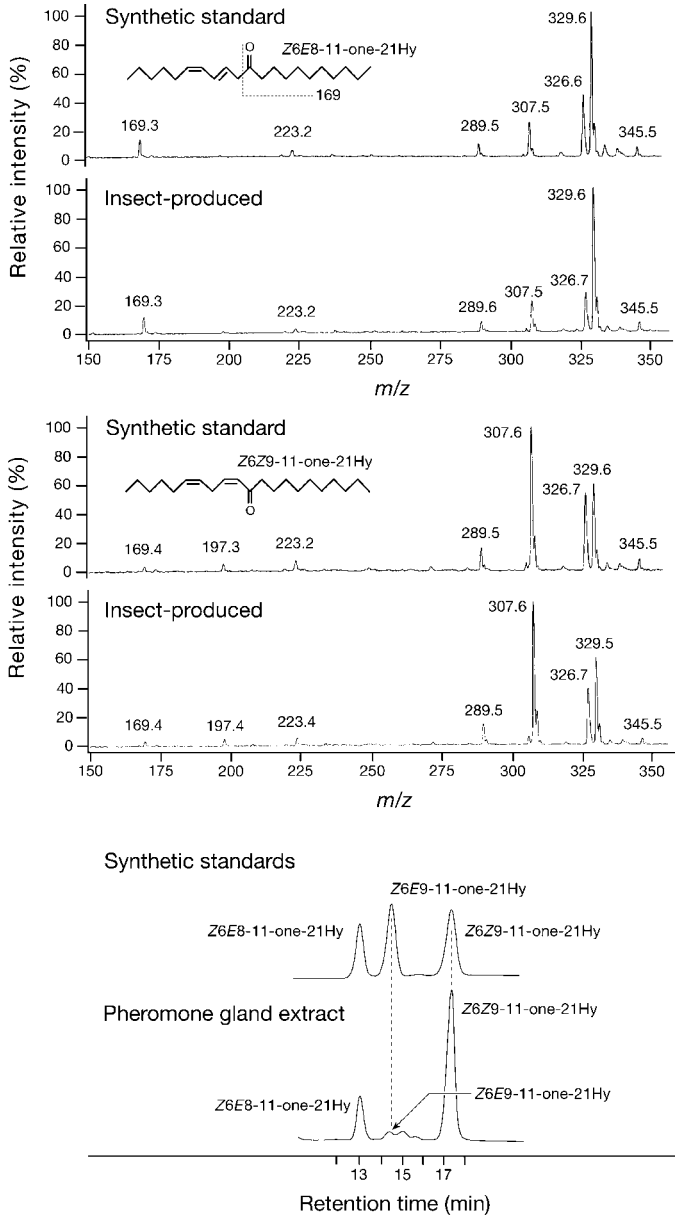

FIG. 3. (*Top*). High-performance liquid chromatography atmospheric pressure electrospray (APES) mass spectra (200 volts) of synthetic and insect-produced (*Z*,*E*)-6,8-heneicosadien-11-one (*Z*6*E*8-11-one-21Hy; ~10 ng/μl), and of (*Z*,*Z*)-6,9-heneicosadien-11-one (*Z*6*Z*9-11-one-21Hy; ~10 ng/μl); (*Bottom*) HPLC chromatograms of synthetic standards and pheromone gland extract of female *T. anartoides*.

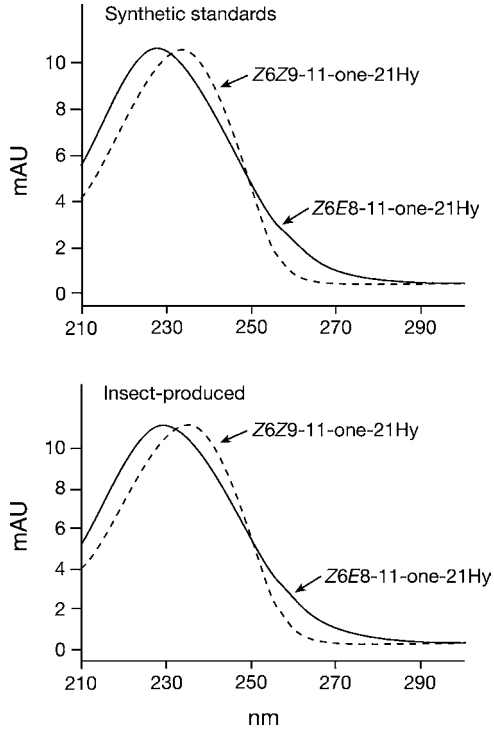

FIG. 4. UV/Visible spectra of synthetic and insect-produced (*Z*,*E*)-6,8-heneicosadien-11-one (*Z*6*E*8-11-one-21Hy) and (*Z*,*Z*)-6,9-heneicosadien-11-one (*Z*6*Z*9-11-one-21Hy). Injection volume on HPLC: 0.5 μl with ~5 ng per compound on column. Note contrasting absorption characteristics and peak maxima (234 nm and 228 nm) for *Z*6*E*8-11-one-21Hy and Z6Z9-11-one-21Hy.

that the second oxygenated group in **M** was either a *cis*-6,7- or *trans*-8,9-epoxide, or, a ketone at C6, C7, C8, or C9. Heneicosan-6,11-dione as our first synthetic candidate had the same mass spectrum and retention times on three columns as the **M** hydrogenation product. In assigning the double bond positions to the target heneicosadien-6,11-dione (**M** in Figure 2), we considered (a) known double bond positions (*Z*6, *E*8, *Z*9, *E*9) in ketodienes from *Orgyia* spp. (Gries et al., 1997; Lui, 1999; Grant et al., 2003), and (b) the large retention indices of **M** (DB-5: 2586, DB-210: 3275) that were indicative of extensive conjugation. (*E*,*E*)-7,9-Heneicosadien-6,11-dione was synthesized as the thermodynamically most stable target and was shown to have the same mass spectral (Figure 6) and retention characteristics as **M** in Figure 2. Moreover, the corresponding 6-hydroxy-(*E*,*E*)-7,9-heneicosadien-11-one met all identification criteria of component **N** in Figure 2.

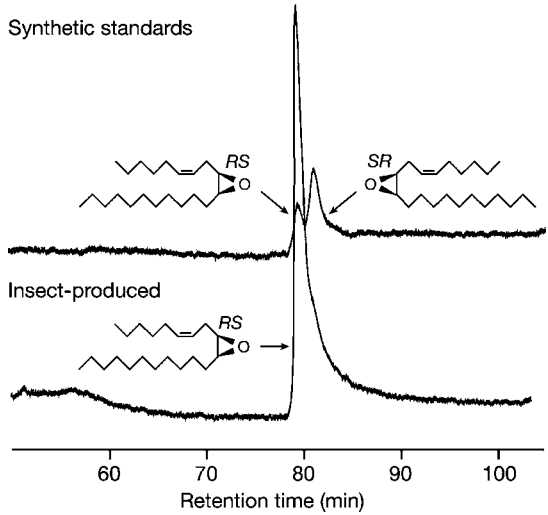

FIG. 5. Chromatogram of a mixture of synthetic enantiomers of (*Z*)-6-*cis*-9,10-epoxy-heneicosene (*top*) and of insect-produced (*Z*6,9*R*,10*S*)-*cis*-9,10-epoxy-heneicosene (50 female equivalents) (*bottom*) on a chiral stationary phase GC column.

Its mass spectrum is shown in Figure 6. Identifications of **K** and **L** were not attempted because both compounds were rearrangement products of *Z*6*Z*9-11-one-21Hy, suggesting that they are not likely part of the PAM pheromone.

GC-EAD analyses of effluvia of calling PAM females (Figure 2) revealed significant antennal responses to *Z*6-9,10-epo-21Hy, *Z*6*Z*9-11-one-21Hy, and *Z*6*E*8-11-one-21Hy (which forms from *Z*6*Z*9-11-one-21Hy during GC analyses), suggesting that the actual pheromone blend might be much simpler than suggested by the pheromone gland extracts.

Behavioral Bioassays. In preliminary wind tunnel bioassay experiments, attraction of male moths to *Z*6*Z*9-11-one-21Hy (the major candidate pheromone component) appeared enhanced by specific HPLC fractions of pheromone gland extracts, or corresponding synthetic candidate pheromone components. Besides *Z*6*Z*9-11-one-21Hy, three components (*Z*6-9*R*10*S*-epo-21Hy, *E*7*E*9-6,11-dione-21Hy, and *E*7*E*9-6-ol-11-one-21Hy) seemed to affect the males' responses and were bioassayed as blends in experiments 1 and 2 (Table 1). When all three of these components were added to *Z*6*Z*9-11-one-21Hy, more PAM males appeared to orient towards the lure, flew midway through the wind tunnel, approached, and contacted the lure (Table 1).

In field trapping experiment 1 in Australia, *Z*6*Z*9-11-one-21Hy as a single component attracted PAM males. Moreover, experiment 1 suggested that the

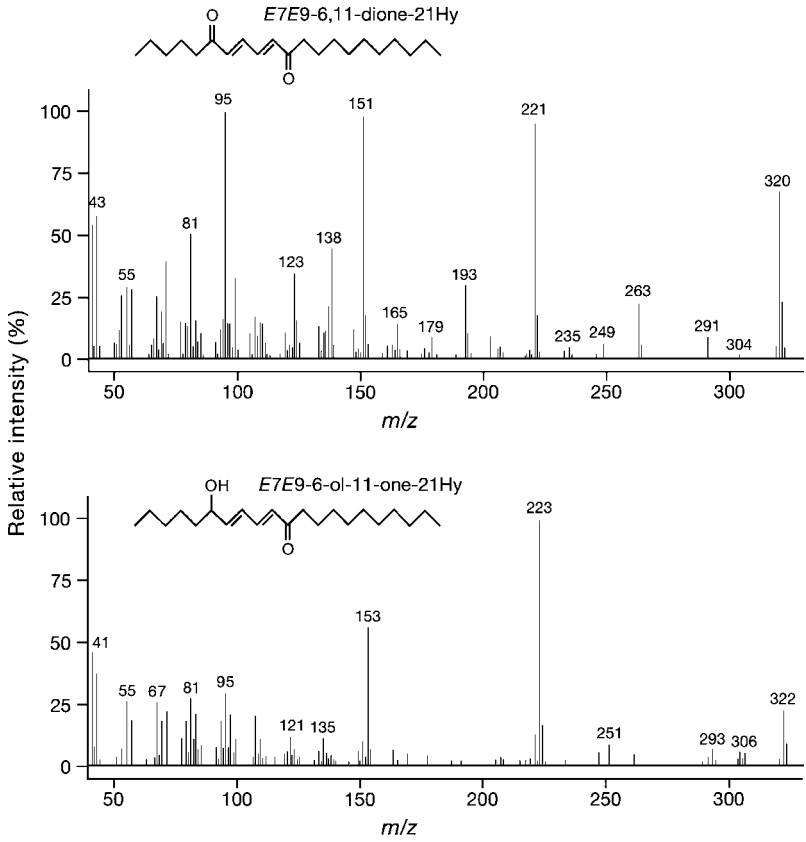

FIG. 6. Ion trap mass spectra of (*E*,*E*)-7,9-heneicadien-6,11-dione (*E*7*E*9-6,11-dione-21Hy) and 6-hydroxy-(*E*,*E*)-7,9-heneicosen-11-one (*E*7*E*9-6-ol-11-one-21Hy) identified in pheromone gland extracts of female *Teia anartoides*. Synthetic standards gave the same spectra.

4-component blend of *Z*6*Z*9-11-one-21Hy, *Z*6-9*R*10*S*-epo-21Hy, *E*7*E*9-6,11-dione-21Hy, and *E*7*E*9-6-ol-11-one-21Hy at a 5:0.5:0.5:0.5 ratio might attract more PAM males than *Z*6*Z*9-11-one-21Hy alone. The 4-component blend at a 5:5:5:5 ratio was not attractive (Figure 7). However, experiments 2–4 failed to demonstrate conclusively whether any of the minor components, singly or in combinations, increased the attractiveness of blends relative to *Z*6,*Z*9-11-one-21Hy alone (Figures 7 and 8). In experiment 5, traps baited with *Z*6*Z*9-11-one-21Hy at 5 μg were more attractive than unbaited control traps, whereas *Z*6 *E*8-11-one-21Hy and *Z*6-9*R*10*S*-epo-21Hy (5 μg each) were not different from controls (data not shown).

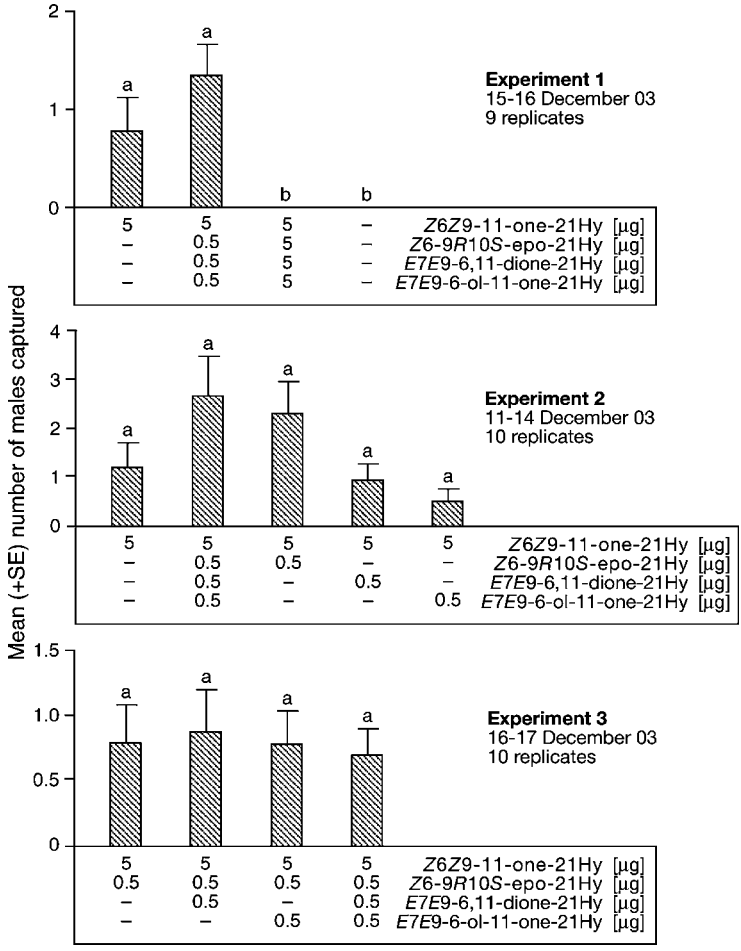

FIG. 7. Captures of male *Teia anartoides* in field experiments 1–3 in sticky traps baited with candidate pheromone components singly and in combinations; all experiments near Campbelltown, N.S.W., Australia. In each experiment, bars with the same letter superscript are not significantly different; $\alpha = 0.05$. Compound abbreviations as in Figure 2.

DISCUSSION

*Z*6*Z*9-11-one-21Hy appears to be the major sex pheromone component of PAM, on the basis of (1) analyses of pheromone gland extracts of PAM females by GC-EAD, GC-MS, HPLC-MS, and HPLC-UV/Visible spectroscopy; (2) GC-EAD analyses of effluvia of PAM females; and (3) wind tunnel and field experiments

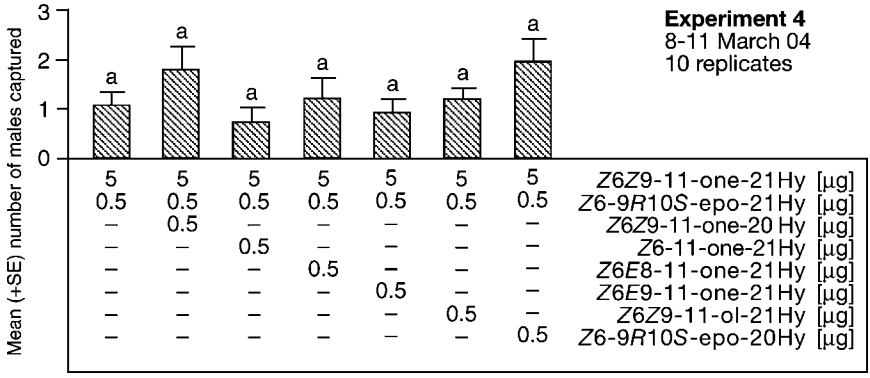

FIG. 8. Captures of male *Teia anartoides* in field experiments 4 in sticky traps baited with candidate pheromone components in various combinations; all experiments near Campbelltown, N.S.W., Australia. Bars with the same letter superscript are significantly different; $\alpha = 0.05$. Compound abbreviations as in Figure 2.

with a synthetic standard. The same compound has been reported as the major sex pheromone component of whitemarked tussock moth, *Orgyia leucostigma* (Liu, 1999; Grant et al., 2003). Whether *Z6E*8-11-one-21Hy is a (secondary) pheromone component of PAM females (Jury et al., 2003) is not easily determined because lures loaded with *Z6Z*9-11-one-21Hy as a single component will, almost immediately, also release the rearrangement product *Z6E*8-11-one-21Hy, thus complicating comparison of such lures with those baited from the beginning with both *Z6Z*9-11-one-21Hy and *Z6E*8-11-one-21Hy.

HPLC-MS was crucial in confirming the presence and ratio of Z6Z9-11-one-21Hy, *Z6E*8-11-one-21Hy, and *Z6E*9-11-one-21Hy in pheromone gland extracts. Aqueous methanol (90% MeOH) provided optimal reverse phase chromatographic separation between *Z6E*8-11-one-21Hy and *Z6Z*9-11-one-21Hy, with retention times reproducible within 0.1 min between runs. Among atmospheric pressure electrospray (APES), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photo ionization (APPI), APES provided the greatest sensitivity and most readily interpretable mass spectra. No APES mass spectra were obtained with acetonitrile as the solvent system. Changes in nebulizer gas temperature from 200°C to 350°C reduced sensitivity but did not result in changes to the mass spectra. A 150-volt fragmentation voltage revealed the major ions 307.5 amu $(MH)^+$ and 329.5 amu $(MNa)^+$ for both compounds, but at 200 volts (Figure 3) the fragmentation ion 169.3 amu and adduct ion 326.5 amu were particularly visible. The fragment ion m/z 169.3 is also observed in corresponding GC-MS analyses (data not shown).

In wind tunnel experiments (Table 1), the 4-component blend of Z6Z9-11-one-21Hy, *Z*6-9*R*10*S*-epo-21Hy, *E*7*E*9-6,11-dione-21Hy, and *E*7*E*9-6-ol-11-one-21Hy appeared more attractive to males than Z6Z9-11-one-21Hy as a single component. However, the biological activity of the former three components was not confirmed in field experiments, possibly as a result of inappropriate blend ratios, release rates, or trap designs. It remains to be determined whether or not Z6-9*R*10*S*-epo-21Hy, *E*7*E*9-6,11-dione-21Hy, and *E*7*E*9-6-ol-11-one-21Hy are indeed PAM sex pheromone components. The epoxide *Z*6-9*R*10*S*-epo-21Hy is the most likely candidate for a secondary pheromone component because it was present in the effluvia of calling females (Figure 2), and appeared to enhance attractiveness of *Z*6*Z*9-11-one-21Hy in field experiment 2 (Figure 7).

Attractiveness of synthetic pheromone lures and of virgin PAM females also will have to be compared in field trials to determine whether synthetic lures could replace PAM females as a trap bait in the program to monitor eradication of PAM in New Zealand.

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IDENTIFICATION OF SEX PHEROMONE COMPONENTS OF THE PAINTED APPLE MOTH: A TUSSOCK MOTH WITH A THERMALLY LABILE PHEROMONE COMPONENT

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Abstract—The sex pheromone of the painted apple moth, *Teia anartoides* (Lymantriidae) was investigated using GC-EAD and GC-MS analysis, derivatization, TLC analysis, and field cage and field trapping bioassays. The major sex pheromone components were identified as (6*Z*,9*Z*)-henicosa-6,9-dien-11-one and (6*Z*,9*Z*)-henicosa-6,9-diene. Other minor components of pheromone gland extracts included (6*Z*)-9*R*,10*S*-epoxyeicos-6-ene, (6*Z*)-9*R*,10*S*-epoxyhenicos-6-ene, (6*Z*,9*Z*)-henicosa-6,9-dien-11-ol, (6*Z*)-henicos-6-en-11-one, and (6*Z*,8*E*)-henicosa-6,8-dien-11-one, but the roles of these minor components remain equivocal. In field cage and field experiments, a blend of all seven identified components [(6*Z*,9*Z*)-henicosa-6,9-dien-11-one (relative amount 100), (6*Z*,9*Z*)-henicosa-6,9-diene (100), (6*Z*)-9*R*,10*S*-epoxyeicos-6-ene (5), (6*Z*)-9*R*,10*S*-epoxyhenicos-6-ene (10), (6*Z*,9*Z*)-henicosa-6,9-dien-11-ol (5), (6*Z*)-henicos-6-en-11-one (1), and (6*Z*,8*E*)-henicosa-6,8-dien-11-one (25)] was as attractive to males as calling females, but tests with blends of the major component(s) with subsets of the minor components did not produce consistent results that unequivocally showed the various minor components to be critical

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components of the active blend. (6*Z*,9*Z*)-henicosa-6,9-dien-11-one is thermally labile and rearranges to (6*Z*,8*E*)-henicosa-6,8-dien-11-one and other products at ambient temperature, rendering the synthetic pheromone lure inactive after two days of field exposure.

Key Words—Pheromone, tussock moth, Lymantriidae, *Teia anartoides*, (6*Z*, 9*Z*)-henicosa-6,9-dien-11-one, (6*Z*,8*E*)-henicosa-6,8-dien-11-one, (6*Z*)-9*R*, 10*S*-epoxyhenicos-6-ene, (6*Z*)-9*R*,10*S*-epoxyeicos-6-ene, (6*Z*,9*Z*)-henicosa-6,9-diene, (6*Z*)-henicos-6-en-11-one, painted apple moth.

INTRODUCTION

The painted apple moth, *Teia anartoides* (Walker) is a native Australian moth that was accidentally introduced into New Zealand in 1999. It was first discovered in Glendene, west Auckland, and then spread to neighboring suburbs. This insect is a minor pest in Australia but has potential for significant economic and ecological damage to New Zealand horticulture and forestry because of its wide host range. Female painted apple moths are wingless, and the ballooning larvae are the main mechanism of dispersal in this species. On discovery of this pest, the New Zealand Ministry of Agriculture and Forestry initiated an eradication program using live female moths to monitor the distribution and dispersal of the moth.

The tussock moths (family Lymantriidae) are represented in Australia by about 70 species in 16 genera and are mainly distributed in the north and east of the continent (Common, 1990). In the Lymantriidae, sex pheromones have been identified for nearly 20 species belonging to six genera (El-Sayed, 2004), representing less than 1% of the 2,500 species in this family. The identified sex pheromones are polyene-derived compounds, mostly epoxides, ketones, and diene or triene hydrocarbons, which are different from the alkenyl acetates, alcohols, and aldehydes found more commonly in other lepidopteran families (El-Sayed, 2004). No sex pheromones have been identified previously for any Australian lymantriids, despite the abundance of this group in the Australian fauna.

The objectives of the work described here were to identify and characterize the sex pheromone of the painted apple moth, *T. anartoides*, and to develop a synthetic pheromone blend to monitor its spread in New Zealand.

METHODS AND MATERIALS

Insects. A colony was established at Mt. Albert Research Center (Auckland) using insects collected from west Auckland. After pupation, the sexes were separated and maintained at 25°C under a 16:8 L:D photoperiod. Pupae required for the field cage experiments were transported to Lincoln Research Center in 9 × 2.5 cm Petri dishes and maintained in quarantine. Pupae and adults were held

under ambient temperature and natural light. All male pupae were sterilized by exposure to 100 Gy using 1.25 MeV gamma rays from a Cobalt^{60} source (Suckling et al., 2002). In the field trial conducted in Auckland, males were marked by allowing adults to emerge in Petri dishes containing fluorescent dyes (DayGlo Corp., Cleveland, OH, USA).

Pheromone Gland Extraction. The sex pheromone glands of 1–2-d-old calling females were removed between 9:00–15:00 hr (diurnal species) and extracted in ca. 10 μl of hexane for 5–10 min. For the preparation of gland extracts used in the field cage experiments, up to 100 sex pheromone glands were removed and extracted in ca. 300 μl of hexane for 10–20 min. Approximately 60 μl of this solution, equivalent to ca. 20 females, were dispensed on each substrate used in the test. Chemical analysis of gland extracts was performed using GC-EAD or GC-MS as described below.

Reduction of Female Extracts. Sex pheromone gland extracts were prepared from the glands of 100 females extracted for 10 min at ambient temperature. The hexane was decanted from the glands and concentrated under a stream of argon. The residue was redissolved in 500 μl of 96% ethanol, and $NaBH_4$ (4 mg, 0.10 mmol) was added. The reaction mixture was shaken for 5 min, and the solids were then removed by filtration through a glass wool plug. The reduced extracts were analyzed by GC-MS.

Chemicals. Chemicals used as authentic standards in chromatographic analyses or in the field experiments were either purchased, obtained as gifts, or synthesized locally. All compounds were >97% pure by GC and were stored at −80°C until used. (6*Z*)-henicos-6-en-11-one (*Z*6-11-one-21Hy) was purchased from Bedoukian Research, Inc. Danbury, CT USA. (6*Z*,9*Z*)-henicosa-6,9-diene (*Z*6,*Z*9-21Hy) was prepared according to Ando et al. (1995) and Jain et al. (1983). ^{1}H NMR ($CDCl_3$, 400 MHz) 5.36 (4H, m), 2.78 (2H, t, $J = 6.4$ Hz), 2.05 (4H,m), 1.4–1.2 (24H, br, m), 0.89 (3H, t, $J = 7.4$ Hz), 0.88 (3H, t, $J = 7.5$ Hz) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz) 130.2, 127.9, 32.0, 31.5, 29.7, 29.7, 29.6, 29.4, 29.4, 29.4, 29.4, 29.3, 27.4, 27.3, 25.6, 22.7, 22.6, 14.2, 14.1 ppm.

Epoxides. (6*Z*)-9*R*,10*S*-epoxyeicos-6-ene (*Z*6-9*R*,10*S*-epo-20Hy), (6*Z*)-9*S*,10*R*-epoxyeicos-6-ene (*Z*6-9*S*,10*R*-epo-20Hy), (6*Z*)-9*R*,10*S*-epoxyhenicos-6-ene (*Z*6-9*R*,10*S*-epo-21Hy), and (6*Z*)-9*S*,10*R*-epoxyhenicos-6-ene (*Z*6-9*S*,10*R*-epo-21Hy) were synthesized according to the methods of Zhang et al. (1999) and Soulie and Lallemand (1995). A sample of *Z*6-9*R*,10*S*-epo-21Hy was provided as a gift from J. G. Millar (University of California, Riverside, CA, USA). *Z*6-9*R*,10*S*-epo-20Hy and *Z*6-9*S*,10*R*-epo-20Hy, ^{1}H NMR ($CDCl_3$, 400 MHz) 5.5–5.3 (2H, m), 2.9–2.8 (2H, m), 2.4–2.3 (1H, m), 2.3–2.2 (1H, m), 2.0–1.9 (2H, m), 1.5–1.3 (24H, br, m), 0.86 (3H, t, $J = 6.7$ Hz), 0.85 (3H, t, $J = 6.6$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) 132.7, 123.8, 57.2, 56.5, 31.9, 31.5, 31.5, 31.5, 29.6, 29.3, 29.2, 27.8, 27.4, 26.6, 26.2, 22.7, 22.6, 22.5, 14.1, 14.0 ppm. *Z*6-9*R*,10*S*-epo-21Hy and *Z*6-9*S*,10*R*-epo-21Hy, ^{1}H NMR ($CDCl_3$, 400 MHz) 5.5–5.3

(2H, m), 2.9–2.8 (2H, m), 2.4–2.3 (1H, m), 2.2–2.1 (1H, m), 2.0–1.9 (2H, m), 1.4–1.1 (26H, m), 0.86 (3H, t, $J = 6.5$ Hz), 0.85 (3H, t, $J = 6.8$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) 132.7, 123.8, 57.2, 56.6, 31.9, 31.5, 29.6, 29.6, 29.6, 29.6, 29.6, 29.3, 29.2, 27.8, 27.4, 26.6, 26.2, 22.7, 22.5, 14.1, 14.0 ppm. All epoxide enantiomers were estimated to be >93% enantiomerically pure (>86% e.e.)

Isomers of 6,8-Diene Ketones. All four isomers of 6,8-diene ketone, (6*Z*, 8*E*)-henicosa-6,8-dien-11-one (*Z*6,*E*8-11-one-21Hy), (6*E*,8*Z*)-henicosa-6,8-dien-11-one (*E*6,*Z*8-11-one-21Hy), (6*Z*,8*Z*)-henicosa-6,8-dien-11-one (*Z*6,*Z*8-11-one-21Hy), and (6*E*,8*E*)-henicosa-6,8-dien-11-one (*E*6,*E*8-11-one-21Hy)-were synthesized according to Comeskey et al. (2004), Muto and Mori (2003), and Jury et al. (2003). *Z*6,*E*8-11-one-21Hy; ^{1}H NMR ($CDCl_3$, 400 MHz) 6.38 (1H, ddq, $J = 15.2, 11.2, 1.2$ Hz), 5.98 (1H, dd, $J = 11.2, 11.2$ Hz), 5.73 (1H, dt, $J = 15.2, 7.4$ Hz), 5.40 (1H, dt, $J = 11.2, 7.7$ Hz), 3.20 (2H, d, $J = 7.4$ Hz), 2.43 (2H, t, $J = 7.5$ Hz), 2.18–2.12 (2H, m), 1.64–1.10 (22H, br, m,), 0.90–0.86 (6H, m, H1 and H21). ^{13}C NMR ($CDCl_3$) 209.2, 132.2, 129.4, 127.8, 125.0, 46.9, 42.4, 31.8, 31.4, 29.7, 29.5, 29.5, 29.4, 29.3, 29.3, 29.2, 23.7, 22.7, 22.5, 14.1, 14.0 ppm; MS: *m*/*z* (rel. int. %) 306 (M^+, 6), 221 (4), 170 (9), 169 (100), 109 (10), 95 (32), 85 (46), 81 (20), 71 (44), 67 (30), 57 (69), 43 (65). E6,*E*8-11-one-21Hy; ^{1}H NMR ($CDCl_3$, 400 MHz) 6.10-5.99 (2H, m), 5.74–5.59 (2H, m), 3.15 (2H, d, $J = 7.2$) 2.42 (2H, t, $J = 7.4$ Hz), 2.06 (2H, m), 1.70–1.60 (2H, m), 1.40–1.20 (20H, m), 0.89 (3H, t, $J = 6.6$ Hz), 0.88 (3H, t, $J = 6.7$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) 209.3, 134.7, 134.2, 129.6, 122.8, 46.7, 42.3, 32.5, 31.9, 31.4, 29.5, 29.4, 29.4, 29.3, 29.2, 28.9, 23.7, 22.6, 22.5, 14.1, 14.0 ppm. E6,*Z*8-11-one-21Hy; ^{1}H NMR ($CDCl_3$, 400 MHz) 6.24–6.09 (2H, m), 5.78–5.71 (1H, m), 5.48–5.42 (1H, m), 3.26 (2H, dd, $J = 7.5, 1.2$ Hz), 2.43 (2H, t, $J = 7.3$ Hz), 2.11 (2H, m), 1.70-1.60 (2H, m), 1.50–1.30 (20H, m), 0.88 (3H, t, $J = 6.8$ Hz), 0.87 (3H, t, $J = 6.8$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) 208.9, 137.1, 131.7, 124.8, 120.0, 42.3, 42.0, 32.0, 31.9, 31.4, 29.5, 29.4, 29.3, 29.2, 28.9, 23.8, 22.7, 22.5, 14.1, 14.0 ppm. *Z*6,*Z*8-11-one-21Hy; ^{1}H NMR ($CDCl_3$, 400 MHz) 6.44 (1H, m), 6.17 (1H, m), 5.61–5.54 (2H, m), 3.29 (2H, dd, $J = 7.5, 1.4$ Hz), 2.43 (2H, t, $J = 7.5$ Hz), 2.17 (2H, m), 1.60–1.40 (2H, m), 1.50–1.20 (20H, m), 0.88 (3H, t, $J = 6.8$ Hz), 0.88 (3H, t, $J = 6.2$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) 208.8, 134.3, 126.5, 122.8, 122.1, 42.4, 41.7, 31.9, 31.4, 29.5, 29.4, 29.4, 29.3, 29.2, 29.2, 27.5, 23.8, 22.6, 22.5, 14.1, 14.0 ppm.

(6Z,8E)-henicosa-6,8-dien-11-ol. (6*Z*,8*E*)-henicosa-6,8-dien-11-ol (*Z*6, *E*8-11-ol-21Hy) has been prepared by Gries et al. (1997), however, this route yielded a mixture of isomeric polyenes requring extensive purification by silver nitrate mediated preparative chromatography. An alternative, more stereoselective route to *Z*6,*E*8-11-ol-21Hy described by Jury et al. (2003) was used to prepare this compound. ^{1}H NMR ($CDCl_3$, 400 MHz) 6.39 (1H, ddd, $J = 1.1, 11.0, 15.1$ Hz), 5.96 (1H, dd, $J = 11.0, 11.0$ Hz), 5.64 (1H, dt, $J = 7.5, 15.0$ Hz), 5.35 (1H, dt, $J = 7.6, 10.8$ Hz), 3.64–3.60 (1H,m), 2.35–2.29 (1H, m), 2.21–2.12 (3H, m),

1.73 (1H, br s), 1.46-1.25 (24H, m), 0.90–0.85 (6H, m) ppm. ^{13}C NMR ($CDCl_3$, 100 MHz) 131.4, 129.5, 129.0, 128.1, 71.1, 40.9, 27.7, 36.8, 31.9, 31.4, 29.6, 29.6, 29.3, 25.7, 22.7, 22.5, 22.5, 22.5, 22.5, 14.0, 14.1 ppm.

(6Z,9Z)-henicosa-6,9-dien-11-ol. (6*Z*,9*Z*)-henicosa-6,9-dien-11-ol (*Z*6, *Z*9-11-ol-21Hy) was prepared as described by Jury et al. (2003). ^{1}H NMR ($CDCl_3$, 400 MHz) 6.39 (1H, ddd, $J = 1.1$, 11.0, 15.1 Hz), 5.96 (1H, dd, $J = 11.0$, 11.0 Hz), 5.64 (1H, dt, $J = 7.5$, 15.0 Hz), 5.35 (1H, dt, $J = 7.6$, 10.8 Hz), 3.64–3.60 (1H, m), 2.35-2.29 (1H, m), 2.21–2.12 (3H, m), 1.73 (1H, br s), 1.46–1.25 (24H, m), 0.90–0.85 (6H, m). ^{13}C NMR ($CDCl_3$, 100 MHz) 131.4, 129.5, 129.0, 128.1, 71.1, 40.9, 27.7, 36.8, 31.9, 31.4, 29.6, 29.3, 25.7, 22.7, 22.5, 22.5, 22.5, 14.0, 14.1 ppm.

(7E,9Z)-henicosa-7,9-dien-11-one and (7E,9E)-henicosa-7,9-dien-11-one. (7*E*,9*Z*)-henicosa-7,9-dien-11-one (*E*7,*Z*9-11-one-21Hy) and (7*E*,9*E*)-henicosa-7,9-dien-11-one (*E*7,E9-11-one-21 Hy) were prepared according to Comeskey and Bunn (2004). *E*7,*Z*9-11-one-21Hy; ^{1}H NMR ($CDCl_3$, 400 MHz) 7.44–7.38 (1H, m), 6.37 (1H, m), 6.17–6.05 (1H, m), 5.92–5.90 (1H, m), 2.45 (2H, t, $J = 7.2$ Hz), 2.18 (2H, m), 1.30–1.10 (24H, m), 0.88 (3H, t, $J = 6.1$ Hz), 0.87 (3H, t, $J = 6.0$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) 201.8, 147.2, 143.2, 127.6, 122.4, 44.3, 33.0, 31.9, 31.9, 31.6, 29.6, 29.5, 29.4, 29.3, 28.9, 28.8, 24.2, 22.7, 22.6, 14.1, 14.0 ppm. *E*7,E9-11-one-21Hy; ^{1}H NMR ($CDCl_3$, 400 MHz) 7.16–7.09 (1H, m), 6.17–6.15 (2H, m), 6.07 (1H, d, $J = 15.4$ Hz), 2.52 (2H, t, $J = 7.3$ Hz), 2.16 (2H, m), 1.50–1.30 (2H, m), 1.30–1.10 (22H, m), 0.87 (3H, t, $J = 6.7$ Hz), 0.86 (3H, t, $J = 7.0$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) 201.2, 145.7, 142.9, 128.8, 127.9, 40.5, 33.1, 31.9, 31.6, 29.6, 29.5, 29.4, 29.3, 29.3, 28.8, 28.7, 24.5, 22.7, 22.5, 14.1, 14.0 ppm.

(6Z,9Z)-henicosa-6,9-dien-11-one (6*Z*,9*Z*)-henicosa-6,9-dien-11-one (*Z*6, *Z*9-11-one-21Hy) was prepared by adding Dess-Martin periodinane (40 mg, 0.094 mmol) to a solution of *Z*6,*Z*9-11-ol-21Hy (10 mg, 0.032 mmol) in dichloromethane (5 ml) at room temperature. The reaction was stirred for 1.5 hr at room temperature. Sodium bicarbonate (5 ml, sat. aq.) was added, and the organic phase was separated. The aqueous phase was extracted with dichloromethane (2×5 ml) and the combined extracts were dried over anhydrous $MgSO_4$. After filtration, the solvent was removed *in vacuo* and the residue was adsorbed onto silica gel. The crude product was purified by silica gel column chromatography (5% EtOAc in petroleum ether) giving pure *Z*6,*Z*9-11-one-21Hy as a pale yellow oil, (8 mg, 0.026 mmol, 81%). The product was immediately cooled and stored at -80°C prior to testing. ^{1}H NMR ($CDCl_3$, 400 MHz) 6.2–5.9 (2H, m), 5.5–5.3 (2H, m), 3.4–3.3 (2H, m), 2.5–2.4 (2H, m), 2.1–1.9 (2H, m), 1.7–1.5 (2H, m), 1.3–1.1 (20H, br, m), 0.87 (3H, t, $J = 6.5$ Hz), 0.84 (3H, t, $J = 6.5$ Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) 201.9, 146.2, 132.0, 126.1, 126.0, 44.3, 31.9, 31.4, 29.5, 29.4, 29.4, 29.3, 29.2, 29.2, 27.8, 27.2, 23.9, 22.6, 22.5, 14.1, 14.0 ppm.

TLC Analysis of Extracts and Synthetic Chemicals. Freshly prepared gland extracts (50 FE/100 μl hexane), *Z*6,*E*8-11-one-21Hy (1 mg/ml in hexane), and *Z*6,*Z*9-11-one-21Hy (1 mg/ml in hexane) were analyzed using Merck Silica Gel 60 F_{254} TLC plates (10% EtOAc in petroleum ether). Visualization was by UV (254 nm) and by development with vanillin spray reagent (prepared from vanillin (3 g), ethanol (500 ml), and conc. H_2SO_4 (10 ml)) followed by heating with a heat gun. Samples of synthetics and female extract were co-spotted and eluted on the same plate for comparisons of Rf values.

TLC Study of Solutions of (6Z,9Z)-henicosa-6,9-dien-11-one under different light conditions. Freshly prepared *Z*6,*Z*9-11-one-21Hy (8 mg) was dissolved in hexane (2 ml), and this solution was divided into two 1 ml portions, each of which were stored in 1 ml volumetric flasks under argon. One of the samples was placed in bright sunshine on the windowsill, and the other was wrapped in aluminum foil and stored in a closed cupboard. Both samples were left overnight at room temperature, and each was analyzed by TLC as described above after 24 hr.

TLC Study of the Effect of BHT on Stability of (6Z,9Z)-henicosa-6,9-dien-11-one. Freshly prepared *Z*6,*Z*9-11-one-21Hy was dissolved in hexane (1 mg/ml). Solutions of BHT (2,6-di-*tert*-butyl-4-hydroxy toluene) were made at the following concentrations: 5% w/v, 1% w/v, 0.1% w/v, and 0.01% w/v in hexane. The TLC mixtures were made as follows: 200 μl of each of the BHT solutions were added to 200 μl of the ketone solution. The mixtures were then flushed with argon and stoppered. Each sample was analyzed by TLC as described above after 24 hr.

Determination of Absolute Configuration of (6Z)-9,10-epoxyhenicos-6-ene. A heptane extract of 90 painted apple moth pheromone glands was fractionated on 1 g of silica gel (Merck, Keiselgel 60) by column chromatography, using a step gradient from heptane to diethyl ether. The heptane/diethyl ether (9:1) fraction contained epoxide and ketone pheromone components by GC-MS. The chirality of (6*Z*)-9,10-epoxyhenicos-6-ene (*Z*6-9,10-epo-21Hy) was determined using a similar method to that applied by Qin et al. (1997) to (6*Z*,9*Z*)-3,4-epoxyoctadeca-6,9-diene. Thus, the fraction containing *Z*6-9,10-epo-21Hy was concentrated under a gentle stream of N_2, in a 6 × 50 mm culture tube. The residue was treated with 3 drops of 14% BF_3/MeOH (Sigma-Aldrich) for 4 hr, diluted with 100 μl heptane, and extracted with 100 μl water. The heptane layer contained 9-hydroxy-10-methoxy and 10-hydroxy-9-methoxy derivatives in a 2:1 ratio, respectively, by GC-MS. These were reacted with 1.2 μl (*S*)-2-acetoxypropionyl chloride (Sigma-Aldrich) in 20 μl pyridine for 16 hr, using a similar method to Slessor et al. (1985), to give two possible diastereoisomers for each alcohol. These were analyzed by GC and GC-MS (BPX-70 column, temperature program: 40°C (1 min hold) to 160°C at 10°C/min, followed by 160°C to 220°C at 4°C/min. Samples of *Z*6-9*S*,10*R*-epo-21Hy and *Z*6-9*R*,10*S*-epo-21Hy, purified by chiral HPLC

(Yamamoto et al., 1999), were derivatized under the same conditions, and the GC results were compared with those for the natural pheromone.

Gas Chromatography-Electroantennogram Detector Studies (GC-EAD). Coupled GC-EAD analysis of pheromone gland extracts and synthetic compounds was conducted on a Varian 3800 GC equipped with a flame ionization detector (FID) and a splitless injector. The column effluent was split 1:1 between the FID and EAD apparatus. Antennal depolarization was detected using a high-resistance EAD Probe, Signal Interface Box Type ID-02, and Intelligent Data Acquisition Controller Type IDAC-02 (Syntech, Hilversum, The Netherlands). Antennae from 2–3 d-old males were excised at the base and attached to the silver electrodes housed in a saline glass electrode using a micromanipulator. Up to 10 antennal preparations were used for GC-EAD analyses. A 30 m × 0.25 mm ID, BPX-70 capillary column (SGE, Ringwood, Victoria, Australia) (this column has higher polarity than a DB-Wax column and is comparable to DB-23) and a 30 m × 0.5 mm ID, DB-5 capillary column (J&W Scientific Folsom, CA, USA) and a Y splitter (Alltech, Deerfield, IL, USA) were used for the analyses. For the BPX-70 capillary column, the oven temperature was programmed from 80°C (held for 1 min) to 240°C at 10°C/min. For the DB-5 capillary column, the oven temperature was programmed from 100°C (held for 1 min) to 210°C at 10°C/min, held for 1 min, and then increased to 240°C at 1.5°C/min. Helium was used as the carrier gas.

Gas Chromatography (GC) Analysis. Gas chromatography of the gland extracts and synthetic standards was performed using three different 30 m × 0.25-mm i.d. capillary columns, DB-5, DB-Wax, and BPX-70. Helium was used as the carrier gas at a flow rate of 1 ml/min. Oven temperature was maintained at 80°C for 1 min, and raised to 240°C at a rate of 10°C/min and held for 30 min.

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis. The gland extracts and synthetic chemicals were analyzed on two different GC-MS systems: a Saturn 2200 GC-MS (Varian Walnut Creek, CA, USA) using an ionization voltage of 70 eV and a mass range of 30 to 500, equipped with a 30 m × 0.25 mm i.d. × 0.5 μm film DB-5 MS capillary column (J&W Scientific) and splitless injection, and a Shimadzu QP5050A (Shimadzu, Kyoto, Japan) equipped with a 30 m × 0.25 mm i.d. × 0.25 um BPX-70 capillary column (SGE) also with splitless injection. In the former machine, the oven was programmed from 100°C (held for 1 min) to 210°C at 10°C/min, held for 1 min, and then increased to 240°C at 1.5°C/min and finally to 300°C at 15°C/min, while in the latter machine, the temperature was programmed from 80°C (held for 2 min) to 240°C at 10°C/min. Compounds were identified by comparing retention times and mass spectra with those of synthetic compounds.

Field Cage Experiments. In the first trapping experiment, we examined the efficacy of different substrates for releasing the unstable *Z*6,*Z*9-11-one-21Hy. Four substrates were used; cigarette filters (Moss Packaging Co. Ltd., Wellington,

New Zealand), 2 ml polyethylene vial closure containing a plug of bleached nonsurgical cotton batting (No. 60975d-3, Kimble Glass, Vineland, NJ, USA) (Grant et al., 2003), dental roll (Roeko, Langenau, Germany), and filter paper (Whatman International Ltd., Maidstone, England). Each substrate was loaded with 20 female equivalents (FE) of freshly prepared female gland extract dissolved in 60 μl hexane.

In the second experiment, different doses (50, 100, 200, and 400 μg) of synthetic *Z*6,*Z*9-11-one-21Hy were tested using the best two substrates in experiment 1 (i.e., cigarette filter and dental roll). In experiment 3, we tested the effect of adding other compounds to *Z*6,*Z*9-11-one-21Hy. The following treatments were compared: (*i*) 50 μg *Z*6,*Z*9-11-one-21Hy; (*ii*) 50 μg *Z*6,*Z*9-11-one-21Hy, 12.5 μg *Z*6,*E*8-11-one-21Hy; (*iii*) 50 μg *Z*6,*Z*9-11-one-21Hy, 12.5 μg *Z*6,*E*8-11-one-21Hy, 0.5 μg *Z*6-11-one-21Hy; (*iv*) 50 μg *Z*6,*Z*9-11-one-21Hy, 12.5 μg *Z*6,*E*8-11-one-21Hy, 0.5 μg *Z*6-11-one-21Hy, 2.5 μg *Z*6,*Z*9-11-ol-21Hy, 5 μg *Z*6-9*R*,10*S*-epo-21Hy, 2.5 μg *Z*6-9*R*,10*S*-epo-20Hy, 50 μg *Z*6,*Z*9-21Hy; (*v*) blank.

In experiment 4, the effects of omitting *Z*6,*Z*9-21Hy and the two ketones *Z*6,*E*8-11-one-21Hy and *Z*6-11-one-21Hy from the best blend (identified in experiment 3) were tested. The rationale for this experiment was a) if it was possible to omit the diene hydrocarbon without loss of activity, then this would significantly reduce the cost of production of a synthetic lure, and b) because synthetic *Z*6,*Z*9-11-one-21Hy rearranges to *Z*6,*E*8-11-one-21Hy and other ketones in the GC-MS system, the presence of these ketones in gland extracts may be due solely to the rearrangement of *Z*6,*Z*9-11-one-21Hy. To address these two issues, the following treatments were tested: (*i*) 50 μg *Z*6,*Z*9-11-one-21Hy; (*ii*) 50 μg *Z*6,*Z*9-11-one-21Hy, 12.5 μg *Z*6,*E*8-11-one-21Hy, 0.5 μg *Z*6-11-one-21Hy, 2.5 μg *Z*6,*Z*9-11-ol-21Hy, 5 μg *Z*6-9*R*,10*S*-epo-21Hy, 2.5 μg *Z*6-9*R*,10*S*-epo-20Hy, 50 μg *Z*6,*Z*9-21Hy; (*iii*) 50 μg *Z*6,*Z*9-11-one-21Hy, 12.5 μg *Z*6,*E*8-11-one-21Hy, 0.5 μg *Z*6-11-one-21Hy, 2.5 μg *Z*6,*Z*9-11-o1-21Hy, 5 μg *Z*6-9*R*,10*S*-epo-21Hy, 2.5 μg *Z*6-9*R*,10*S*-epo-20Hy; (*iv*) 50 μg *Z*6,*Z*9-11-one-21Hy, 2.5 μg *Z*6,*Z*9-11-ol-21Hy, 5 μg *Z*6-9*R*,10*S*-epo-21Hy, 2.5 μg *Z*6-9*R*,10*S*-epo-20Hy; 50 μg *Z*6,*Z*9-21Hy (*v*) blank.

In the fifth experiment, the two enantiomers of the two epoxides (6*Z*)-9,10-epoxyeicos-6-ene (*Z*6-9,10-epo-20Hy) and *Z*6-9,10-epo-21Hy were tested to determine the active enantiomers. The following treatments were tested: (*i*) 50 μg *Z*6,*Z*9-11-one-21Hy, 12.5 μg *Z*6,*E*8-11-one-21Hy, 0.5 μg *Z*6-11-one-21Hy, 2.5 μg *Z*6,*Z*9-11-ol-21Hy, 5 μg *Z*6-9*R*,10*S*-epo-21Hy, 2.5 μg *Z*6-9*R*,10*S*-epo-20Hy, 50 μg *Z*6,*Z*9-21Hy; (*ii*) 50 μg *Z*6,*Z*9-11-one-21Hy, 12.5 μg *Z*6,*E*8-11-one-21Hy, 0.5 μg *Z*6-11-one-21Hy, 2.5 μg *Z*6,*Z*9-11-ol-21Hy, 2.5 μg *Z*6-9*R*,10*S*-epo-21Hy, 2.5 μg *Z*6-9*S*,10*R*-epo-21Hy, 1.25 μg *Z*6-9*R*,10*S*-epo-20Hy, 1.25 μg *Z*6-9*S*,10*R*-epo-20Hy, 50 μg *Z*6,*Z*9-21Hy; (*iii*) 50 μg *Z*6,*Z*9-11-one-21Hy, 12.5 μg *Z*6,*E*8-11-one-21Hy, 0.5 μg *Z*6-11-one-21Hy, 2.5 μg *Z*6,*Z*9-11-ol-21Hy, 5 μg *Z*6-9*S*,10*R*-epo-21Hy, 2.5 μg *Z*6-9*S*,10*R*-epo-20Hy, 50 μg

*Z*6,*Z*9-21Hy; (*iv*) 50 μg *Z*6,*Z*9-11-one-21Hy, 12.5 μg *Z*6,*E*8-11-one-21Hy, 0.5 μg *Z*6-11-one-21Hy, 2.5 μg *Z*6,*Z*9-11-ol-21Hy, 50 μg *Z*6,*Z*9-21Hy; (*v*) blank.

In the final field cage experiment, the attractiveness of virgin females and the best synthetic pheromone blend were compared. A 1–2 d old virgin female was placed in a 6 cm long × 4 cm diam plastic cage that was closed at each end with a metal screen (8 × 8 mm mesh), while the synthetic pheromone contained the following compounds: 50 μg *Z*6,*Z*9-11-one-21Hy, 12.5 μg *Z*6,*E*8-11-one-21Hy, 0.5 μg *Z*6-11-one-21Hy, 2.5 μg *Z*6,*Z*9-11-ol-21Hy, 5 μg *Z*6-9*R*,10*S*-epo-21Hy, 2.5 μg *Z*6-9*R*,10*S*-epo-20Hy, 50 μg *Z*6,*Z*9-21Hy. A blank trap was used as a control.

In all field experiments, the ratio of *Z*6,*Z*9-11-one-21Hy was arbitrarily set to 100%, while the relative ratio of *Z*6,*E*8-11-one-21Hy was based on the ratio of the *Z*6,*E*8-11-ol-21Hy to *Z*6,*Z*9-11-ol-21Hy in the reduced gland extract. The relative ratios of the other five compounds (*Z*6-11-one-21Hy, *Z*6,*Z*9-11-ol-21Hy, *Z*6-9*R*,10*S*-epo-20Hy, *Z*6-9*R*,10*S*-epo-21Hy, and *Z*6,*Z*9-21Hy) were based on their original amounts relative to the rearrangement products of *Z*6,*Z*9-11-one-21Hy in the non-reduced gland extracts. All field cage experiments were carried out in a field cage (8 m long × 8 m wide × 3 m high), covered with shade cloth (mesh size 3 × 3 mm). Compounds were applied to the substrates in 50–200 μl of analytical grade hexane in a fume hood. Immediately after preparation, lures were stored on dry ice and transported in an ice chest to the field cage for testing. White plastic delta traps (Suckling and Shaw, 1991) were placed approximately 2 m from the ground and 2 m apart from each other. Trap positions were rotated every day (four in total) of an experiment. Sterilized 1–2 d old painted apple moth males were placed in the field cage between 9.30 and 12.00 hr. A total of ca. 3,900 males were released in all of the field cage experiments. The significance of differences between treatments was identified using ANOVA. Significantly different treatment means were identified using Fisher's Protected Least Significant Difference Test (SAS Institute Inc., 1998).

Field Trapping Experiments. In experiments conducted in the field at Edenvale Park, Auckland from February 17–22, 2004, we tested the following synthetic blends in delta traps: (*i*) 50 μg *Z*6,*Z*9-11-one-21Hy; (*ii*) 50 μg *Z*6,*Z*9-11-one-21Hy, 12.5 μg *Z*6,*E*8-11-one-21Hy, 0.5 μg *Z*6-11-one-21Hy; (*iii*) 50 μg *Z*6,*Z*9-11-one-21Hy, 12.5 μg *Z*6,*E*8-11-one-21Hy, 0.5 μg *Z*6-11-one-21Hy, 2.5 μg *Z*6,*Z*9-11-ol-21Hy, 5 μg *Z*6-9*R*,10*S*-epo-21Hy, 2.5 μg *Z*6-9*R*,10*S*-epo-20Hy, 50 μg *Z*6,*Z*9-21Hy; (*iv*) 50 μg *Z*6,*Z*9-11-one-21Hy, 12.5 μg *Z*6,*E*8-11-one-21Hy, 0.5 μg *Z*6-11-one-21Hy, 2.5 μg *Z*6,*Z*9-11-ol-21Hy, 5 μg *Z*6-9*R*,10*S*-epo-21Hy, 2.5 μg *Z*6-9*R*,10*S*-epo-20Hy; (*v*) 50 μg *Z*6,*Z*9-11-one-21Hy, 5 μg *Z*6-9*R*,10*S*-epo-21Hy, 2.5 μg *Z*6-9*R*,10*S*-epo-20Hy; (*vi*) 50 μg *Z*6,*Z*9-11-one-21Hy, 2.5 μg *Z*6,*Z*9-11-ol-21Hy; (*vii*) live female; (*viii*) blank. Five replicates of the eight treatments were placed in the area, with about 25 m

separation between replicates. During the experiment, approximately 6,000 sterile, dyed males were released near the trapping site as part of a Sterile Insect Technique (SIT) program to eradicate the painted apple moth (Suckling, 2003). The effect of treatment on the mean number of *T. anartoides* captured was tested using ANOVA (SAS Institute Inc., 1998) after variances were stabilized using the $\sqrt{x}$ transformation. Significantly different treatment means were identified using Fisher's Protected Least Significant Difference Test (SAS Institute Inc., 1998).

RESULTS

Identification of Pheromone Gland Components. Analysis of pheromone gland extracts by GC-EAD using polar (BPX-70), and non-polar capillary columns (DB-5) revealed that nine compounds consistently elicited antennal responses from male antennae (Figures 1 and 2). The mass spectrum of component 1 had the diagnostic ions *m/z* 292 $[M]^+$, 194 $[M\text{-}\ C_7H_{14}]^+$, 81 $[C_6H_9]^+$, and 67 $[C_5H_7]^+$ and was identical to the mass spectrum of the authentic standard *Z*6,*Z*9-21Hy. On GC analysis of the gland extracts on three different columns (BPX-70, DB-WAX, DB-5), component 1 and the authentic standard *Z*6,*Z*9-21Hy showed the same

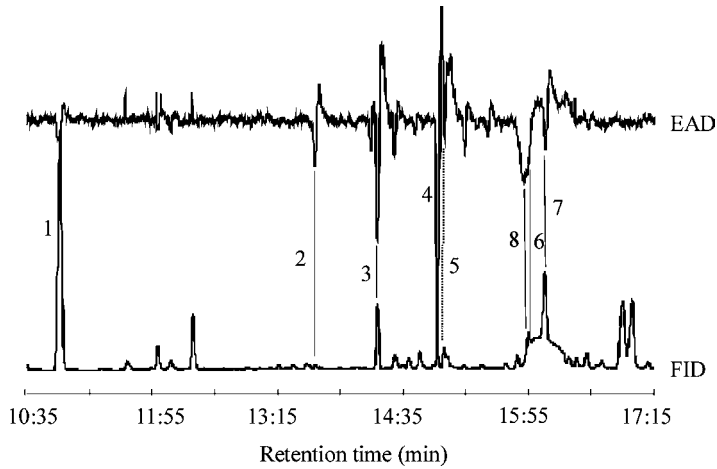


FIG. 1. Coupled GC-EAD responses of antenna of male painted apple moth, *T. anartoides*, to female gland extract. Peak identification: (6*Z*,9*Z*)-henicosa-6,9-diene (1), (6*Z*)-9*R*,10*S*-epoxyeicos-6-ene (2), (6*Z*)-9*R*,10*S*-epoxyhenicos-6-ene (3), (6*Z*)-henicos-6-en-11-one (4), (6*Z*,9*Z*)-henicosa-6,9-dien-11-ol (5), (6*E*,8*Z*)-henicosa-6,8-dien-11-one (6), (6*Z*,8*E*)-henicosa-6,8-dien-11-one (7), and (7*E*,9*Z*)-henicosa-7,9-dien-11-one (8). Chromatographic column and conditions: BPX-70, 30 m × 0.25 mm i.d.; program 80°C/1 min, 10°C/min to 240°C, 240°C/30 min.

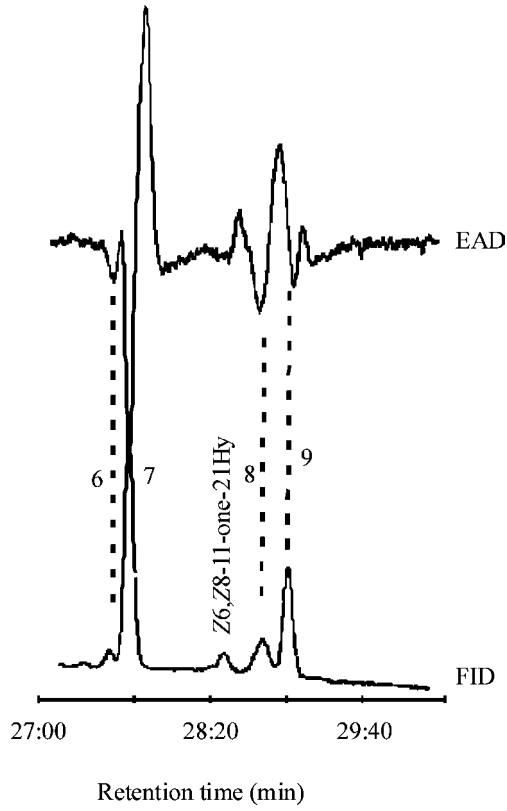


FIG. 2. Coupled GC-EAD responses of antenna of male painted apple moth, *T. anartoides* to female gland extract. Peak identification: (6*E*,8*Z*)-henicosa-6,8-dien-11-one (6), (6*Z*,8*E*)-henicosa-6,8-dien-11-one (7), (7*E*,9*Z*)-henicosa-7,9-dien-11-one (8), and (6*E*,8*E*)-henicosa-6,8-dien-11-one (9). Chromatographic column and conditions: DB-5, 30 m × 0.25 mm i.d.; program 100°C/1 min, 10°C/min to 210°C, isothermal for 1 min and 1.5°C/min to 240°C, then 15°C/min to 300°C, held for 30 min.

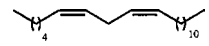
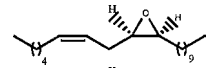
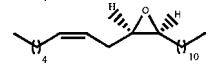
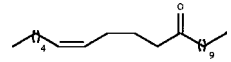
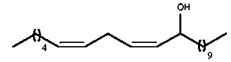
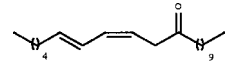
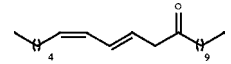
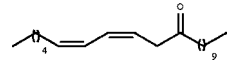
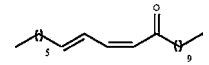
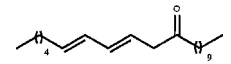
retention time. Mass spectra of components 2 and 3 were similar for most of the *m/z* values, such as 81 $[C_6H_9]^+$, 124 $[C_9H_{16}]^+$, and 153 $[C_{10}H_{17}O]^+$, except for their respective molecular ions at *m/z* 294 $[M]^+$ and 308 $[M]^+$, suggesting two mono-unsaturated epoxides with 20 and 21 carbon atoms, respectively. The mass spectra and GC retention times (three capillary columns) of components 2 and 3 were identical to those of the authentic standards *Z*6-9,10-epo-20Hy and *Z*6-9,10-epo-21Hy, respectively. On the DB-5 column, *Z*6-9,10-epo-21Hy (component 3) co-eluted with *Z*6-11-one-21Hy (component 4). The absolute configuration of

the *Z*6-9,10-epo-21Hy was determined to be 9*R*,10*S* by micro-derivatization (see below). Because of the small quantity present, derivatization was not possible for the *Z*6-9,10-epo-20Hy obtained from the gland extracts. However, field trapping experiments testing the two enantiomers (9*S*,10*R*; 9*R*,10*S*) confirmed that *Z*6-9*R*, 10*S*-epo-20Hy was indeed the active enantiomer (experiment 5).

The mass spectrum of component 4 had the diagnostic ions *m/z* 309 (M+ H^+), 291 [M+1-H_2O], 223 $[C_{15}H_{27}O]^+$, 169 $[C_{11}H_{21}O]^+$, and 167 $[C_{11}H_{19}O]^+$ suggesting a possible mono-unsaturated 21-carbon ketone. The spectrum of component 4 and its retention times on BPX-70 and DB-WAX were identical to those of the authentic standard *Z*6-11-one-21Hy. Component 5 showed diagnostic ions at *m/z* 308 $[M]^+$, 290 [M-H_2O]$^+$, 233 $[C_{17}H_{29}]^+$, 177 $[C_{13}H_{21}]^+$, 163 $[C_{12}H_{19}]^+$, 135 $[C_{10}H_{15}]$ suggestive of a di-unsaturated 21-carbon alcohol. The spectrum of component 5 and its retention times on three different columns were identical to those of authentic *Z*6,*Z*9-11-ol-21Hy. The mass spectra of components, 6, 7, and 9 were similar and had diagnostic ions at *m/z* 307 $[M+H]^+$ and 169 $[C_{11}H_{21}O]^+$ suggesting different stereoisomers of a di-unsaturated C21 ketone with a carbonyl group on C11. On a BPX-70 capillary column, the diene ketones were not fully separated (Figure 1) whereas in another GC-MS analysis aimed at improving the observed resolution and fully separating the diene ketones, a non-polar column (DB-5) and a slow temperature program were used, and the diene ketones were fully separated (Figure 2). GC and GC-MS analyses of gland extracts and the authentic standards *E*6,*Z*8-11-one-21Hy, *Z*6,*E*8-11-one-21Hy, *Z*6,*Z*8-11-one-21Hy, and *E*6,*E*8-11-one-21Hy on the DB-5 capillary column confirmed that components 6, 7, and 9 were *E*6,*Z*8-11-one-21Hy, *Z*6,*E*8-11-one-21Hy, and *E*6,*E*8-11-one-21Hy, respectively. The stereoisomer *Z*6,*Z*8-11-one-21Hy was also present in the gland extracts, although this compound did not elicit any antennal response (Figure 2). The mass spectrum of component 8 had diagnostic ions at *m/z* 307 $[M+H]^+$, 165 $[C_{11}H_{17}O]^+$, 169 $[C_{11}H_{21}O]^+$, and a base peak at 221 $[C_{15}H_{25}O]^+$ suggesting a different regioisomer of a C21 ketone. GC and GC-MS analysis of gland extracts, and the authentic compounds *E*7,*Z*9-11-one-21Hy and *E*7, *E*9-11-one-21Hy on BPX- 70 and DB-5 capillary columns confirmed that component 8 was *E*7,*Z*9-11-one-21Hy.

*Z*6,*E*8-11-one-21 Hy and *Z*6,*Z*9-21Hy were the major components present in the pheromone gland extracts. The relative amounts of the other seven components ranged from 0.5–100% of *Z*6,*E*8-11-one-21Hy (Table 1). *E*6,*Z*8-11-one-21Hy, *Z*6,*E*8-11-one-21Hy, *E*7,*Z*9-11-one-21Hy, and *E*6,*E*8-11-one-21Hy elicited antennal responses, whereas *Z*6,*Z*8-11-one-21Hy did not (Figure 2). Of the nine compounds detected by GC-EAD, four diene ketones (*Z*6,*E*8-11-one-21Hy, *E*6,*Z*8-11-one-21Hy, *E*6,*E*8-11-one-21Hy, *E*7,*Z*9-11-one-21Hy), and a monoene ketone (*Z*6-11-one-21Hy) elicited the strongest antennal responses. The two epoxides, *Z*6-9*R*,10*S*-epo-20Hy and *Z*6-9*R*,10*S*-epo-21Hy, elicited medium responses, and the dienic hydrocarbon, *Z*6,*Z*9-21Hy and the

TABLE 1. COMPOUNDS IDENTIFIED BY GC-MS ANALYSIS[a] AND THEIR APPARENT RATIO IN THE SEX PHEROMONE GLAND EXTRACTS AND EFFLUVIA OF FEMALE PAINTED APPLE MOTH, *Teia anartoides*

Compound Name (abbreviation)	Chemical structure	Relative ratio
(6*Z*,9*Z*)-henicosa-6,9-diene (*Z*6,*Z*9-21Hy)		100
(6*Z*)-9*R*,10*S*-epoxyeicos-6-ene (*Z*6-9*R*,10*S*-epo-20Hy)		5
(6*Z*)-9*R*,10*S*-epoxyhenicos-6-ene (*Z*6-9*R*,10*S*-epo-21Hy)		11
(6*Z*)-henicos-6-en-11-one (*Z*6-11-one-21Hy)		0.5
(6*Z*,9*Z*)-henicosa-6,9-dien-11-ol (*Z*6,*Z*9-11-ol-21Hy)		5
(6*E*,8*Z*)-henicosa-6,8-dien-11-one (*E*6,*Z*8-11-one-21Hy)		3
(6*Z*,8*E*)-henicosa-6,8-dien-11-one (*Z*6,*E*8-11-one-21Hy)		100
(6*Z*,8*Z*)-henicosa-6,8-dien-11-one (*Z*6,*Z*8-11-one-21Hy)		4
(7*E*,9*Z*)-henicosa-7,9-dien-11-one (*E*7,*Z*9-11-one-21Hy)		5
(6*E*,8*E*)-henicosa-6,8-dien-11-one (*E*6,*E*8-11-one-21Hy)		4

[a]DB-5 capillary column, initial temperature 100°C for 1 min, raised to 210°C at 10°C/min, isothermal 1 min, raised to 240°C at 1.5°C/min and then to 300°C at 15°C/min, and then held for 30 min.

secondary alcohol, *Z*6,*Z*9-11-ol-21Hy elicited the lowest antennal responses (Figure 1).

Absolute Configuration of (Z6)-9,10-epoxyhenicos-6-ene. BF_3-catalyzed methanolysis of synthetic samples of *Z*6-9*S*,10*R*-epo-21Hy and *Z*6-9*R*,10*S*-epo-21Hy, followed by esterification with (*S*)-2-acetoxypropionyl chloride [(*S*)-AP Cl], resulted in three peaks when analyzed by GC and GC-MS. The peak with the lowest retention time corresponded to the (*S*)-AP ester of 6*Z*-(9*R*,10*R*)-9-hydroxy-10-methoxyhenicosene, whereas the peak with the longest retention time corresponded to the (*S*)-AP ester of 6*Z*-(9*S*,10*S*)-9-hydroxy-10-methoxyhenicosene (mass spectra with diagnostic ions *m*/*z* (rel. int.) 343 (10) [M-C_8H_{15}]$^+$, 322 (12) [M- (*S*)-AP acid]$^+$, 251 (12) [M- (*S*)-AP acid–C_5H_{11}]$^+$, 199

(31) $[C_{13}H_{27}O]^+$, 167 (100) [M- (*S*)-AP acid $-C_{11}H_{23}]^+$). The central peak was due to the (*S*)-AP esters of both 9*S*,10*S*- and 9*R*,10*R*-9-methoxy-10-hydroxy derivatives, which overlapped (mass spectra with diagnostic ions *m*/*z* (rel. int.) 343 (100), 322 (5), 155 (12) $[C_{10}H_{19}O]^+$. The (*S*)-AP esters derived from both *Z*6-9*S*,10*R*-epo-21Hy and *Z*6-9*R*,10*S*-epo-21Hy showed a small peak corresponding to the derivative from the epoxide of opposite configuration, even though they were purified by HPLC, indicating that approximately 10% racemization occurred during BF_3-catalyzed methanolysis, similar to the result of Qin et al. (1997). Comparison of the results from derivatization of the natural pheromone component, with those of the standards, indicated that the natural *Z*6-9,10-epo-21Hy had the 9*R*,10*S* configuration.

Identification of (6Z,9Z)-henicosa-6,9-dien-11-one. Initial wind tunnel bioassays in the laboratory and field trapping experiments with various blend combinations of the nine compounds that elicited antennal responses resulted in very few males responding to the synthetic blends, and then only in wind tunnel experiments. Therefore, we hypothesized that an essential compound (s) was/were missing, and that, based on the experience of Grant et al. (2003) with *Orgyia leucostigma* (J. E. Smith) (Lymantriidae), this/these compound (s) may be thermally labile, and rearrange to other compounds in the heated injection port of the GC-MS system. We therefore treated female gland extracts with $NaBH_4$ so that any ketones present in the extracts would be reduced to secondary alcohols, and analyzed the reduced extracts by GC and GC-MS. GC-MS analysis of a non-reduced extract suggested that *Z*6,*E*8-11-one-21Hy was the major dienic ketone in the gland extracts (Figure 3A). If this were true, then *Z*6,*E*8-11-ol-21Hy should be the major product in the reduced gland extract. However, *Z*6,*Z*9-11-ol-21Hy was found to be the major secondary alcohol in the reduced gland extracts (Figure 3B), with a lesser amount (>10% of *Z*6,*Z*9-11-ol-21Hy) of *Z*6,*E*8-11-ol-21Hy, demonstrating that *Z*6,*Z*9-11-one-21Hy was in fact the main dienic ketone in the gland extracts. The identity of *Z*6,*Z*9-11-ol-21Hy in the reduced gland extracts was confirmed based on the comparison of its retention time and mass spectral data with an authentic standard of *Z*6,*Z*9-11-ol-21Hy on two different capillary columns. Injection of synthetic *Z*6,*Z*9-11-one-21Hy in the GC-MS system confirmed the thermal rearrangement of this compound to the five diene ketones, *E*6,*Z*8-11-one-21Hy, *Z*6,*E*8-11-one-21Hy, *Z*6,*Z*8-11-one-21Hy, *E*7,*Z*9-11-one-21Hy, and *E*6,*E*8-11-one-21Hy, with identical ratios and patterns as observed in the GC-MS analyses of the gland extracts (Figure 4). Upon TLC analysis of gland extracts and the two authentic standards, *Z*6,*Z*9-11-one-21Hy and *Z*6,*E*8-11-one-21Hy, *Z*6,*Z*9-11-one-21Hy was visible for the first time in the gland extract as compared to synthetic *Z*6,*Z*9-11-one-21Hy and *Z*6,*E*8-11-one-21Hy (Figure 5). A small amount of *Z*6,*E*8-11-one-21Hy was present in the gland extracts along with a larger amount of *Z*6,*Z*9-21Hy (Figure 5). All these results confirm that *Z*6,*Z*9-11-one-21Hy is present in

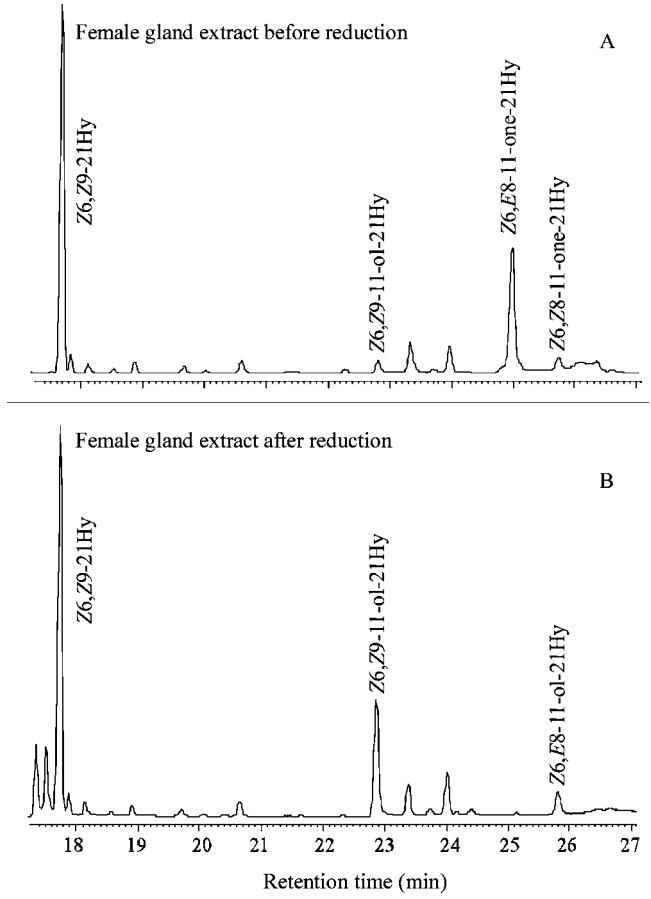


FIG. 3. Coupled GC-MS analysis of female painted apple moth pheromone gland extracts before (A) and after (B) reduction. Chromatographic column and conditions identical to Figure 2.

the gland extracts and rearranges to five diene ketones upon analysis by GC or GC-MS.

Field Cage Experiments. In the first trapping experiment, traps baited with 20 FE of the female gland extracts loaded on cigarette filters captured significantly more males than traps baited with 20 FE of the female gland extracts loaded on the three other substrates (i.e., dental rolls, polyethylene closures, and filter papers) (Figure 6). Among the four substrates tested, filter paper was the least effective substrate (Figure 6). Regardless of the type of substrate used, traps baited with the female gland extracts only caught moths on the first day of the trial.

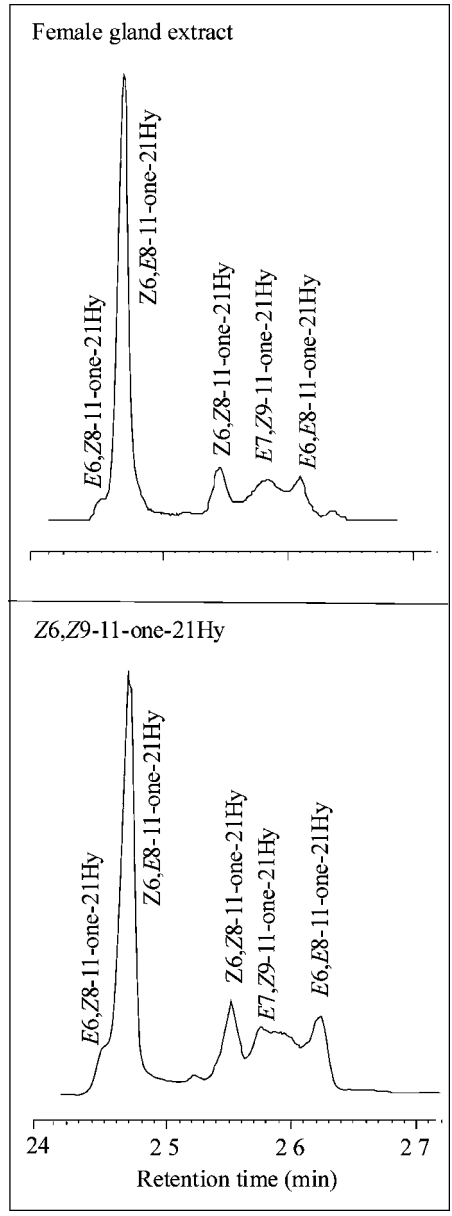


FIG. 4. Coupled GC-MS analysis of female painted apple moth pheromone gland extract and synthetic *Z*6,*Z*9-11-one-21Hy. Chromatographic column and conditions identical to Figure 2.

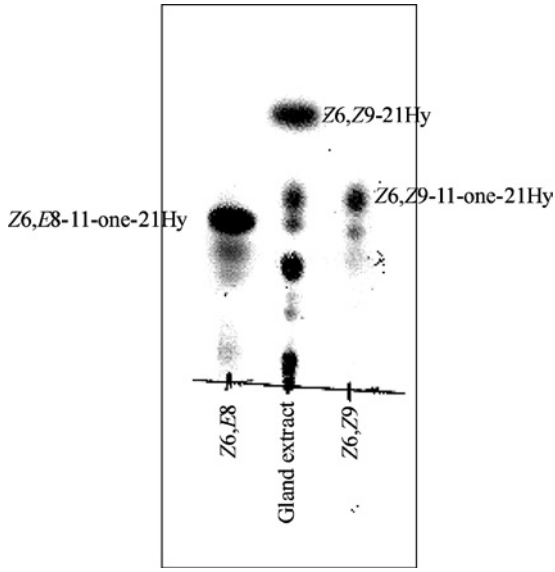


FIG. 5. TLC analysis on a Merck Silica Gel 60 F_{254} plate of female painted apple moth pheromone gland extract, synthetic *Z*6,*Z*9-11-one-21Hy, and *Z*6,*E*8-11-one-21Hy.

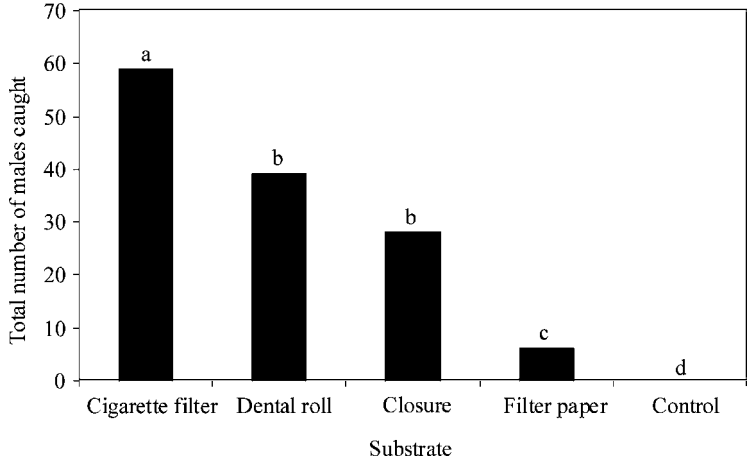


FIG. 6. Total number of *T. anartoides* males caught in traps baited with pheromone gland extract (20 FE) loaded on four different substrates in a field cage experiment. Treatments labeled with the same letters are not significantly different ($P > 0.05$).

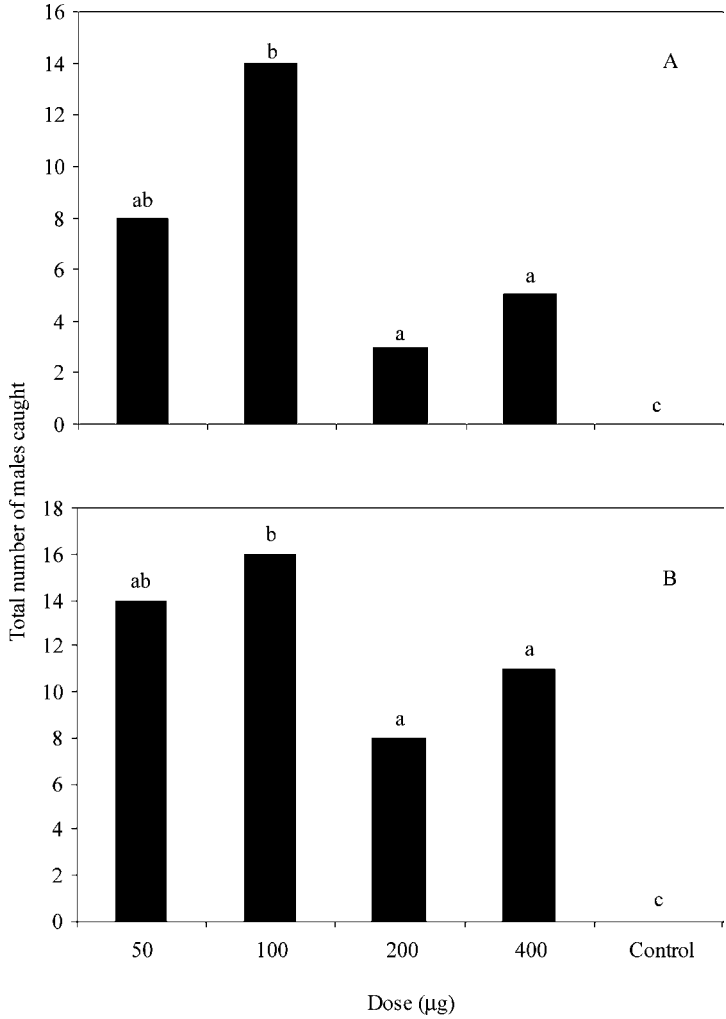


FIG. 7. Total number of *T. anartoides* males caught in traps baited with different doses of synthetic *Z*6,*Z*9-11-one-21Hy loaded on dental rolls (A) and cigarette filters (B) in a field cage experiment. Treatments labeled with the same letters are not significantly different ($P > 0.05$).

The second field cage trial, testing different doses of synthetic *Z*6,*Z*9-11-one-21Hy, showed that the 100 μg dose caught the greatest number of male moths regardless of whether the dental roll or the cigarette filter were used as releasing substrates (Figures 7A and 7B), although the numbers were not significantly

different from traps baited with 50 μg of this component. Increasing the dose to 200 or 400 μg resulted in a significant reduction in the number of males caught (Figures 7A and 7B). In the third field cage experiment, the addition of *Z*6,*E*8-11-one-21Hy or *Z*6,*E*8-11-one-21Hy and *Z*6-11-one-21Hy to 50 μg of *Z*6,*Z*9-11-one-21Hy did not enhance the attractiveness of lures compared to the major component alone. However, the blend containing the 7 compounds that elicited EAG responses gave the greatest catches of males ($P < 0.05$, Figure 8). In a further experiment, omission of *Z*6,*Z*9-21Hy or the two ketones *Z*6,*E*8-11-one-21Hy and *Z*6-11-one-21Hy caused a significant reduction in males caught ($P < 0.05$, Figure 9). These results suggest that these three compounds are part of the sex pheromone blend of the painted apple moth.

In the fifth field cage experiment, traps baited with the (9*R*,10*S*) enantiomer of *Z*6-9,10-epo-20Hy and *Z*6-9,10-epo-21Hy, respectively, captured more males than any treatment tested in this experiment (Figure 10). Traps baited with the racemic mixtures of the C20 and C21 epoxides captured significantly more males than traps baited with the (9*S*,10*R*) enantiomer of *Z*6-9,10-epo-20Hy and

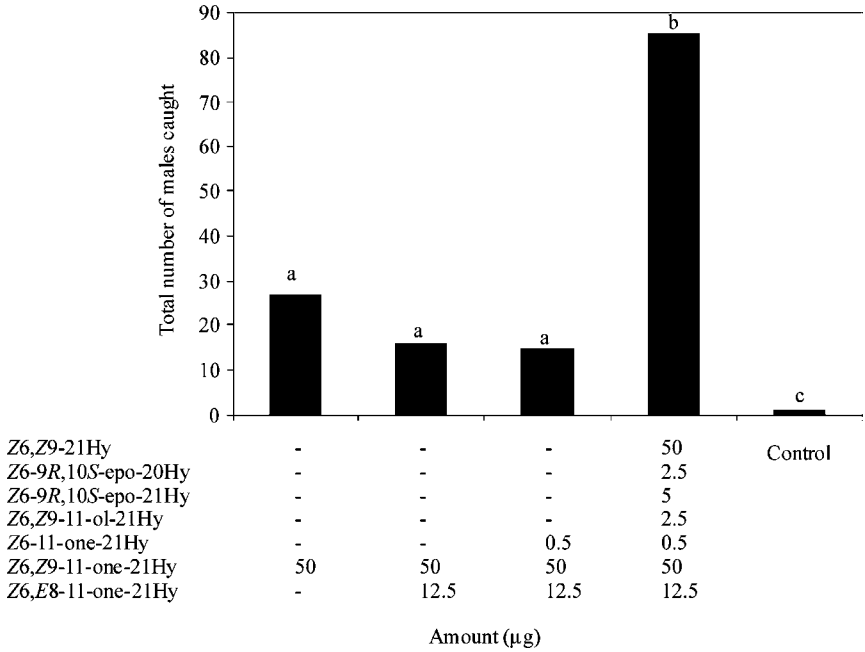
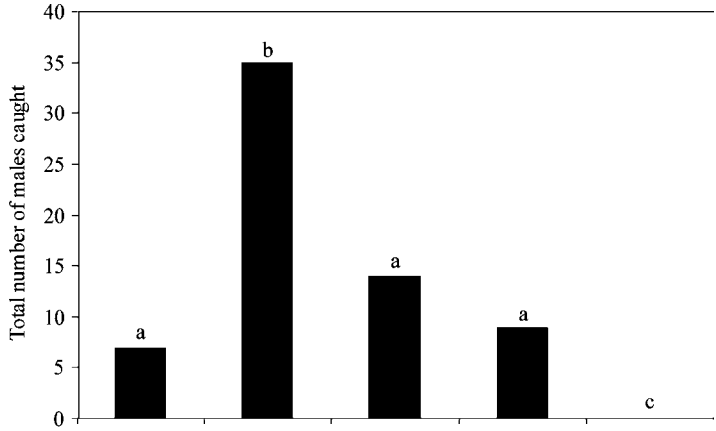


FIG. 8. Total number of *T. anartoides* males caught in traps baited with different blends of synthetic pheromone compounds loaded on cigarette filters in a field cage experiment. Treatments labeled with the same letters are not significantly different ($P > 0.05$).



*Z*6,*Z*9-21Hy	-	50	-	50	Control
*Z*6-9*R*,10*S*-epo-20Hy	-	2.5	2.5	2.5	
*Z*6-9*R*,10*S*-epo-21Hy	-	5	5	5	
*Z*6,*Z*9-11-ol-21Hy	-	2.5	2.5	2.5	
*Z*6-11-one-21Hy	-	0.5	0.5	-	
*Z*6,*Z*9-11-one-21Hy	50	50	50	50	
*Z*6,*E*8-11-one-21Hy	-	12.5	12.5	-	

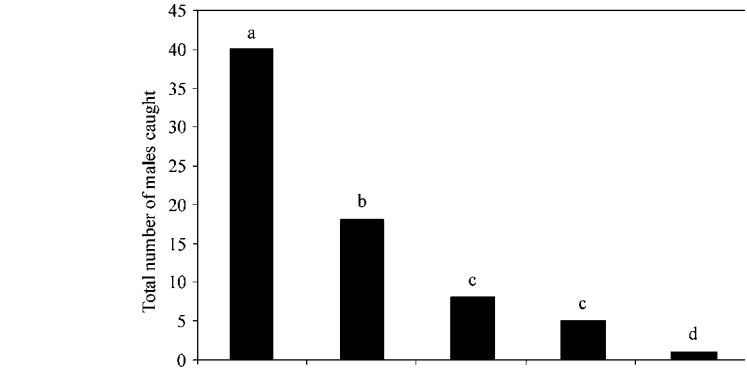
Amount (μg)

FIG. 9. Total number of *T. anartoides* males caught in traps baited with different blends of synthetic pheromone compounds loaded on cigarette filters in a field cage experiment. Treatments labeled with the same letters are not significantly different ($P > 0.05$).

*Z*6-9,10-epo-21Hy, the catches of which were equivalent to catches in traps without either of these epoxides (Figure 10). In the final field cage experiment, traps baited with the seven-component blend caught 9 males, whereas traps baited with one virgin female caught 6 males ($P > 0.05$). Trap catches in this experiment were low due to low ambient temperature (ca. 16°C).

Field Trapping Experiment. The full 7-component synthetic blend was the most effective synthetic blend, and caught more males than did traps baited with virgin females ($P < 0.05$, Figure 11). Traps baited with synthetic pheromones also caught moths for two consecutive days, with highest catch taking place in the first day.

Degradation Experiments. TLC analysis of synthetic *Z*6,*Z*9-11-one-21Hy over time showed that this compound disappeared after 24 hr when maintained in dilute solution at ambient temperature. In contrast, synthetic *Z*6,*E*8-11-one-21Hy was reasonably stable as compared to *Z*6,*Z*9-11-one-21Hy. Comparison of these TLC samples with other reference materials suggested that one of



*Z*6,*Z*9-21Hy	50	50	50	50	Control
*Z*6-9*R*,10*S*-epo-20Hy	2.5	1.25	-	-	
*Z*6-9*S*,10*R*-epo-20Hy	-	1.25	2.5	-	
*Z*6-9*R*,10*S*-epo-21Hy	5	2.5	-	-	
*Z*6-9*S*,10*R*-epo-21Hy	-	2.5	5	-	
*Z*6,*Z*9-11-ol-21Hy	2.5	2.5	2.5	2.5	
*Z*6-11-one-21Hy	0.5	0.5	0.5	0.5	
*Z*6,*Z*9-11-one-21Hy	50	50	50	50	
*Z*6,*E*8-11-one-21Hy	12.5	12.5	12.5	12.5	

Amount (μg)

FIG. 10. Total number of *T. anartoides* males caught in traps baited with different blends of synthetic pheromone compounds loaded on cigarette filters in a field cage experiment. Treatments labeled with the same letters are not significantly different ($P > 0.05$).

the decomposition products was a related ketone, namely *Z*6,*E*8-11-one-21Hy, along with several other unidentified products. TLC analysis of the two *Z*6, *Z*9-11-one-21Hy solutions maintained under complete darkness or bright sunshine revealed that both samples had decomposed to an identical extent to a mixture of products, with only a trace of the original ketone present. This experiment, coupled with the fact that our synthetic pheromone lure was active for only two days suggested that thermal degradation rather than hydrolysis or photodegradation of this material was occurring. The addition of BHT has no effect on the stability of the ketone and the sample degraded to the same extent as a control sample without BHT.

DISCUSSION

We have identified seven possible components of the sex pheromone of *T. anartoides*, from extracts of the sex pheromone gland, including *Z*6,*Z*9-11-one-21Hy, *Z*6,*E*8-11-one-21Hy, *Z*6-11-one-21Hy, *Z*6,*Z*9-11-ol-21Hy, *Z*6-9*R*,10*S*-epo-21Hy, *Z*6-9*R*,10*S*-epo-20Hy, and *Z*6,*Z*9-21Hy. The efficacy of the blend of

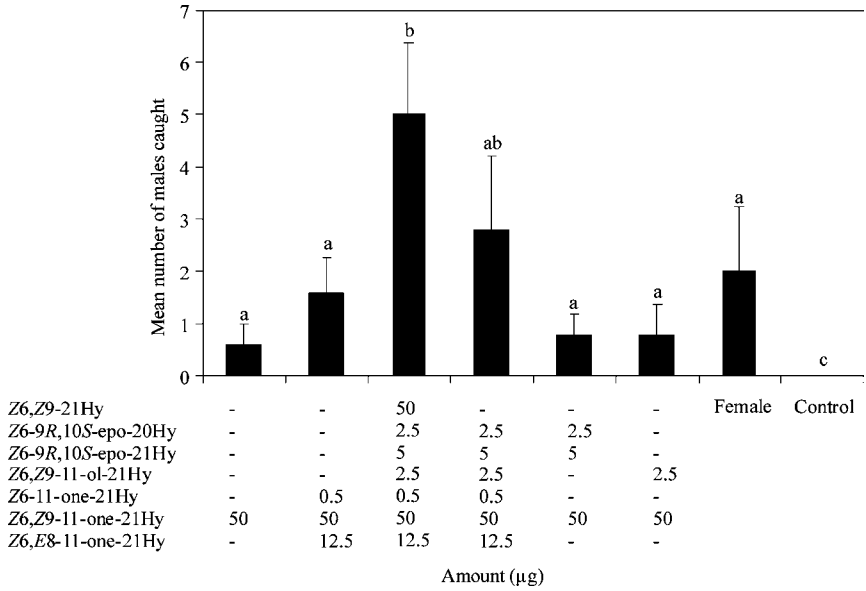


FIG. 11. Mean number of *T. anartoides* males caught in traps baited with different blends of synthetic pheromone compounds loaded on cigarette filters, or virgin females, in a field trapping experiment conducted in Auckland. Treatments labeled with the same letters are not significantly different ($P > 0.05$).

seven components was confirmed in a field trial in which traps baited with this blend caught significantly greater numbers of males than did traps baited with virgin females. Testing of the pheromone blend was complicated by the thermal lability of the major component, *Z*6,*Z*9-11-one-21Hy. In field trials, traps baited with the synthetic blends only caught males for one or two days. Grant et al. (2003) recently identified *Z*6,*Z*9-11-one-21Hy as the major sex pheromone compound of the whitemarked tussock moth, *O. leucostigma*, and also reported that the compound was extremely unstable, with a tentative half life of 12 hr at room temperature, due to the proximity of the carbonyl group to the homoconjugated diene system. To date, four dienone pheromones have been identified from lepidoptera, including (3*E*,6*Z*)-henicosa-3,6-dien-11-one in the douglas fir tussock moth, *Orgyia pseudotsugata* (McDunnough) (Lymantriidae) (Smith et al., 1978), (6*Z*, 9*Z*)-nonadeca-6,9-dien-3-one in the willow beauty, *Peribatodes rhomboidaria* (Schiffermüller) (Geometridae) (Buser et al., 1985), *Z*6,*E*8-11-one-21Hy in the douglas fir tussock moth, *O. pseudotsugata* (Gries et al., 1997), and *Z*6,*Z*9-11-one-21Hy in the whitemarked tussock moth, *O. leucostigma* (Grant et al., 2003).

A small amount of *Z*6,*E*8-11-ol-21Hy was present in the reduced female gland extracts, suggesting that the corresponding dienic ketone *Z*6,*E*8-11-one-21Hy might also be present. However, it was not clear whether *Z*6,*E*8-11-one-21Hy was produced by the female or was present only as a rearrangement product of *Z*6,*Z*9-11-one-21Hy. Furthermore, in two field cage experiments (Figures 8 and 9) and a field bioassay (Figure 11), addition of *Z*6,*E*8-11-one-21Hy and *Z*6-11-one-21Hy to *Z*6,*Z*9-11-one-21Hy did not increase trap catches significantly.

The di-unsaturated secondary alcohol, *Z*6,*Z*9-11-ol-21Hy, has been recently identified as a major sex pheromone compound in the tussock moth, *Orgyia detrita* (Guerin-Meneville) Lymantriidae (Gries et al., 2003). In the study reported here, this compound was identified as a minor component in the pheromone gland extract, but a blend of *Z*6,*Z*9-11-ol-21Hy with *Z*6,*Z*9-11-one-21Hy did not increase trap catches relative to *Z*6,*Z*9-11-one-21Hy as a single component (Figure 11).

Two monoepoxyalkenes, *Z*6-9*R*,10*S*-epo-20Hy and *Z*6-9*R*,10*S*-epo-21Hy, were also identified as minor components of female painted apple moth pheromone gland extracts, with both compounds eliciting strong antennal responses in coupled GC-EAD analyses. It is likely that both are important to the pheromone blend because dropping both compounds from the full seven-component mixture in a field cage test resulted in decreased trap captures. However, in a field trial, the three-component blend of the epoxides with *Z*6,*Z*9-11-one-21Hy was no more attractive than *Z*6,*Z*9-11-one-21Hy alone (Figure 11). Confirmation of the structure and absolute configuration of *Z*6-9*R*,10*S*-epo-21Hy was accomplished by derivatization and comparison with authentic samples, whereas *Z*6-9*R*,10*S*-epo-20Hy was present in quantities that were insufficient for the derivatization method. Unsaturated epoxides have been reported frequently as pheromone components of arctiid, lymantriid, geometrid, and some noctuid species (El-Sayed, 2004). Because these epoxides have two enantiomeric forms, each of which serves as a unique structure at the receptor level, male moths can be selective in their responses to the two enantiomers. For example, male mulberry looper, *Hemerophila atrilineata* (Butler) (Geometridae), respond mainly to (6*Z*)-9*S*,10*R*-epoxyoctadec-6-ene, whereas the other enantiomer of this compound is not attractive (Tan et al., 1996). Similarly, male crocus geometer, *Xanthotype sospeta* (Drury) (Geometridae), respond selectively to the (6*Z*)-9*S*,10*R*-epoxynonadec-6-ene and not the (9*R*,10*S*)-enantiomer (Millar et al., 1991). In our study, in one field cage trial (Figure 10), male painted apple moths appeared to respond selectively to *Z*6-9*R*,10*S*-epo-20Hy and *Z*6-9*R*,10*S*-epo-21Hy, whereas *Z*6-9*S*,10*R*-epo-20Hy and *Z*6-9*S*,10*R*-epo-21Hy elicited neither positive nor negative responses. Both of these epoxides have been previously reported as lepidopteran pheromone components. Racemic *Z*6-9,10-epo-20Hy has been reported as an attractant for *Hypenomorpha calamina* (Butler) (Ando et al., 1995). *Z*6-9,10-epo-21Hy of unknown absolute configuration was reported as a possible sex pheromone component of the ruby tiger moth,

Phragmatobia fuliginosa (Linnaeus) (Déscoins and Frérot, 1984), whereas the fruit-piercing moth, *Oraesia excavata* (Butler) uses *Z*6-9*S*,10*R*-epo-21Hy in its pheromone blend (Yamamoto et al., 1999).

We have fairly strong evidence that *Z*6,*Z*9-21Hy, identified in the sex pheromone gland of female painted apple moth, is indeed a pheromone component because omission of this component from the synthetic blend resulted in consistent decreases in trap catch in several trials. This compound has been previously identified in the sex pheromone glands of a number of geometrid, arctiid, and noctuid moths (Millar, 2000), and it has been implicated or proven to be a pheromone component in a number of these species (e.g., *Synegia esther* (Butler) (Ando et al., 1995)).

Overall, our data demonstrate that *Z*6,*Z*9-11-one-21Hy and *Z*6,*Z*9-21Hy appear to be critical components of the painted apple moth pheromone blend. However, the roles of the other components identified from pheromone gland extracts are not yet clear, because field cage bioassays and a field trial to test the effects of these components did not provide consistent and unequivocal results. Whereas the GC-EAD experiments show that the male moths detect these compounds, their possible roles as minor components of the pheromone blend remain to be elucidated.

Of the seven electrophysiologically active components observed, four chemical classes are represented: hydrocarbon *Z*6,*Z*9-21Hy, ketones *Z*6-11-one-21Hy, *Z*6,*E*8-11-one-21Hy, *Z*6,*Z*9-11-one-21Hy, epoxides *Z*6-9*R*,10*S*-epo-20Hy and *Z*6-9*R*,10*S*-epo-21Hy, and secondary alcohol *Z*6,*Z*9-11-ol-21Hy making the extracts of the sex pheromone gland of the painted apple moth a diverse chemical blend. All seven compounds appear to be derived from methylene-interrupted polyenes exhibiting, with the exception of *Z*6,*Z*9-21Hy and *Z*6,*Z*9-11-one-21Hy, a single oxidative modification/ rearrangement of one of the carbon-carbon double bonds.

In an attempt to evaluate the identified pheromone compounds for control of this pest, Suckling et al. (2002) investigated the potential use of the minor pheromone component *Z*6-11-one-21Hy alone for mating disruption. Only a moderate degree of mating disruption was achieved, suggesting that the major components or a more complete blend might be required in order to achieve an acceptable level of control. However, the instability of the major pheromone component may make it difficult to use sex pheromone based mating disruption against this pest. Currently, traps baited with live females are being used to monitor the distribution and dispersal of this insect in New Zealand. In this study, the synthetic blend was attractive for only two days, whereas live females remained attractive for up to seven days (Suckling et al., unpublished). Therefore, in the absence of a more stable formulation, live females should continue to be used for monitoring and detection of this pest in New Zealand. Work is underway to develop a more stable formulation of the synthetic pheromone.

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IDENTIFICATION OF A SEX PHEROMONE COMPONENT OF THE GEOMETRID MOTH *Milionia basalis pryeri*

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Abstract—A single component in extracts of virgin female *Milionia basalis pryeri* moths elicited responses from male moth antennae. This compound (ca. 7 ng/female) was identified as (*Z*,*Z*)-(3*S*,4*R*)-3,4-epoxynonadeca-6,9-diene by GC-MS and NMR analyses, microchemical reactions, and comparative chiral HPLC. In a field test, synthetic (*Z*,*Z*)-(3*S*,4*R*)-3,4-epoxynonadeca-6,9-diene attracted male moths. The opposite enantiomer, the racemic mixture, and virgin female moths held in small cages attracted no more moths than the solvent controls.

Key Words—Sex pheromone, *Milionia basalis pryeri*, Geometridae, (*Z*,*Z*)-(3*S*,4*R*)-3,4-epoxynonadeca-6,9-diene.

INTRODUCTION

The geometrid moth *Milionia basalis pryeri* Druce (Lepidoptera: Geometridae) is found in Japan in southern Kyushu and the Amami and Ryukyu Islands (Okinawa),

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and in Taiwan. This diurnal moth has a metallic blue body, blackish wings with an orange stripe on the forewings, and another orange stripe with black spots on the hindwings. The larvae of this species feed exclusively on the podocarp tree, *Podocarpus macrophyllus* (Podocarpaceae). *P. macrophyllus* is used as a building material, or as a street and garden tree in southern Japan. This tree contains substances toxic to some insect species (Ito and Kodama, 1976; Yasui, 2001), but *M. b. pryeri* has adapted to this plant and will occasionally cause significant damage to trees.

Control of this species is problematic. Large doses of synthetic insecticides are currently used (Gushiken et al., 1993). Biological control is likely to be ineffective because the larvae sequester toxic chemicals from the host plant (Yasui, 2001), and predation is rare. Therefore, we sought to identify the sex pheromone for potential use in control. In this paper, we report on the identification of the major sex pheromone component of *M. b. pryeri*, and the results of field trials with synthetic pheromone.

METHODS AND MATERIALS

Insects. M. b. pryeri larvae were collected from podocarp trees, *P. macrophyllus*, on Okinawa Is., Japan. The larvae were reared on podocarp leaves at 25°C and a 16L:8D photoperiod in the laboratory. Pupae were sexed and maintained in separate containers until emergence.

Extraction. Abdominal tips, including the pheromone gland, of 283 females at two or three days after emergence were excised with fine tweezers and soaked with hexane at ca. 10 tips/0.5 ml for 15 min. The extracts were filtered through a small wad of cotton, the residue was rinsed twice with the same volume of hexane, and the rinses were added to the extract. The extracts were accumulated and stored below −20°C until use.

Column Chromatography. Column chromatography was conducted on a silica gel column (particle size: 75–150 μm, Wako gel C-200, Wako Pure Chem. Ind., Ltd., Osaka, Japan). The crude extract from 270 female equivalents (FE) was concentrated to 150 μl and applied to 0.5 g of silica gel. Compounds were successively eluted with 2.5 ml of hexane, 2%, 5%, 15%, and 50% ether in hexane, and ether.

Gas Chromatography (GC) and Electroantennographic Detection (EAD). GC analyses were conducted on a Hewlett-Packard (HP) 5890 Series II (for GC-EAD) or HP 6890 (for quantitative analysis) gas chromatograph equipped with a flame ionization detector (FID). Injection was made with an on-column injector programmed at oven temperature plus 3°C. For GC-EAD, an HP-1 or HP-INNOWax fused silica column (15 m × 0.25 mm ID × 0.25 μm film thickness) was used with helium as the carrier gas at a column head pressure of 55 kPa

or 50 kPa, respectively. The GC oven temperature was held at 50°C for 1 min, increased from 50°C to 150°C at a rate of 20°C min^{-1}, raised to 270°C (HP-1) or 240°C (HP-INNOWax) at a rate of 5°C min^{-1} and then held at 270°C or 240°C for 5 min. The electroantennographic (EAG) response was obtained simultaneously with FID recording. The EAD was set up according to Struble and Arn (1984). For quantitative analysis, the HP-1 column was used with helium at an initial column head pressure of 67 kPa. The GC oven temperature was kept at 50°C for 1 min, increased from 50°C to 220°C at a rate of 25°C min^{-1}, and then held at 220°C for 2 min.

Coupled Gas Chromatography-Mass Spectrometry (GC-MS). GC-MS analyses were conducted with an HP 6890 gas chromatograph interfaced to a JEOL JMS SX-102A double focusing magnetic sector mass spectrometer (EI mode, 70 eV) and operated with an HP model 715/64 computer. The carrier gas was helium. The GC was operated under the same conditions and columns as GC-EAD, but in constant flow mode (1.6 ml/min). In some analyses, a mixture of normal alkanes with even numbers of carbon atoms from dodecane to octacosane was coinjected for calculation of retention indices.

High Performance Liquid Chromatography (HPLC). A) *Purification of the EAG-Active Component(s).* The EAG-active fraction (5% ether in hexane) of the female extract (220 FE) was further separated on an HP 1050 Series HPLC, equipped with a Chiralpak AD column (10 × 250 mm, Daicel Chemical Industry Co., Ltd., Tokyo, Japan), at room temperature. Peaks were monitored by UV-absorbance at 210 nm. The mobile phase was 0.1% 2-propanol in hexane, and the flow rate was 2.2 ml min^{-1}. Fractions were collected every 2 min from 2.0 min to 22.0 min. The fractions were analyzed with GC-MS. B) *Determination of the Stereochemistry of the EAG-Active Component.* The EAG-active fraction of the female extract (15 FE), synthetic (*Z*,*Z*)-(3*S*,4*R*)-3,4-epoxynonadeca-6,9-diene [(3*S*,4*R*)-isomer] (150 ng), and (*Z*,*Z*)-(3*R*,4*S*)-3,4-epoxynonadeca-6,9-diene [(3*R*,4*S*)-isomer] (150 ng) were analyzed on a Shimadzu 10ADVp system HPLC, equipped with a Chiralpak AD column (4.6 × 250 mm). Analysis was performed under the same conditions as above) but the flow rate was 0.45 ml min^{-1}. The absolute configurations of the first and second eluted compounds were assigned to be (3*S*,4*R*) and (3*R*,4*S*), respectively, by comparing the previously reported elution order using the same chiral HPLC column and conditions (Qin et al., 1997). The (3*S*,4*R*)- and (3*R*,4*S*)-isomers were eluted at 14.7 min and 15.4 min, respectively.

Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR spectra were recorded at 303 K on a Bruker AVANCE800 spectrometer operating at 800.20 MHz (^1H) equipped with a 5 mm triple-resonance inverse probehead [$^1H/(^{13}C, ^{15}N)$]. ^1H-^1H-homodecoupling one-dimensional experiments were performed with presaturation technique. Spectra were measured in pyridine-d_5, using a 5-mm symmetrical

micro tube matched with methanol-d_4 (0.25 ml, Shigemi, Inc., Tokyo, Japan). The concentration of the sample was estimated to be ca. 1.5 μg/0.27 ml by comparison of the GC peak areas with docosane. The ^{1}H chemical shifts are given on the δ scale and referenced to the residual signal of partly un-deuterated solvent: δ (C_5HD_4N, H-2 and H-6) = 8.71 ppm.

Microchemical Reactions. A) *Diimide Reduction.* Polyenyl compounds were hydrogenated according to the method described by Yamaoka et al. (1976). A hexane solution containing the major EAG-active compound (ca. 20 FE), concentrated to about 5 μl in a 1-ml taper-bottomed vial, was combined with 10 μl each of hydrazine and hydrogen peroxide solution in ethanol (N_2H_4: 0.3 ml of hydrazine hydrate in 10 ml of ethanol, H_2O_2: 0.04 ml of 31% H_2O_2 in 10 ml of ethanol). The mixture was maintained at 55°C for 1 hr for complete hydrogenation. Then 2 μl of the reaction mixture was injected into the GC-MS. B) *Microozonolysis.* Microozonolysis was conducted according to Beroza and Bierl (1967). Ozone was bubbled into a ca. 10 μl hexane solution, containing the major EAG-active component (20 FE) cooled to −78°C, through a glass capillary tube for 1 min. Two μl of the reaction mixture was immediately injected into the GC-MS.

Chemicals. Racemic (*Z*,*Z*)-*cis*-3,4-epoxynonadeca-6,9-diene was synthesized from linolenic acid, via epoxidation of (*Z*,*Z*,*Z*)-nonadeca-3,6,9-triene, (Ando et al., 1993). The (*Z*,*Z*)-*cis*-3,4-epoxynonadeca-6,9-diene was separated from regioisomers with silica gel chromatography (Wako gel C-200, hexane, 0.5%, 0.7%, 1%, 1.5% ether in hexane, and the compound was eluted with 1% ether in hexane and later fractions).

Field Attraction. Field tests were conducted along podocarp hedges around a field of the Nago Branch of OPAES, in Nago, Okinawa, on 7 May, 2003. Tent-shaped sticky traps (white type, SE trap of Sankei Chem. Co., Kagoshima, 29 × 32 × 8 cm) with a 24 × 30-cm sticky bottom were used for field attraction.

Synthetic Chemicals. (*Z*,*Z*)-(3*R*,4*S*)-3,4-Epoxynonadeca-6,9-diene, its (3*S*,4*R*)-isomer, and the racemic mixture dissolved in hexane were dispensed onto one end, over a ca. 2 cm^2 area, of a 1 × 3-cm piece of filter paper (Toyo No. 2, Toyo Roshi Kaisya, Tokyo). A filter paper of the same size was treated with the same volume of hexane as an unbaited control. Immediately after the solvent evaporated, the treated papers were suspended about 2 cm above the sticky bottoms in traps at about 15:00 hr because the attraction of *M. b. pryeri* males occurred around 15:00–19:00 hr ($N = 5$). Virgin females in small mesh cages suspended in traps ($N = 3$) were used as positive controls. The traps were placed on stems of the podocarp trees, about 1 m above the ground, at 5-m intervals. Trap catches (X) were transformed to $(X + 0.5)^{1/2}$ and submitted to one-way analysis of variance. The means were ranked by Tukey's method.

RESULTS

GC and GC-EAD Analyses of the Female Gland Extract. The crude extract of 270 females was concentrated to ca. 150 μl, and ca. 0.5 μl of the extract (1 FE) was injected into the GC-EAD system. One distinct EAG-active component (peak A) was observed in conjunction with a number of FID peaks (Figure 1). The remaining crude extract was chromatographed on a silica gel column. The EAG-active component was eluted with 5% ether in hexane (the EAG-active fraction). In separate GC analyses, the amount of the component in the extracts was estimated to be 7 ng/female by comparison of FID peak areas, with 5 ng of docosane as an internal standard.

Identification of EAG-Active Component. The EAG-active fraction of the female extract (ca. 1 FE) was analyzed by GC-MS. The mass spectrum of the active compound showed a molecular ion at *m/z* 278 (relative intensity 7%, base peak: *m/z* 80), and diagnostic fragment ions at *m/z* 262 ($[M-16]^+$, 2%) and 260 ($[M-18]^+$, 8%), which suggested an unsaturated epoxide (Figure 2). Micro-scale reduction of ca. 100 ng of the component with diimide produced a single compound with a molecular ion at *m/z* 282 (M^+, 3%, base peak: *m/z* 83), 266 ($[M-16]^+$, 3%) and 253 ($[M-CH_3CH_2]^+$, 70%). The last ion indicated the position of an epoxy ring at C-3 and -4. The mass spectrum and retention values of the product were identical to those of authentic *cis*-3,4-epoxynonadecane. The ozonolysis of the EAG-active fraction containing ca. 100 ng of the EAG-active component and subsequent GC-MS analysis showed a mass spectrum typical of an aliphatic aldehyde, *m/z* 138 ($[M-18]^+$, 12%, base peak: *m/z* 57) and 128 ($[M-28]^+$, 14%), which was identical to that of decanal. Therefore, the position of one double bond was determined to be between C-9 and C-10.

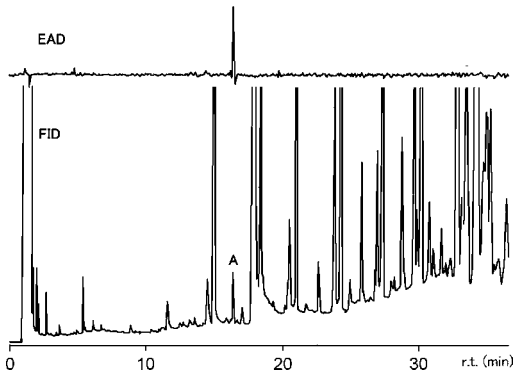

FIG. 1. GC-EAD chromatograms from analysis of hexane extract of abdominal tips of *M. b. pryeri* virgin females. A: FID peak corresponding to potent EAG response. HP-1 column, 15 m, FID:EAD = 1:1 split.

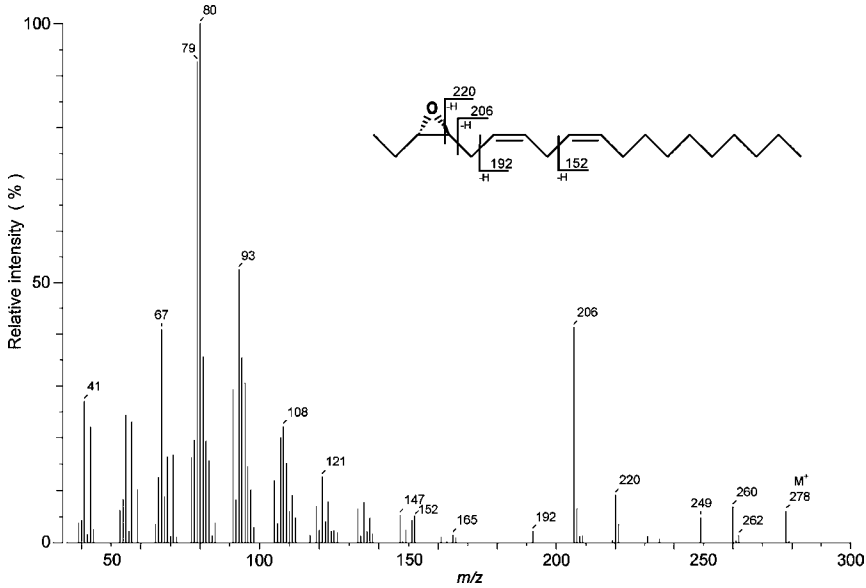

FIG. 2. EI mass spectrum and chemical structure of the major sex pheromone component in extracts of virgin *M. b. pryeri* females.

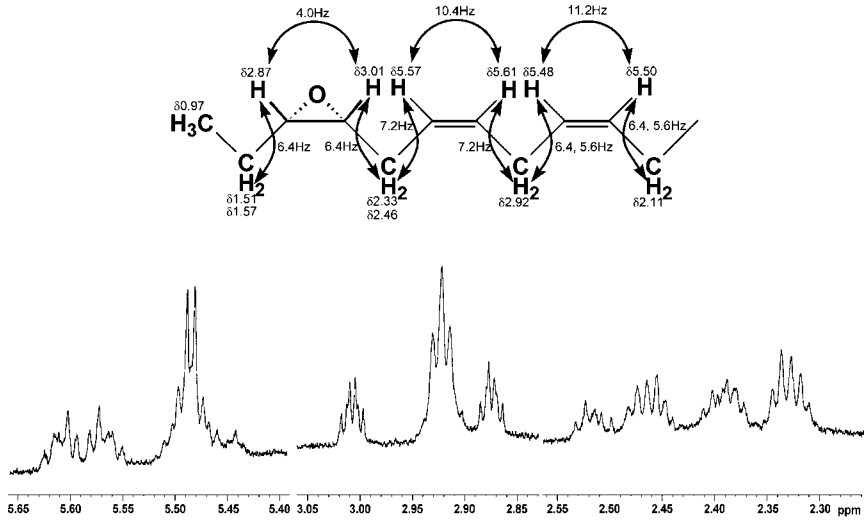

FIG. 3. ^{1}H NMR spectrum of the insect-produced pheromone component and the resulting partial structure. Signals at δ 2.39 and δ 2.52 are unrelated.

The ^{1}H NMR spectrum of the EAG-active component (220 FE, ca. 1.5 μg/pyridine-d_5 0.27 ml, Figure 3) indicated four olefinic protons (δ 5.45 − 5.65), in two pairs, with protons in each pair coupled to each other. The coupling constants between olefinic protons observed at δ 5.48 and δ 5.50, and δ 5.57 and δ 5.61, were determined to be 11.2 and 10.4 Hz, respectively, which indicated that the two double bonds had (*Z*)-configuration. The spectrum also showed the presence of two epoxide protons at δ 2.87 (1H, *dt*) and 3.01 (1H, *dt*), in which the coupling constant of 4.0 Hz confirmed a *cis*-epoxide structure (Silverstein et al., 1991). The decoupling experiments for all the olefinic protons, epoxide protons, and methylene protons next to double bonds or an epoxy ring revealed a partial structure with two double bonds and one epoxy ring (Figure 3). The correlation, starting from the epoxy ring, was as follows: a methylene group (δ 2.33 and δ 2.46) next to the epoxy ring (δ 2.87 and δ 3.01), then one double bond (δ 5.57 and δ 5.61) next to the methylene group (δ 2.33 and δ 2.46), another methylene group (δ 2.92, 2H) next to the double bond (δ 5.57 and δ 5.61), and then another double bond (δ 5.48 and δ 5.50) next to another methylene group (δ 2.92, 2H). The only possible structure of the active compound was, thus, (*Z*,*Z*)-*cis*-3,4-epoxynonadeca-6,9-diene. The NMR and mass spectra of the natural epoxide were identical to those of the synthetic compound. The retention index values of the synthetic compound [KI = 2023 (HP-1), KI = 2386 (HP-INNOWax)] matched those of the natural component [KI = 2025 (HP-1), KI = 2386 (HP-INNOWax)].

Stereochemistry of Epoxydiene. (*Z*,*Z*)-*cis*-3,4-Epoxynonadeca-6,9-diene has two chiral centers, such that (3*S*,4*R*)- and (3*R*,4*S*)-enaitiomers are possible. Therefore, chiral HPLC analysis was performed to determine the stereochemistry of the natural component. The insect-produced epoxide eluted at 14.80 min, whereas the synthetic (3*S*,4*R*)- and (3*R*,4*S*)-enantiomers eluted at 14.75 and 15.25 min respectively. The retention times of the natural component and two synthetic enantiomers were replicated three times, confirming the natural component as the (3*S*,4*R*)-enantiomer.

Field Attraction of M. b. pryeri. Field tests were conducted with the synthetic (3*R*,4*S*)-enantiomer, (3*S*,4*R*)-enantiomer, the racemic mixture, virgin females, and a solvent control. The (3*S*,4*R*)-enantiomer attracted male moths, whereas all other treatments were no different from solvent controls (Figure 4). These results supported the identification of the (3*S*,4*R*)-enantiomer as the major sex pheromone component of *M. b. pryeri*. The male catch fluctuated greatly, for unknown reasons. The trap locations and/or conditions of females may have been factors.

DISCUSSION

Because *M. b. pryeri* is brightly colored and diurnal it was thought to use visual rather than olfactory cues in the mating system, as most butterflies

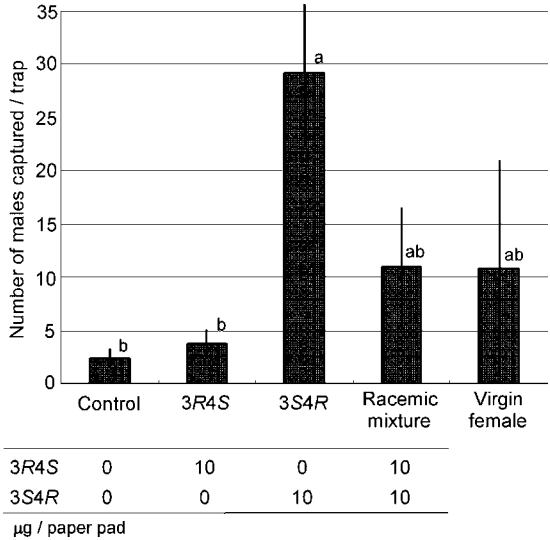

FIG. 4. Captures of male *M. b. pryeri* with synthetic (*Z*,*Z*)-(3*R*,4*S*)-3,4-epoxynonadeca-6,9-diene, (3*S*,4*R*)-enantiomer, the racemic mixture, and virgin females (in Okinawa, 2003). $N = 5$, virgin female: $N = 3$. Bar: S.E. Trap catches (X) were transformed to $(X + 0.5)^{1/2}$ and submitted to one-way ANOVA. The means capped with the same letters are not significantly different at the 5% level by Tukey's method.

do (Smith, 1989). In this study, however, we found that this species uses a sex pheromone analogous to other cryptically colored geometrid moths. (*Z*,*Z*)-(3*S*,4*R*)-3,4-Epoxynonadeca-6,9-diene appeared to be the only component in pheromone gland extracts that elicited EAG responses from antennae of males, and this compound attracted male moths. This compound is also a sex pheromone of other geometrid moths. For example, (*Z*,*Z*)-*cis*-3,4-epoxynonadeca-6,9-diene has been reported as a major sex pheromone component of several species of geometrid moths (Becker et al., 1990; Millar et al., 1990; Cossé et al., 1992; Szöcs et al., 1993; Ando et al., 1997). The (3*S*,4*R*)-enantiomer has been identified as a sex pheromone component of *Agriopis aurantiaria* (Szöcs et al., 1993) and the giant looper, *Boarmia (Ascotis) selenaria* (Becker et al., 1990; Cossé et al., 1992). Those two species and *M. b. pryeri* use the same pheromone component, but have disjunct geographic distributions: *A. aurantiaria* is found in Central Europe (Szöcs et al., 1993), *B. selenaria* is found in Israel (Cossé et al., 1992), and *M. b. pryeri* is native to south Japan and Taiwan.

In contrast, with *A. aurantiaria*, the pure (3*S*,4*R*)-enantiomer attracted males, but a racemic mixture or the (3*R*,4*S*)-isomer attracted few males (Szöcs et al., 1993). In another example, male *Probole amicaria* moths were more attracted

when a second component was added to racemic (*Z*,*Z*)-*cis*-3,4-epoxynonadeca-6,9-diene (Millar et al., 1990). The possible effect of minor components in pheromone gland extracts on mating behavior of *M. b. pryeri* is now under investigation.

Furthermore, the (3*R*,4*S*)-enantiomer is a sex pheromone component of the Japanese giant looper, *Ascotis selenaria cretacea* (Ando et al., 1997). Although *A. s. cretacea* females produce both the (3*S*,4*R*)- and (3*R*,4*S*)-enantiomers in a 53:47 ratio, only the (3R,4S)-enantiomer appears to act as a sex pheromone.

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THE AGGREGATION PHEROMONE OF *Diorhabda elongata*, A BIOLOGICAL CONTROL AGENT OF SALTCEDAR (*Tamarix* SPP.): IDENTIFICATION OF TWO BEHAVIORALLY ACTIVE COMPONENTS[1]

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Abstract—The leaf beetle *Diorhabda elongata* Brullé (Coleoptera: Chrysomelidae) has been introduced as a biological control agent for saltcedars, *Tamarix* spp., an exotic, invasive weedy tree in the western United State. Gas chromatographic (GC) analysis of volatiles collected from feeding male or female beetles, or saltcedar foliage alone, showed two components produced almost exclusively by males. These compounds elicited responses from antennae of male and female beetles in GC-electroantennographic detection (EAD) analyses. The compounds were identified as (2*E*,4*Z*)-2,4-heptadienal (**1**) and (2*E*,4*Z*)-2,4-heptadien-1-ol (**2**) by GC-mass spectrometry (MS), and confirmed with authentic standards. The two compounds were also detected at trace levels from feeding females and foliage controls, but the amounts from feeding males were 8–40 times higher, typically 55–125 ng per day per male. The amounts of **1** and **2** in collections from females did not differ significantly from amounts collected from control foliage. In field trials, **2** as a single component was as attractive as a 1:1 blend of **1** and **2**. Compound **1** as a single component was more attractive than controls, but much less attractive than **2** or the blend. Males and females

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[1]Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

were attracted in about equal numbers, indicating that this is an aggregation pheromone.

Key Words—*Diorhabda elongata*, Coleoptera, Chrysomelidae, aggregation pheromone, saltcedar, *Tamarix ramosissima*, (2*E*,4*Z*)-2,4-heptadienal, (2*E*,4*Z*)-2,4-heptadien-1-ol, biological control.

INTRODUCTION

Saltcedar (*Tamarix* spp.) is an exotic invasive weedy tree causing up to $285,000,000 of damage per year in water loss due to transpiration and flood damage due to stream channel alteration (Zavaleta, 2000). The invasion of *Tamarix* into riparian areas in the western United States has also caused ecosystem damage that is more difficult to quantify, such as decreased habitat quality for wildlife and native plant species, increased soil salinity, and increased fire risk (Deloach et al., 2000). Efforts to control saltcedar through conventional means such as herbicides or physical removal have been expensive and of limited success. For this reason, a multistate biological control program was initiated in the 1980's (Deloach, 1989). Currently, the only approved and released biological control agent is the leaf beetle *Diorhabda elongata* Brullé (Coleoptera: Chrysomelidae). A release made at Lovelock, Nevada, during 2001 has become particularly well established.

The beetles overwinter as adults in leaf litter and emerge in the spring (typically in May at Lovelock, NV) when saltcedar starts to produce new foliage. Eggs are laid on the foliage. The larvae feed on the leaves, and then pupate in the soil. There are two generations per year at Lovelock, with new adults emerging in July and again in August. Adults from the second generation enter overwintering diapause (DWB, unpublished; Deloach et al., 2004).

One important need in a biological control program is to be able to monitor the beetle populations accurately in the field, and a tool based on the pheromone of the beetles could serve this need. A pheromone-based monitoring system could be useful for studying population attributes such as dispersal rate and distance, local survival, abundance relative to habitat properties, and times of adult activity during the year. Such information can be difficult to obtain with standard sampling methods, especially when the population is sparse. Even in areas with high population density and heavy defoliation, pheromone-based monitoring in surrounding areas could yield information on the behavior of dispersing beetles.

Only a small number of chrysomelid beetle pheromones have been identified from the more than 1,400 chrysomelid species known from North America north of Mexico (Arnett, 1993), but both male- and female-produced long-range pheromones exist within the family. The pheromones are chemically diverse and can consist of either single compounds or blends. The first chrysomelid pheromone

to be chemically identified was that of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Guss et al., 1982). This pheromone is emitted by female beetles and attracts only males. Chemically related, female-produced sex pheromones were subsequently defined for eight *Diabrotica* species and subspecies (summarized by Krysan et al., 1989). These pheromones all consist of methyl-branched esters or ketones.

In contrast, recent studies of chrysomelid chemical communication systems have found only male-produced aggregation pheromones. For example, six male-specific sesquiterpenes were identified from the flea beetle *Phyllotreta cruciferae* Goeze (Bartelt et al., 2001), and a blend of the synthetic compounds was shown to be attractive under field conditions both in Canada and Hungary (J. Soroka, M. Tóth, and R. Bartelt, unpublished). Male-produced aggregation pheromones have been subsequently reported from two other chrysomelid species, the cereal leaf beetle, *Oulema melanopus L.* (Cossé et al., 2002, Rao et al., 2003), and the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Dickens et al., 2002). Chemically, the latter pheromones are unusual hydroxy ketones. In addition, male-produced sesquiterpenes that elicit antennal responses have been identified in three *Aphthona* species (Bartelt et al., 2001)

In the present study we report the identification of two male-produced components of the aggregation pheromone in *D. elongata*.

METHODS AND MATERIALS

Insects. D. elongata adults used in this study were obtained from a colony maintained at the USDA-ARS Exotic and Invasive Weed Research Unit, Albany, CA, USA. The beetles originated from sites near Fukang, in the Xinjiang province of northwestern China (DeLoach et al., 2003, Lewis et al., 2003). Upon arrival in Peoria, IL, adult beetles were kept at 25°C under a 17L:7D h photoperiod. *Tamarix ramosissima* Ledebour (Tamaricaceae) was grown year round in a greenhouse facility at NCAUR to provide food for the beetles.

Volatiles Collections. Initial volatiles collections were made to demonstrate and identify the pheromone, followed by additional collections to quantitate the pheromone emissions.

Beetles (7 to 10 d old) were sexed by inspection of the ventral side of the abdominal tip. A dark internal spot is visible through a sternite in females, and males have a v-shaped indentation at the apex of the last abdominal segment. Volatiles collections were made from individuals and groups of 10 male or female beetles feeding on saltcedar foliage, and from foliage alone. The beetles were placed into a horizontal glass tube (5 cm ID $\times$ 20 cm in length) containing a piece of saltcedar foliage (approx. 15 cm long) with the cut end in a small water vial. A Teflon seal in the cap of the vial kept the water from spilling. Collector equipment

and general volatiles collection procedures were reported earlier (Cossé et al., 2002).

Collections were made in an incubator (25°C, 17L:7D) and volatiles were collected on Super-Q traps (80–100 mesh, Alltech, Deerfield, IL) with an airflow-rate of 100 ml/min. Foliage was replaced daily, and any dead beetles in the collectors were replaced with fresh ones. Collected volatiles were eluted from the traps into vials using methylene chloride (300 μl) every 2–3 d. 1-Octanol (10 μl of a hexane solution containing 25 ng/μl) was added to each vial as a quantitative internal standard. Early in the project, trap breakthrough was evaluated by placing a second Super-Q trap in series with the first. Only compounds of six or fewer carbons were detected in the breakthrough trap.

Electrophysiology. Coupled gas chromatographic-electroantennographic (GC-EAD) analyses were made by methods and equipment generally described by Cossé and Bartelt (2000). GC-EAD connections were made by inserting a glass pipette silver-grounding electrode into the back of an excised beetle head. A second glass pipette silver-recording probe was placed in contact with the distal end of one antenna. Both pipettes were filled with Beadle-Ephrussi saline (Ephrussi and Beadle, 1936).

Instrumentation. Extracts were analyzed by GC with flame-ionization detection (GC-FID) and coupled GC-mass spectrometry (GC-MS). Samples were injected splitless using Hewlett Packard 6890 (Palo Alto, CA) instruments fitted with 30 meter DB-1 or DB-5 capillary columns (0.25 mm I.D., 0.25 μm or 1.0 μm film thickness, J&W Scientific, Folsom, CA). Temperature programs were from 50°C to 275°C at 10°C per min. Inlet temperatures were maintained at 250°C and GC-EAD effluent interface from the post-column splitter was kept at 275°C. Mass spectrometry was performed using an HP 5973 instrument (electron impact, 70 eV). The Wiley mass spectral library, with 275,821 spectra, was available on the MS data system (Wiley, 1995).

HPLC purification was carried out on synthetic heptadienals to separate geometrical isomers. A silica column (Econosphere Silica, 5-μm particle size, 4.6 × 250 mm, Alltech, Deerfield, IL), treated with silver nitrate (Heath and Sonnet, 1980), was used for this purpose. The solvent was benzene:hexane (1:1). The pump was a Waters 515 (Milford, MA) (flow-rate 1 ml/min), and the detector was a Waters R401 differential refractometer.

^{1}H-NMR spectra ($CDCl_3$) were obtained on a Bruker Avance 400 MHz instrument (Billerica, MA).

Analysis of Commercial Heptadienals. Initial GC-MS analysis suggested that one male-specific compound was identical to a minor (ca. 5%) constituent of commercial (2*E*,4*E*)-2,4-heptadienal. A sample of this minor compound was purified by HPLC (250 μg per injection, 10 injections) for identification by NMR. The target compound was recognized in HPLC fractions by GC retention. In preparation for NMR, the combined sample in benzene/hexane was applied to a

small open-column of silica gel (0.5 × 3 cm), which was then eluted with pure hexane to remove the benzene and finally with redistilled diethyl ether to recover the aldehyde. The ether solution was evaporated under a gentle stream of nitrogen and $CDCl_3$ was added.

Quantitation. The amounts of (2*E*,4*Z*)-2,4-heptadienal (**1**) and (2*E*,4*Z*)-2,4-heptadien-1-ol (**2**) in the volatiles collections were quantified by GC-MS in selected ion monitoring (SIM) mode. Two ions were monitored for both **1** and **2** (*m*/*z* 81 and 110 for **1**; *m*/*z* 83 and 112 for **2**). The selected ions for the internal standard, 1-octanol, were *m*/*z* 70 and 84. Quantitation was based on ions *m*/*z*, 110, 112, and 84, whereas ions at *m*/*z* 81, 83, and 70 served as qualifiers (proper ion ratios would support compound identity and purity in the GC peaks). Serially diluted solutions of synthetic **1**, **2**, and 1-octanol (0.01 ng/μl −30 ng/μl) were analyzed by GC-MS in SIM mode to obtain linear calibration curves (log dose vs. log abundance), which served as the basis of the quantitation. In SIM mode, **1** and **2** were still easily detectable at concentrations as low as 10 pg/μl.

Chemicals. Compounds **1** and **2** were synthesized according to Petroski (2003). The purities were checked by GC and found to be >95%; impurities were other geometrical isomers. Samples of (2*E*,4*E*)-2,4-heptadienal and (2*E*,4*E*)-2,4-heptadien-1-ol were obtained from Bedoukian (Danbury, CT).

Field Lures. The lures for 2003 consisted of a 1:1 mixture of **1** and **2** (500 μg of each component). These were prepared by adding solutions (50 μg/μl) of synthetic **1** and **2** in methylene chloride to mineral oil (250 μl) in 1.5-ml glass vials. Vials were sealed with Teflon disks, held in place by caps with central holes for use with septa (SRI, Eatontown, NJ). The vials were wrapped in aluminum foil to afford UV protection. A wire, wrapped around at the base of the cap, was used to secure the lure to a trap. Prior to field deployment, the Teflon disks were pierced with a single pinhole to allow compound release from the headspace. Vials were kept upright in the field. The lures for 2004 were prepared as above except that **1** and **2** were used at 2.5 mg each per vial, and additional lures had just **1** alone (2.5 mg) or **2** alone (2.5 mg).

The emission rate of **1** and **2** was measured from a freshly prepared lure (1:1 mixture of **1** and **2**, 500 μg of each component) in an incubator. The vial was placed in a volatiles collector at 25°C with an air flow-rate of 100 ml/min. Volatiles were collected daily for a period of 5 d and the release rates of **1** and **2** were measured by GC-FID using 1-octanol as the quantitative internal standard. Compounds were released in a ratio of nearly 1:1 and at a rate of about 7 μg/day for each compound. Emission was consistent over 5 d. Less than 1% degradation of the aldehyde to the more stable all-*E* isomer was found.

Field Study. The experiments were carried out in saltcedar stands along the Humboldt River (40°01′N, 118°31′W) 17 Km southwest of Lovelock, NV, where a population of *D. elongata* has been established (DeLoach et al., 2004). Two types of experiments were carried out.

The first set was a pair-wise comparison of a 1:1 mixture of **1** and **2**, using five lures of 500 μg of each component, and unbaited controls, on July 15 and August 10, 2003, when newly emerged beetles were present. The 2003 experiments were carried out in saltcedar stands with relatively little defoliation. This experiment was repeated, using single lures of the same total dosage, on May 4, 2004, when the overwintered beetles were becoming active. The 2004 site had experienced complete defoliation late in 2003, and little foliage was present on these weakened trees during the pheromone trial. The numbers of replications per treatment for the three experiments were 20, 15, and 22, respectively.

The second experiment measured the effects of the individual compounds and was set up on May 5, 2004. Treatments were a 1:1 mixture of **1** and **2**, **1** alone, **2** alone (all at 2.5 mg per component), and an unbaited control. A randomized complete block design was used (four traps per block), and there were 20 replicates of each treatment.

Traps were placed in trees that were about 10 m apart and similar in size, foliage density, and in accessibility to beetles flying from downwind (the prevailing wind at the site was from the west). There were always other saltcedar trees in the vicinity of the trap trees and usually between them as well. Yellow sticky traps (15.5 $\times$ 30.5 cm, AgriSense, Pontypridd, UK) were attached to branches (oriented vertically) in the upper half of the trees, typically at a height of 2–4 m, and every effort was made to place traps at nearly the same height. The higher locations were accessed by bending branches to within reach. Foliage or defoliated branches surrounding the traps were removed as needed so that there was a clear "flight path" to the trap. The protective paper was removed only from one side of the trap, so that the sticky side was oriented downwind. The choice of which trap would receive a particular treatment was made randomly by coin flip. The dispensers were activated when they were attached to the traps by making a pin hole in the Teflon seal.

Traps were set out in mid afternoon, when flight activity usually began to increase. They were then examined at intervals of about 2 h, and counts of beetles were recorded. The next morning, the traps were removed. Final counts were made and the beetles trapped on July 16 were saved for determination of sex.

Statistics. Amounts of compounds **1** and **2** collected from the beetles and foliage controls were submitted to analysis of variance (ANOVA) using Statistix for Windows software (Analytical Software, 1998). The amount data were transformed to a log scale to stabilize variance. The composition data (amount of **1** as a percentage of **1** + **2**) were submitted to ANOVA without transformation. For the field test, counts of *D. elongata* adults on traps were transformed using log (X + 1) to stabilize variance. The data from the pair-wise experiments were analyzed by paired *t*-tests, and data from the randomized complete block design were submitted to analysis of variance (ANOVA) and means were compared using the least significant difference (LSD) test.

RESULTS

Volatiles Collections. By GC comparison of volatiles extracts, males of *D. elongata* feeding on saltcedar emitted two compounds, **1** and **2**, that were not immediately obvious in volatiles extracts from females feeding on saltcedar or saltcedar foliage alone (Figure 1). The unlabeled peaks in Figure 1 were primarily saltcedar volatiles, based on GC-MS comparisons to uninfested plant materials.

Spectral Analysis and Identification. Mass spectra of the two male-emitted compounds indicated molecular weights of 110 for **1** and 112 for **2**. A search of the mass spectral library indicated close matches with (2*E*,4*E*)-2,4-heptadienal and (2*E*,4*E*)-2,4-heptadien-1-ol for **1** and **2**, respectively. Commercial samples of the aldehyde and alcohol closely matched the mass spectra of **1** and **2**, but their retention times were slightly later than the natural materials, indicating that **1** and **2** were probably geometrical isomers of the all-*E* standards. Fortuitously, the commercial sample of (2*E*,4*E*)-2,4-heptadienal contained an impurity (approx. 5%) whose mass spectrum, polarity (elution from open silica column with 10% ether in hexane) and retention time matched that of the natural aldehyde **1** exactly.

The impurity was isolated by HPLC ($AgNO_3$ on silica) and analyzed by ^{1}H-NMR. The configurations of the double bonds in the aldehyde impurity were determined based on vicinal coupling constants for the olefinic protons. The

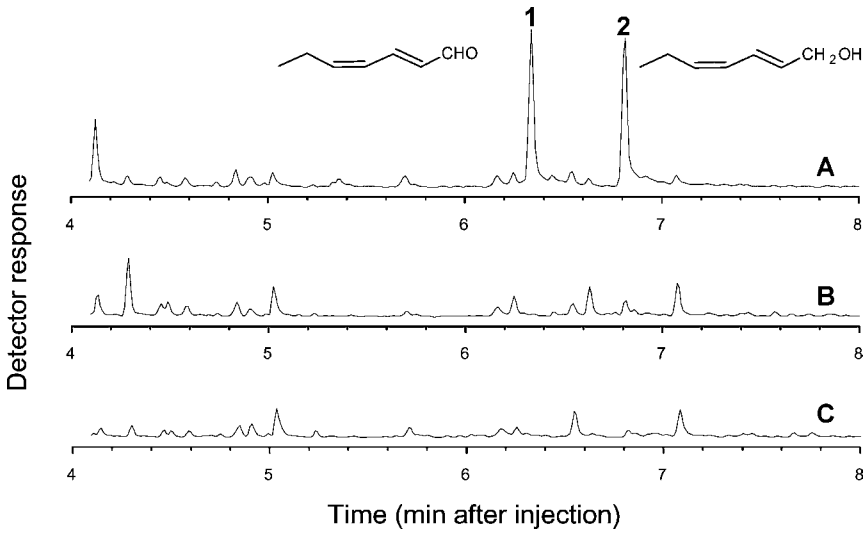

FIG. 1. Gas chromatographic profiles of volatiles collected from feeding *D. elongata*. A) males feeding on saltcedar foliage; B) females feeding on saltcedar foliage; C) saltcedar foliage control.

aldehydic proton (δ 9.61, 1H, d, $J_{1-2} = 8.0$, H-1) was coupled to one of these (δ 6.15, 1H, dd, $J_{1-2} = 8.0$, $J_{2-3} = 15.2$, H-2), and the magnitude of J_{2-3} established that the double bond in the 2 position had the *E* configuration (Williams and Fleming, 1980). The configuration of the second double bond was determined from the olefinic proton adjacent to the terminal ethyl group (δ 5.99, 1H, dt, $J_{4-5} = 10.7$, $J_{5-6} = 7.8$, H-5), and the magnitude of J_{4-5} indicated the *Z* configuration (Williams and Fleming, 1980). The other two olefinic protons (δ 7.44, 1H, dd, $J_{2-3} = 15.2$, $J_{3-4} = 11.5$, H-3; and δ 6.23, 1H, apparent br t, J_{3-4} and $J_{4-5} \cong 11$, H-4) were consistent with the above conclusions. The value of J_{3-4} also supported the conjugated diene system (Williams and Fleming, 1980). Thus, the configuration of the impurity and, therefore, of **1**, was 2*E*,4*Z*. Several of the olefinic proton signals were broadened by long-range allylic coupling. The ethyl group protons were observed (δ 2.39, 2H, apparent quin, J_{5-6} and $J_{6-7} \cong 7.5$, H-6; δ 1.11, 3H, t, $J_{6-7} = 7.5$, H-7).

The synthetic (2*E*,4*Z*)-2,4-heptadienal (**1**) was reduced with $LiAlH_4$ to give (2*E*,4*Z*)-2,4-heptadien-1-ol (**2**). The mass spectrum and retention time of this alcohol matched those of natural **2** exactly. Therefore, the two male-specific compounds were identified as (2*E*,4*Z*)-2,4-heptadienal (**1**) and (2*E*,4*Z*)-2,4-heptadien-1-ol (**2**) and subsequent synthesis of the two compounds further confirmed these identifications (Petroski, 2003).

A few of the extracts of male volatiles that showed relatively large amounts of **1** and **2** indicated the presence of at least two additional male-specific compounds (<1% of the total of **1** + **2**). These compounds eluted earlier from the GC columns than **1** and **2**, but they did not elicit antennal responses, and the small amounts available did not allow a more detailed examination.

Further examination of volatiles extracts from female beetles feeding on foliage and foliage controls by GC-MS did reveal trace amounts of **1** and **2**, based on both GC retention times (5.04 and 5.20 min, respectively) and mass spectra.

Electrophysiology. GC-EAD analysis of extracts of volatiles from male beetles feeding on saltcedar foliage showed that natural **1** and **2** were readily detected by the antennae of both sexes. Similar results were obtained for the synthetic materials (Figure 2). GC-EAD analysis supported the existence of **1** in volatiles from uninfested and female-infested saltcedar foliage. Antennal responses at the retention time for **1** (5.04 min) was observed in several cases in the initial analyses of female- and foliage-derived samples, and when such samples were combined and concentrated, antennal responses to **1** were observed more consistently. However, the presence of **2** (retention time, 5.20 min) in collections from foliage or feeding females was not verified by GC-EAD, presumably because the even lower amounts were below the antennal response threshold.

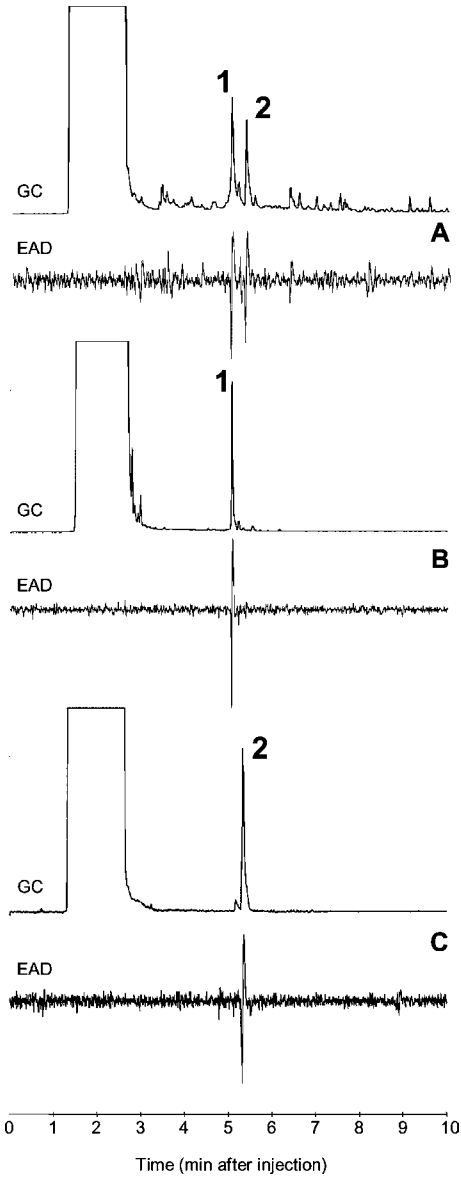

FIG. 2. Simultaneously recorded gas chromatogram (GC) and electroantennographic detection (EAD) of: A) female antenna responding to volatiles collected from male *D. elongata* feeding on saltcedar, including **1** and **2**; B) male antenna responding to synthetic **1**; C) male antenna responding to synthetic **2**.

Synthetic (2*E*,4*E*)-2,4-heptadienal and (2*E*,4*E*)-2,4-heptadien-1-ol were also detected by the antennae of both sexes (GC-EAD) when sufficient amounts of these isomers were injected (25 ng for each compound).

Emission Rates. Amounts of compounds **1** and **2** collected per day are shown in Table 1 for both sexes, and both densities (grouped and single) and for uninfested foliage. The amounts in the table are expressed both on a per-tube and a per-beetle basis, for comparison. Compounds **1** and **2** were obvious within days of placing new male beetles in the collection apparatus, and emission continued as long as the beetles lived (approx. 30 d) with fairly constant component ratios.

On a per-beetle basis, amounts of **1** and **2** from tubes with males were always significantly higher than from tubes with females (LSD, $P < 0.05$ level). Groups of males emitted significantly more of **1** and **2** compared to single males, but the trace amounts of **1** and **2** collected from tubes with grouped females did not differ significantly from those kept as individuals. The maximum amount of **1** and **2** collected from a single male was 1.57 μg/d.

Expressing amounts of **1** and **2** on a per-tube basis allowed the effect of foliage to be considered in comparisons, as well as beetles. The amounts of **1** and

TABLE 1. EMISSION OF (2*E*,4*Z*)-2,4-HEPTADIENAL (**1**) AND (2*E*,4*Z*)-2,4-HEPTADIEN-1-OL (**2**) BY SINGLE AND GROUPED (USUALLY 10) *D. elongata adults*

		Emission rate (ng/tube/day)			Emission rate (ng/beetle/day)	
Component	*N*	Mean[1]	Confidence interval[2]	**1** as % of (**1** + **2**)	Mean[1]	Confidence interval[2]
Male Beetles						
Grouped						
1 + **2**	31	860[a]	225–3320	61[a]	125[a]	46–330
Single						
1 + **2**	37	55[b]	8.7–350	60[a]	55[b]	8.7–350
Female Beetles						
Grouped						
1 + **2**	15	28[bc]	13–60	93[b]	3.1[c]	1.8–5.5
Single						
1 + **2**	14	7.1[d]	2.8–18	92[b]	7.1[c]	2.8–18
Foliage						
1 + **2**	5	8.1[cd]	4.4–15	84[b]	NA	NA
F statistics		37.2 ($df = 4,97$) $P < 0.001$		22.2 ($df = 4,97$) $P < 0.001$	33.2 ($df = 3,93$) $P < 0.001$	

[1]Untransformed means, means within a column followed by the same letter do not differ by LSD test, $P < 0.05$.
[2]Untranformed (log mean ± one standard deviation).

2 emitted by grouped males were dramatically higher than all other treatments, and the amounts from tubes with single males were higher than all treatments with females or foliage alone, except that the difference from grouped females was not significant. The amounts of **1** and **2** from tubes with females did not differ from those collected from the saltcedar foliage.

Considering composition, the percentage of **1** (relative to **1** plus **2**) was significantly lower in extracts from males than with the female or foliage-only extracts (Table 1). However, the percentage of **1** in extracts from females did not differ significantly from that found with uninfested foliage.

Field Study. Trapping experiments demonstrated the attractiveness of the two male-specific compounds to *D. elongata* in the field on four different dates with two different populations (Table 2). Significantly more adult *D. elongata* were trapped on traps baited with **1**, **2**, and a 1:1 mixture of **1** and **2** compared to the control traps.

However, compound **2** as a single component was as attractive as the 1:1 blend of the two components, whereas compound **1** as a single component was only weakly attractive (Table 2).

Male and female beetles were about equally attracted to the two component mixture. July 16, 2003 saw a total of 945 adults on the baited traps, with a male: female sex ratio of 1.1: 1; control traps caught a total of 351 beetles with a male: female sex ratio of 1.4: 1. Nearly all of the trapped beetles were caught between mid-afternoon and twilight, during the peak of flight activity. There was no evidence for beetles being caught between twilight and the final trap count the following morning.

TABLE 2. MEAN NUMBER OF *D. elongata* ADULTS PER STICKY TRAP BAITED WITH SYNTHETIC (2*E*,4*Z*)-2,4-HEPTADIENAL (**1**), (2*E*,4*Z*)-2,4-HEPTADIEN-1-OL (**2**), AND A 1:1 MIXTURE OF **1** AND **2**, IN LOVELOCK, NEVADA SALTCEDAR STANDS DURING 2003 AND 2004

	Mean number of beetles/trap			
	2003		2004	
Treatment	July 16	Aug. 11	May 5	May 6
1 + **2**	32.1**	13.7**	14.4**	76.7[a]
Control	7.9	4.0	1.4	5.3[c]
1	—	—	—	10.4[b]
2	—	—	—	65.5[a]
t statistics	4.63 (*df* = 19)	6.00 (*df* = 14)	5.58 (*df* = 21)	—
F statistics	—	—	—	69.79 (*df* = 3,57)
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

DISCUSSION

Analysis of extracts of volatiles demonstrated that male *D. elongata* emitted two components, (2*E*,4*Z*)-2,4-heptadienal (**1**) and (2*E*,4*Z*)-2,4-heptadien-1-ol (**2**), which were sensed by antennae of both males and females.

However, in field trials, **2** was much more attractive than **1**, and **2** as a single component was as attractive as a 1:1 blend of **1** and **2**. Thus, the role of **1** as a component of the pheromone blend is not yet clear. Further trials with different blend ratios may reveal an additive or synergistic role for it in the blend. The synthetic pheromone attracted approximately equal numbers of males and females, indicating that it is an aggregation pheromone rather than a sex pheromone.

These compounds are quite different from previously described chrysomelid pheromones (see introduction), being smaller in size and simpler in structure (i.e., without branches or rings). Indeed, the compounds were overlooked in the early stages of this study because of their similarity to the well known, six-carbon "green leaf volatiles" (Visser and Avé, 1978).

An unusual result in this study is that compounds identical to the pheromone components could be found at trace levels in collections from feeding females and from the host plant. Of these two, the data suggest that the foliage is the actual source, rather than the females, because the collections from single, feeding females were essentially the same as collections from uninfested foliage. The collections from groups of females had significantly greater total amounts of the compounds than collections from single females, but on a per-beetle basis, the group collections did not differ significantly from the single beetles. A likely explanation is that the greater amount of feeding activity by groups of females led to a greater release of plant volatiles, including **1** and **2**. Further evidence for a foliar origin of the compounds in the collections from females is that a ratio of **1** and **2** was essentially the same as in the foliage, but was quite different from the collections from males.

It is not surprising that damaged foliage might emit aldehyde **1** because this compound is known to be an autoxidation product of the ubiquitous biochemical, linolenic acid (Frankel, 1998 and references therein). The more remarkable situation is that the amounts were so much higher when males were feeding on that foliage, and that the component ratios of **1** and **2** were so different from when males are absent, indicating that males exert a great deal of control over the emission of the compounds. Beyond the greater amounts and different ratios noted above, compound emission is closely linked to the physiology of the males and to their environment. For example, males induced to enter overwintering reproductive diapause by short daylength do not emit or induce the production of **1** and **2** (although they continue to feed), and a day/night emission rhythm has been observed in reproductive males. Furthermore, strains of beetles of various geographic origins also produce different, characteristic ratios of components **1**

and **2**. These (presently unpublished) examples of control over emission lead us to believe that males produce the pheromone within their bodies rather than inducing the host plant to emit them, despite the superficial similarity of the pheromone components to green leaf volatiles.

In the future, different doses and ratios of the two components should be tested in the field to determine whether alternative responses are possible. In addition, the blend/single component experiment should be repeated in areas with various degrees of defoliation, various population densities, and at various times of the year to further assess the effects of the single components and competition from natural sources of **1** and **2**. Also, volatiles collections in which components **1** and **2** were relatively abundant had additional, minor male-specific compounds, and the possible behavioral effects of these additional components remains to be evaluated. Finally, GC-EAD analysis indicated that some saltcedar derived compounds elicited antennal responses. Whether host odor is necessary for optimal field response will have to be studied, particularly as synergism between aggregation pheromones and host-related volatiles is known in the Chrysomelidae (Metcalf and Metcalf, 1992; Pivnick et al., 1992).

A pheromone-based monitoring system will be useful for studying several population attributes of *D. elongata*. The field results demonstrated the potential of using the synthetic pheromone for monitoring field populations of *D. elongata*, with the added benefit that both sexes can be monitored.

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RESPONSES OF THE CRAB *Heterozius rotundifrons* TO HETEROSPECIFIC CHEMICAL ALARM CUES: PHYLOGENY VS. ECOLOGICAL OVERLAP

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Abstract—The big-handed brachyuran crab *Heterozius rotundifrons* extends the time spent in its anti-predator posture, limb extended posture, if exposed to chemical cues from crushed conspecifics. In this study, we tested whether crabs also respond to chemical cues from crushed heterospecific crabs, and if so, whether phylogenetic relations or ecological overlap is more important in influencing the duration of the anti-predator posture. Chemical cues from two other brachyuran crabs (*Cyclograpsus lavauxi* and *Hemigrapsus sexdentatus*), which do not overlap directly in ecological distribution with *H. rotundifrons*, elicited a duration of the anti-predator posture that was indistinguishable from that produced by conspecific chemical cues. In contrast, chemical cues from two anomuran crabs (*Petrolisthes elongatus* and *Pagurus novizealandiae*) that overlap in ecological distribution with *H. rotundifrons*, elicited durations of the antipredator posture that were significantly shorter than those of either conspecifics or more closely related crab species. Thus, phylogenetic relationship seems to be more important than ecological overlap in influencing anti-predator behavior in *H. rotundifrons*.

Key Words—*Heterozius rotundifrons*, alarm cues, heterospecific, ecological context, phylogenetic history, crabs

INTRODUCTION

Virtually all species of animals tested show an increase in anti-predator behaviors when chemical cues from an injured or crushed conspecific are detected (Smith,

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1992; Chivers and Smith, 1998; Wisenden, 2000). Because the execution of anti-predator behavior results in a decrease in successful predation (Mathis and Smith, 1993; Mirza and Chivers, 2001), it is not surprising that selection has favored lineages that respond to cues from damaged conspecific individuals. In some species, responses to cues from damaged heterospecific individuals also occur (Wisenden et al., 1999; Chivers, 2002; Mirza and Chivers, 2003). Given the generalized diet of many predators, it is reasonable to predict that prey would be selected to utilize any information that indicates elevated risk of predation.

While it is clear that some prey learn to recognize heterospecific cues (Brown and Chivers, In Press), the literature on the use of heterospecific cues is mixed as to whether phylogenetic relationship or ecological overlap is more important in determining what cues are utilized by prey. That is, do prey treat cues from more closely related animals as good indicators of predation risk or is the extent to which animal species overlap ecologically a stronger determinant of cue utilization? Learning cues would mandate experience, and, thus, ecological overlap, while related species might release similar cues to those released by damaged conspecifics, and be innately recognized.

Individuals of the Australian hermit crab *Clibanarius infraspinatus* responded to haemolymph from two congeners (*C. taeniatus* and *C. virescens*) just as strongly as to conspecific haemolymph but responded more weakly to haemolymph from the distantly related diogenid hermit crab *Diogenes avarus* (Hazlett, 1996) even though *D. avarus* overlaps in microdistribution with *C. infraspinatus* and *C. virescens* does not. Rittschof et al. (1992) reported individuals of the hermit crab *C. vittatus* did not respond at all to cues from *Pagurus* species (different families) even though they overlap in microdistribution. The *Pagurus* species each responded most strongly to fluids from conspecifics, less strongly to congeneric fluids and even less to fluids from *C. vittatus* (Rittschoff et al., 1992).

In contrast, Mirza and Chivers (2003) found that minnows respond to cues from injured damselfly larvae (obviously unrelated) but only when the minnows are small and in the same prey guild as damselfly larvae. In this case, ecological context appears more important than phylogenetic relatedness. Stenzler and Atema (1977) found that three species of the mud snail *Nassarius* responded to each others alarm substance, responded strongest to conspecific substance, but did not respond to cues from other gastropod genera. However, one species, *N. trivittatus*, responded to the sympatric, but taxonomically not closely related, *Urosalpinx cinerea* (Stenzler and Atema, 1977). Mirza and Chivers (2003) cite a number of cases in both aquatic and terrestrial systems where similarities in ecology appear to be more important than degree of relatedness.

Individuals of crayfish species in Australia (Gherardi et al., 2002), Italy (Hazlett et al., 2003) and North America (Hazlett, 2000), show distinct differences in whether or not they respond as strongly to heterospecific cues as to conspecific cues. However, the pattern of response does not seem to relate to phylogeny but

rather to whether species have evolved in specious regions and are successful invaders or being displaced. Those species that have recently successfully expanded their ranges respond more strongly to heterospecific danger signals, and this pattern of responses is not linked to how closely related the crayfish species are.

Heterozius rotundifrons is a New Zealand endemic crab that has an unusual anti-predator behavior. When stimulated by tactile input, crabs assume a limb-extended posture in which the cheilpeds are spread almost 180° apart (Field, 1990) and remain motionless in this posture for a number of minutes. This posture is an effective defense against fish predators (Hazlett and McClay, 2000), and the duration of the posture is extended upon introduction of fluids from crushed conspecifics (Hazlett and McClay, 2000).

The purpose of this study was to see if individuals of *H. rotundifrons* responsed to cues generated from crushed heterospecific crabs and, if so, which was more important in determining the strength of responses, phylogenetic relationship or ecological overlap.

METHODS AND MATERIALS

Heterozius rotundifrons A. Milne Edwards, 1867 used in this study were collected from the vicinity of the Edward Percival Field Station in Kaikoura, New Zealand and transported to the salt-water facility of the School of Biological Sciences, University of Canterbury, Christchurch, New Zealand. The experiments were conducted in May 2003 at the University of Canterbury. Individuals of *Heterozius rotundifrons* were tested with alarm cues prepared from one of five crustacean species, conspecific cues, and four other New Zealand species. We tested 20 different individuals of *H. rotundifrons* per species.

In all cases, the number of seconds spent in the limb-extended posture were recorded following induction of this defensive posture by grasping a crab, turning it upside-down and then placing it right-side up on the layer of gray sand in a plastic container with 750 ml of sea water and 25 ml of alarm cue. Field (1990) has shown that this method of tactile induction is the most reliable way to induce this behavior. The end of about of the limb-extended posture was recognized by the movement of any ambulatory leg or cheliped.

The five species used to prepare the alarm cues were *Heterozius rotundifrons*, *Cyclograpsus lavauxi*, *Hemigrapsus sexdentatus*, *Petrolisthes elongatus*, and *Pagurus novizealandiae*. *C. lavauxi* and *H. sexdentatus* are brachyurans, as is *H. rotundifrons*, but do not overlap directly in distribution in the intertidal, as these grapsids occur higher in the intertidal (McLay, 1988). In contrast, individuals of *P. elongatus* and *P. novizealandiae* overlap extensively in distribution with *H. rotundifrons*, but are anomurans (McLay, 1988). The number of individuals used to prepare the alarm cue varied but was equivalent in weight to two medium-size H. *rotundifrons*. For each preparation, the crustaceans used for making the

alarm cue solutions were thoroughly crushed and mixed in 200 ml of sea water and filtered with coarse filter paper. Twenty-five ml of the crushed crustacean solution were added to the plastic container in which the test individual of *H. rotundifrons* was placed.

A one-way ANOVA was used to compare the difference between number of seconds spent in the limb-extended posture by the 20 crabs placed in the different alarm cue preparations. The five test preparation values were compared with Tukey tests.

RESULTS

There was significant variation in the duration of the limb-extended posture among the five alarm cue preparations (ANOVA $F = 3.37$, $df = 4,95$, $P = 0.013$). As shown in Figure 1, the time spent in the posture was similar for the three brachyuran crab preparations (*H. rotundifrons*, *C. lavauxi*, and *H. sexdentatus*), and the Tukey test values comparing those three indicated no difference between them (*H. rotundifrons–C. lavauxi* $P = 0.809$, *H. rotundifrons–H. sexdentatus* $P = 0.962$). The number of seconds spent in the posture when the test crabs were placed in either of the anomuran crab preparations was significantly

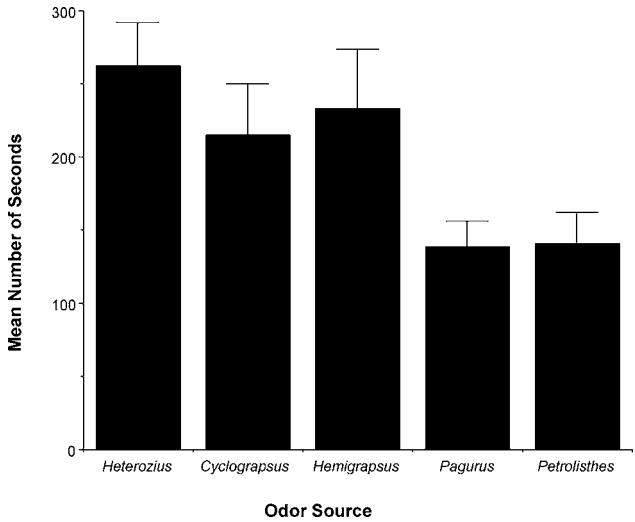

FIG. 1. Number of seconds (means + s.e.) spent in the limb-extended posture by individuals of *Heterozius rotundifrons* when placed in sea water containing chemical cues prepared with one of five crustacean species: *H. rotundifrons*, *Petrolisthes elongatus*, *Cyclograpsus lavauxi*, *Hemigrapsus sexdentatus*, and *Pagurus novizealandiae*.

lower (*H. rotundifrons–P. elongates* $P = 0.045$, *H. rotundifrons–P. novazealandiae* $P = 0.039$). These values were similar to the values reported in other studies when only tactile stimulation and no chemical cues were added (Hazlett and McLay, 2000, In Press).

DISCUSSION

Individuals of *H. rotundifrons* responded to the cues of crushed heterospecific crabs, but those in the same broad taxonomic group produced a significantly greater response. Their response to cues from species with whom they overlap extensively in ecological micro-distribution but that are more distantly related was significantly less than to related crabs. In previous investigations, the control group of physically stimulated crabs that received no chemical cues from damaged conspecifics showed durations of response similar to those seen here to the non-related crabs (Hazlett and McLay, 2000). This suggests that the shorter responses seen for the non-related crabs could, in fact, be no response to chemicals at all, just the response to being physically stimulated. *Heterozius* is a monotypic genus placed in the Family Bellidae, closely allied to the Family Xanthidae. Thus, while it is in the same order (Brachyura) as the two grapsid species used in this study, the three brachyruans are not closely related. The crabs in the Order Anomura are only distant phylogenetically. While tests with a range of concentrations would provide additional details and an assessment of the ecological reality of responding to these cues in the much lower concentrations that might be encountered in the field, the responses of *Heterozius* to approximately equal concentration of cue from two closely related vs. two distantly related crabs is consistent.

The results of this study suggest a priority of phylogeny over ecological overlap in determining the cues to which a potential prey species will respond. This finding is consistent with the majority, but not all, of similar studies using crabs. Of course individuals of a species could respond to chemical cues more or less strongly based upon both phylogeny and ecology (Stenzler and Atema, 1977). Certainly, ecological overlap sets the stage for learning to use heterospecific cues. However, individuals of *H. rotundifrons* responded less to cues from unrelated than to cues from related crabs, and given that previous studies with this species (Hazlett and McClay, 2000) show that tactile stimulation without any chemical alarm cues can cause anti-predator posture duration that is about equal to our treatments with unrelated crab cues, it is possible that there was no response to unrelated species. It is conceivable that *H. rotundifrons* does not share predators with the porcellanid, *P. elongatus*, or hermit, *P. novizelandaiae*, crabs. If that were the case there would be no advantage in utilizing cues resulting from the crushing/predation of individuals of those species. Indeed, Mirza and Chivers (2003) showed that the minnow, *Pimephales promelas*, responded to cues from

attacks on damselfly larvae, *Enallagma boreale* only when minnow size dictated the two species were in the same prey guild.

As has been pointed out elsewhere (Thacker, 1994), one might expect phylogenetic relationship to be important in determining cue use, because of the similarity of the chemicals released from the bodies of related species. Thus, individuals of *H. rotundifrons* could be responding to a cue that is common to all brachyurans but absent from anomurans. Unless a species evolved under predation pressure from a taxonomically very specialized predator, selection may favor use of a generalized cue.

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LETTER FROM THE EDITOR-IN-CHIEF

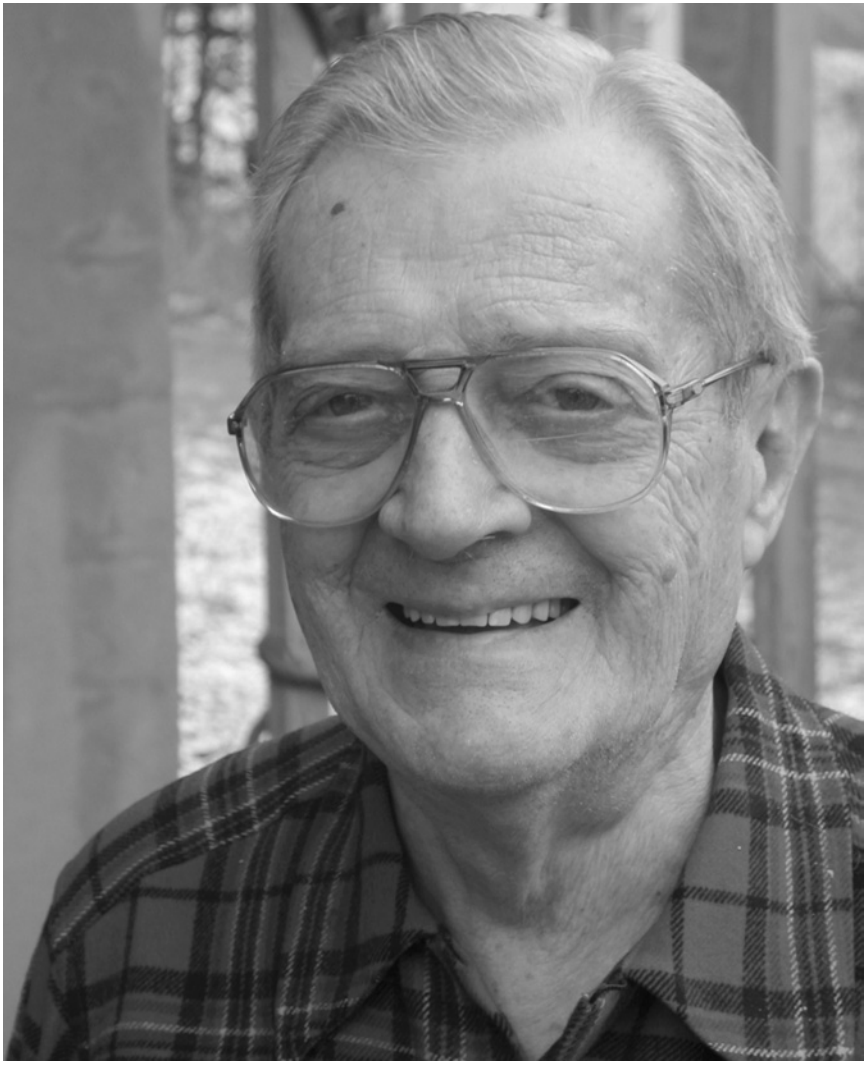
JOHN T. ROMEO (FOR THE ASSOCIATE EDITORS
AND EDITORIAL BOARD)

Everyone associated with the *Journal of Chemical Ecology* is saddened by the passing of John Simeone, one of the founding editors of the *Journal*. The *Journal* he envisioned with Robert Milton Silverstein was established in 1975, and the International Society of Chemical Ecology, which they also helped found, in 1983. John was a thinker ahead of his time; a tireless caretaker who devoted many years of his life to the *Journal*; and a warm human being who was the inspiration for countless "students" of our discipline.

The following tribute by Jim Nation, to whom John and Milt entrusted the *Journal* when the time came, reminds us of many of the important events in John's life, his gentleness and firmness, and his legacy. There will be appropriate forums in the future for those of you who wish to express your own feelings.

The Silverstein/Simeone award, which is awarded annually by the International Society of Chemical Ecology and funded by Springer Publishing, recognizes a scientist who is advancing the discipline of chemical ecology with innovative research.

We dedicate this issue of the *Journal* with respect and gratitude to John.



John B. Simeone 1919–2005

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JOHN B. SIMEONE 1919–2005

JAMES L. NATION (PROFESSOR EMERITUS)

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All of us in chemical ecology and entomology were saddened to learn of the death of John B. Simeone. John passed away at home on January 10, 2005, after a short period of illness. John was born in Providence, Rhode Island, November 20, 1919. Etta, his wife of many years, two sisters, and nieces and nephews survive John. We will all miss him greatly. John was a friend, mentor, editor, and scientific colleague to many of us. Younger chemical ecologists and forest scientists who may not have gotten to know John truly missed one of the warmest personalities in our science. John did undergraduate work in biology at the University of Rhode Island, a master's degree in forestry at Yale School of Forestry, and a PhD from Cornell University. It was at Cornell in the fall of 1957 that I met John. We were assigned to be lab partners in the graduate course in insect physiology. I was a very green, first semester graduate student. John was about 38 years old then, and had served in World War II as a naval officer, participating in the landing at Normandy and other amphibious operations. He stayed in the naval reserves after the war, and eventually retired with the rank of commander. John always had a gentle, but commanding presence that was clearly evident to all who knew him. We often joked about which of us got the other through that graduate course; but when John said to get my portion of a lab report done, I did it. We both learned physiology from Professor Bob Patton, who taught the course, but I learned a lot from John and formed a life-long friendship that I have valued very highly. After John finished his PhD at Cornell, he returned to Syracuse University, where he had started his career in entomology in 1948 as a young entomologist in the Entomology Department. During a long career at Syracuse, he taught a wide variety of entomology and ecology courses, but his special interest was in the ecology and biology of wood destroying insects. In 1962, not long after returning with his PhD, John was asked to chair the Entomology Department. Some time about the middle of the 1960s, he teamed with Dr. Ernest Sondheimer to teach a seminar in chemical ecology at Syracuse. In 1970, Academic Press published these lectures in a book entitled *Chemical Ecology*, edited by John B. Simeone and Ernest Sondheimer.

In the meantime, Robert Milton Silverstein, an organic chemist and a specialist in instrumental methods of chemical identification, had moved from Stanford Research Institute to join the Chemistry Department at University of Syracuse. Milt not only had a premier reputation in organic chemistry, but he and David Wood at Berkeley had already initiated studies of the pheromone of the bark beetle *Ips confusus* (now *Ips paraconfusus*). They identified the pheromone as a three-component pheromone in 1966, which helped to dispel the early idea that each species might produce a single, but different pheromonal compound.

It is extraordinarily fortunate for the field of chemical ecology that Milt and John teamed with Plenum Publishing Corp. to publish the *Journal of Chemical Ecology*, the first journal devoted to chemical ecology. The first issue appeared in January 1975. They co-edited it for the next 20 years.

John continued as Chairman of Entomology until 1977, when he was asked to become Chairman of the newly formed Department of Environmental and Forest Biology at Syracuse. This was an amalgam of the departments of Botany/Plant Pathology, Zoology, and Entomology. John served as Chairman until 1983, when he retired. Well, not really; he continued to go to his office regularly, and helped in insect ID and biology of forest insects, interacted with colleagues and graduate students, and continued with Milt to edit the *Journal of Chemical Ecology* through 1994. Through the *Journal of Chemical Ecology*, John and Milt played a major role in defining, encouraging, and molding the field of chemical ecology. By the early 1980s scientists all over the world were devoting major efforts to understanding chemical ecology of insects, plants, vertebrates, terrestrial, fresh water and marine organisms and publishing their work in the *Journal of Chemical Ecology*. The time had come for a professional society, and John, Milt, Lincoln Brower, Jean Langenheim, Michael Martin, and Gerald Rosenthal founded the International Society of Chemical Ecology. The Society was incorporated in 1983, and its constitution approved in 1984 at the business meeting of the first annual meeting of ISCE at the University of Texas, Austin.

John taught, conducted research, chaired departments, and informally interacted with students and colleagues for more than 55 years at Syracuse. John and Etta established a Graduate Fellowship in Forest Entomology at Syracuse to support graduate students in entomology. John's many contributions to entomology were recognized in the awarding of the prestigious L.O. Howard Distinguished Achievement Award by the Entomological Society of America and by election as an Illustrious Emeritus Professor.

I am indebted to the obituary published in the *Post-Standard*, Syracuse, NY, to Etta Simeone, and to an article on John written by Cynthia King for information.

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SEASONAL CHANGES IN FOLIAR TERPENES INDICATE SUITABILITY OF DOUGLAS-FIR BUDS FOR WESTERN SPRUCE BUDWORM

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Abstract—The terpene composition of current-year buds of Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, was analyzed from before budburst to after buds were fully flushed. Terpene composition was measured at weekly intervals for several seasons at eight different locations in the southern interior of British Columbia, Canada. Discriminant functions relating terpene composition to suitability of buds for newly emerged western spruce budworm, *Choristoneura occidentalis* Freeman, were developed based on terpene profiles of the buds and bioassays measuring the suitability to budworms of a sister group of buds. Changes in percent composition of bud terpenes before and during budburst were closely associated with changes in the suitability of the buds to utilization by budworms at both the tree and site by date levels. Use of a degree-day scale for bud suitability removed much of the year-to-year variation, but remaining differences among sites suggest additional sources of variation influencing the insect–host plant relationship. The success of correctly classifying bud suitability using terpene profiles demonstrates the value of foliar terpenes as indicators of seasonal changes in suitability of Douglas-fir foliage during the critical spring emergence period of western spruce budworm. This indicator could be used to screen individual trees susceptible to budworm damage and identify sites at high risk of damaging defoliation.

Key Words—Douglas-fir, *Pseudotsuga menziesii*, western spruce budworm, *Choristoneura occidentalis*, foliar terpenes, host suitability, phenology, seasonal development, chemical indicators.

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INTRODUCTION

The western spruce budworm, *Choristoneura occidentalis* Freeman, is an important native defoliator of Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, in western North America. This insect–host plant association extends over highly varied climatic zones. Analysis of historical defoliation and impact records suggest that regional-scale outbreaks are associated with areas of optimal synchrony between bud development of the host tree and budworm-feeding activity (Thomson et al., 1984). Synchrony between budworm activity and foliage development also influences local patterns of defoliation. Differences in relative severity of defoliation of putatively "resistant" vs. "susceptible" Douglas-fir during outbreaks are the result of intertree differences in foliage phenology and relative synchrony with local, developing budworms (Chen et al., 2003). Thus, the relationship between phenology of budburst of Douglas-fir and of western spruce budworm development may provide insight into the host–plant relationship at several ecological scales.

Thomson and Moncrieff (1982) produced a robust, empirical method of predicting budburst in Douglas-fir, but their model was not referenced directly to insect performance. Shepherd (1983) used a system of photographs to classify seasonal development of Douglas-fir buds into nine stages ranging from fully closed winter buds to expanded summer shoots. He then identified the critical stages of bud development in terms of survival of western spruce budworm as: (1) the overwintering bud stage when budworms could not penetrate the hard bud; (2) the swollen, soft bud stage that could be penetrated by budworms; and (3) the stages following bud flush when budworms became exposed feeders (Shepherd, 1992). His analysis of budworm survival focused on stages following bud penetration by the budworm and during the period of exposed feeding. Similarly, investigations of the role of associated changes in foliage nutrients and terpene composition in budworm fitness have been restricted to the period of development following budworm penetration of the current-year buds (Wagner et al., 1989; Cates and Zou, 1990; Chen et al., 2002). Little information has been available on foliar chemistry or budworm survival early in the season as buds are just becoming available as a resource to foraging budworms (Clancy et al., 1993). Host–plant relationships at this early seasonal stage play an important role in the population ecology of conifer-feeding budworms (Campbell, 1993; Nealis, 2003).

Nault (2003) examined seasonal changes in terpene composition of Douglas-fir foliage over a 3-year period at several sites in British Columbia (BC), Canada. Measurements began in early spring before budburst and included the earliest phases of bud development identified by Shepherd (1983). The results revealed highly dynamic shifts in relative concentrations of different terpenes during the early spring period in contrast to their relative stability over

the remainder of the growing season (Nault, 2003). Seasonal changes in several terpenes characteristic of Douglas-fir buds resembled seasonal changes in the suitability of vegetative buds to spring-emerging larvae in other budworm systems (Nealis and Lomic, 1994). It seemed likely that these changes in terpenes could serve as indicators of bud suitability for western spruce budworm. Such an indicator would be less subjective than methods that rely on photographic interpretation (Shepherd, 1983) and would extend our understanding of the relationship between budworms and foliage chemistry to the critical period when budworms are foraging for feeding sites immediately following spring emergence.

This paper describes a set of discriminant functions based on terpene composition of current-year buds of Douglas-fir that accurately classify the suitability of those buds to exploitation by newly emerged western spruce budworm in early spring. The accuracy of predictions using terpenes as indicators of bud suitability is compared to predictions based on a degree-day model. Use of foliage chemistry results in models that are robust over a wide range of environmental conditions and allow rapid assessment of foliage suitability. This indicator can be used to quantify synchrony between local trees and budworms to interpret risk rating at the stand level as well as to identify relative susceptibility to defoliation of individual trees within a site.

METHODS AND MATERIALS

General. We used plant material collected directly from field sites. Eight sites were selected over a range of elevations where Douglas-fir dominates the forest community (Table 1). Sites 1 to 5 were in the Nicola Valley near Merritt, BC, and sites 6 to 8 were in the Okanagan Valley near Peachland, BC. All sites were in the Interior Douglas-fir (IDF) zone except site 5 which is an enclave of Douglas-fir within the Ponderosa Pine (PP) zone (Meidinger and Pojar, 1991). Temperature profiles for these sites for 1998 to 2003 were constructed from records obtained from the Meteorological Service of Canada for the two weather stations closest to the study sites: (1) Merritt STP (station #1125079, 50°7′N, −120°48′, 588 m); and (2) Peachland (station #1126070, 49°47′N, −119°43′, 345 m). Temperature regimes for each site were calculated using the program BioSIM (Régnière, 1996), which uses these nearest weather stations and adjusts for differences in location and elevation at the target sites based on estimated thermal gradients (Régnière and Logan, 2003). Daily degree-day accumulations above a temperature threshold of 4°C were calculated using daily maximum and minimum temperatures.

In seven sites, 10 codominant Douglas-fir trees were chosen at ~10- to 20-m intervals along a 200-m transect. Only five trees were similarly selected at

TABLE 1. LATITUDE, LONGITUDE, AND ELEVATION OF SAMPLE SITES AND FREQUENCY OF SAMPLING FOR BIOASSAYS AND FOLIAR CHEMICAL ANALYSIS IN TWO REGIONS OF THE SOUTHERN INTERIOR OF BRITISH COLUMBIA, CANADA

Region and site	Latitude (N), Longitude (W)	Elevation (m)	Sampling frequency (April to July)			
			1998	1999	2000	2003
Nicola valley						
1	50°08′, 120°45′	1100	3	–	–	3
2	50°14′, 120°49′	950	4	–	–	4
3	50°11′, 120°40′	1050	4	8	5	4
4	50°14′, 120°39′	1350	4	12	7	5
5	50°11′, 120°40′	750	4	10	6	3
Okanagan valley						
6	49°45′, 119°48′	600	5	10	4	–
7	49°47′, 119°46′	850	5	10	3	–
8	49°47′, 119°46′	1050	4	10	4	–

site 5 in 1998 and 1999, but this was increased to 10 trees after 1999. Samples were taken approximately weekly and consisted of a single 45-cm branch tip removed without regard to directional quadrant from the midcrown of each tree at each sample date using pole pruners equipped with a cutting head. Branches were bagged, labeled, and returned to the laboratory where they were stored at 2°C in the dark until buds could be excised, usually within 3 days. Defoliation estimates were made by applying the method of Fettes (1950) to branches sampled in August after feeding was complete. Defoliation estimates were expressed as percentage defoliation of current-year foliage for each tree in all years (V.G. Nealis, unpublished data).

Western spruce budworm used in bioassays were derived from wild populations collected as late-instar larvae or pupae in the previous season. These insects were reared to adult and allowed to mate in the laboratory. Female moths were provided cut host foliage on which to lay egg masses. Egg masses were placed into a Petri dish covered with parafilm to which a triple-layer patch of cheesecloth had been attached to the inside surface, facing the egg masses. The dish was placed into a paper envelope with a window cut to expose the cheesecloth patch and incubated at 20°C L:D 16:8 hr. As budworms hatched, they moved toward the light, became embedded in the cheesecloth, and spun hibernacula. After 2 wks at 20°C, hibernating larvae were stored at 2°C to satisfy their diapause requirements until needed the following spring. To activate the budworms, the cheesecloth patch with the budworms was saturated with water, placed into a dry glass tube, and incubated at 20°C.

Bioassays to Determine Bud Suitability. Current-year buds and 2-years' previous shoot growth were excised from each branch on each sample date to

determine their current suitability for western spruce budworm. The cut end of the shoot was inserted into a hole in a cork (3-cm diam). The cork served as a stand for the shoot. Freshly emerged western spruce budworms that were not fed yet were introduced to the base of the shoot at a ratio of one budworm per bud. Five buds per tree and five insects were used on samples from the earliest dates in the season. This was increased to 10 buds and 10 insects during the period of budburst and expansion. Thus, there were five or 10 buds tested for each of 10 trees on each sampling data at the site level.

A glass tube (3.5 cm in diam 15 cm long) was placed over the set-up to cage the budworms and the buds. These units were placed at 20°C, L:D 16:8 hr for 2 days to allow budworms to forage. After that time, the number of budworms that had successfully penetrated a bud was scored. This number was converted into a proportion of the cohort, ρ, that successfully established in buds and used as a measure of bud suitability for each tree on each sample date. An analogous measure for the site, P, was obtained by pooling across all trees within a site on each sample date.

Foliage Chemistry Assays. Detailed methods used to extract and analyze foliar terpenes are described by Nault (2003). Briefly, current-year vegetative buds were cut from the same branches as bioassay material and stored at −5°C until terpenes could be extracted (less than 1 mo). Sufficient buds from each branch sample were selected randomly to ensure a minimum total mass of ~0.3 g. To extract terpenes, tissues were homogenized in a solution of cold water, methanol, hexane, and an internal standard (methyl palmitate in iso-octane). The homogenized samples were centrifuged and the terpenes recovered in the hexane layer. Extracted terpenes in solution were analyzed on a gas chromatograph. Individual terpenes were identified by peak retention times. Identifications were confirmed by gas chromatography—mass spectrometry and gas chromatography—Fourier transform infrared spectroscopy. In all cases, blank samples without tissues were used to verify solvent purity. Individual terpenes were calculated as percent contribution to the total peak area recorded on the chromatogram and as milligrams per gram of green sample.

Analysis. Discriminant function analysis (STATISTICA, version 6.0; Stat-Soft Inc., Tulsa, OK) was used to develop classification algorithms distinguishing samples in which buds from individual trees were either unsuitable ($\rho < 0.5$) or suitable ($\rho \geq 0.5$) for budworms. The frequency of sampling and often rapid transition of buds from unsuitable to suitable (Figure 1A) precluded using more than two categories.

We explored several possible discriminant functions relating bud suitability to its terpene profile by dividing the available data into various, mutually exclusive training and test sets. To form a training set, the sample set was first divided into six, ordinal categories based on ρ values between 0 and 1. In order to avoid overrepresentation of extreme values ($\rho = 0.0$ or 1.0) and to ensure adequate

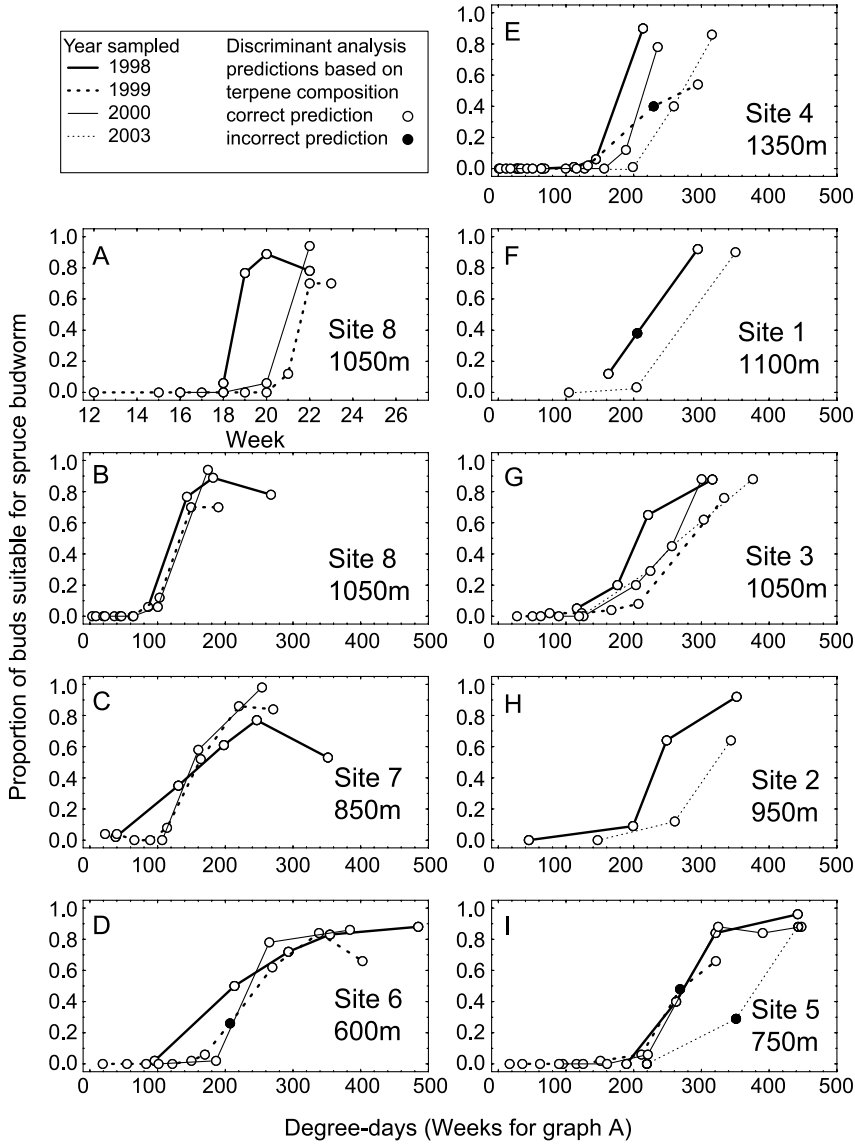

FIG. 1. Seasonal changes in bud suitability, *P*, for western spruce budworm **A** by week at site 8 and **B**–**I** by accumulated degree days above 4°C at all sites between 1998 and 2003. Open symbols indicate correct classifications and closed symbols indicate incorrect classifications using discriminant function B (Tables 2 and 3).

representation of observations throughout the range of ρ values, an equal number of samples was then randomly chosen from each of these six categories. Several discriminant functions were developed and tested as follows:

(1) Function A. Data were stratified by year(s) (1998–2000 vs. 2003) pooled over all sites (Table 2). The training set consisted of an equal number of samples from all sites from the 1998 to 2000 group selected as described above. The test set used all samples from all sites in 2003. The procedure was repeated using 2003 data to compile the training set and all 1998 to 2000 data as the test set. The discriminant functions distinguished suitable and unsuitable buds based upon percent composition of terpenes. Results are presented for each procedure as well as the sum of the two test sets combined. The identical procedures using actual terpene concentrations instead of percent composition of terpenes as the measure returned similar, but slightly less accurate, predictions and are not reported further.
(2) Function B. Data were stratified by sites pooled over all years (Table 2). The training set consisted of samples randomly chosen from four sites (two from each valley) in all years, and the test set was comprised of samples from the remaining four sites in all years. As in (1), the procedure

TABLE 2. CLASSIFICATION FUNCTIONS STRATIFIED BY YEAR OR SITE USING EITHER PERCENT COMPOSITION OF TERPENES OR DEGREE DAYS ABOVE 4°C AS VARIABLES

Classification				Percent correct predictions for test sets[a]	
Function	Stratum	Training set	Test set	Tree	Site by date
Variables are percent composition of terpenes					
A	Year	1998, 1999, 2000	2003	92.7 (179)	100 (18)
A	Year	2003	1998, 1999, 2000	88.7 (1010)	94.2 (120)
A	Year		**Combined**	**89.3 (1189)**[b]	**94.9 (138)**
B	Site	2, 5, 6, 7	1, 3, 4, 8	91.6 (669)	97.3 (73)
B	Site	1, 3, 4, 8	2, 5, 6, 7	82.5 (520)	95.4 (65)
B	Site		**Combined**	**87.6 (1189)**	**96.4 (138)**
Variable is degree days					
C	Year	1998, 1999, 2000	2003	73.7 (179)	72.0 (18)
C	Year	2003	1998, 1999, 2000	79.5 (1010)	81.7 (120)
C	Year		**Combined**	**78.6 (1189)**	**80.4 (138)**
D	Site	2, 5, 6, 7	1, 3, 4, 8	84.6 (669)	89.0 (73)
D	Site	1, 3, 4, 8	2, 5, 6, 7	82.9 (520)	86.2 (65)
D	Site		**Combined**	**83.9 (1189)**	**87.7 (138)**

[a] Percent correct predictions given for indicated test sets by sample tree and by averages per site by date
[b] Sample sizes given in parentheses. Bold figures represent mean for combined sets.

distinguished suitable and unsuitable buds based on percent composition of terpenes. Also, as in (1), the training and test sets were reversed and the procedure was repeated. Results are presented for each procedure as well as the sum of the two test sets combined.

(3) Function C. The same stratification and procedure were used as in Function A except degree-day data rather than terpene composition was used to develop the discriminant function.

(4) Function D. The same stratification and procedure were used as in Function B except degree-day data rather than terpene composition was used to develop the discriminant function.

To evaluate the performance of these discriminant functions at the site by date, rather than the tree level, an average bud suitability, P, and an average classification [unsuitable ($P < 0.5$) or suitable ($P \geq 0.5$)] for each site on each date was calculated from the test sets. These were compared to classifications at the site by date level just as was done for classifying suitability of trees (ρ).

To examine the possibility that fewer terpenes may provide equally good predictive value, training and test sets were developed using Function B, but selecting only the two best terpenes as determined by the stepwise procedure in STATISTICA for prediction.

RESULTS

Foliar Terpenes and Bud Suitability. Classifications using percent terpene composition predicted bud suitability for western spruce budworm correctly more than 80% of the time at the tree level and more than 90% of the time at the site by date level irrespective of whether data were stratified by year (Function A) or by site (Function B) (Table 2). Thus, terpene composition of buds provided highly accurate indicators of bud suitability for western spruce budworms over a wide range of site conditions with all of their attendant seasonal and annual variations in weather.

Analysis of misclassifications of individual samples ($N = 147$) revealed that misclassified samples were not necessarily statistical outliers (i.e., two or more standard deviations from the mean for a given site and date). Of more ecological interest, there was no difference in previous defoliation levels of misclassified and correctly classified samples [mean (SD) percent defoliation 30% (29%) vs. 31% (32%), respectively]. Thus, defoliation by the western spruce budworm does not seem to influence the reliability of terpenes as indicators of bud suitability. This confirms similar results of Chen et al. (2002) and further generalizes the utility of the indicator.

Each of the training sets in Table 2 provides a distinct set of coefficients to discriminate between unsuitable and suitable buds. The most accurate discrimination of bud suitability that we found was Function B using site = 2, 5, 6, 7 as the training set (Table 2). The coefficients for Function B using that training set along with the corresponding mean percent composition of the various terpenes are given in Table 3. To use for predictions, observed percent composition of each terpene is multiplied by its corresponding coefficients in Table 3, one for an unsuitable score and one for a suitable score. These products are summed to provide separate scores for unsuitable and suitable categories. The greater of the two scores is accepted as the prediction (suitable vs. unsuitable). Table 2 indicates the expected accuracy of such a prediction.

Comparison of percent terpene composition between suitable and unsuitable buds (Table 3) provides additional insight into the patterns of particular terpenes as buds change from unsuitable to suitable for western spruce budworm. The two dominant terpenes, α-pinene and β-pinene, reverse their relative positions with α-pinene more abundant in unsuitable buds and β-pinene more abundant in suitable buds. Some of the largest relative differences, however, are in the less dominant terpenes. For example, the percent composition of Δ-3-carene is, on average, almost twice as high in unsuitable than in suitable buds, while percent composition of camphene is almost twice as high in suitable than in unsuitable buds (Table 3).

TABLE 3. COEFFICIENTS FOR DISCRIMINANT FUNCTION B (TABLE 2, TRAINING SET IS SITE = 2, 5, 6, 7) AND MEAN (SD) PERCENT COMPOSITION OF TERPENES IN BUDS THAT WERE UNSUITABLE OR SUITABLE FOR WESTERN SPRUCE BUDWORM[a]

	Coefficients		Mean percent composition (SD)	
Terpene	Unsuitable	Suitable	Unsuitable	Suitable
Tricyclene	–	–	1.4 (0.8)	1.9 (0.9)
α-Pinene	0.902	0.755	35.3 (12.4)	24.3 (4.7)
Camphene	–	–	7.6 (5.5)	11.9 (5.9)
Sabinene	–	–	4.7 (4.2)	4.5 (5.3)
β-pinene	0.704	0.734	25.8 (10.9)	35.6 (9.5)
Myrcene	1.055	0.951	5.0 (3.0)	3.8 (1.8)
Δ-3-Carene	1.309	1.123	2.8 (3.5)	1.4 (1.6)
Limonene	1.123	0.985	10.9 (5.1)	9.7 (4.2)
β-Phellandrene	5.323	4.102	1.2 (0.6)	1.0 (0.2)
Terpinolene	–	–	2.7 (2.2)	1.8 (1.8)
Bornyl acetate	–	–	2.7 (1.9)	4.2 (2.7)
Constant	−39.425	−32.342		

[a] Blanks indicate cases where discriminant function did not utilize that particular terpene.

We found that good predictions could be obtained by using fewer terpenes. The best single terpene to use was α-pinene, a relatively dominant terpene that decreases rapidly in its contribution to total terpenes early in the season as budburst proceeds (Nault, 2003) and buds become suitable to budworms (Table 3, Figure 1). Using training and test sets described under Function B, predictions using α-pinene alone were 78.5% accurate at the tree level and 89.9% accurate at the site by year level compared to 87.6% accurate and 96.4% accurate, respectively, using as many as six terpenes (Tables 2, 3). Further increasing the accuracy of prediction using the single terpene by adding a second or third terpene was not straightforward because the second-best terpene added in stepwise development of the discriminant function varied with the selection of the training set. In other words, one would need to measure α-pinene plus several other candidate terpenes to obtain accuracies approaching those provided by all terpenes. More practically, there is no gain in either efficiency or cost by selecting a subset of terpenes to measure because the collecting, processing, and analytical procedures described above must be followed whether one is measuring one or many terpenes. Therefore, we consider the larger set of terpenes to be the preferred basis for prediction using the coefficients in Table 3.

Influence of Temperature. Classifications based upon accumulated degree days were not as accurate as classifications based on percent terpene composition whether stratified by year or site (Table 2: compare corresponding predictions from Functions A vs. C and B vs. D, respectively). Nonetheless, predictions based on degree days were also acceptably accurate as might be expected by the strong correlation between cumulative degree days and percent composition of individual terpenes (Nault, 2003). Analysis of cases of misclassification at the site by date level revealed that errors using terpenes tended to occur within a narrow range of bud suitability ($0.26 < P < 0.48$) whereas misclassifications using degree days covered almost the entire range ($0.0 < P < 0.94$).

The relative success of using terpene composition over degree days for classification suggests that terpenes are a more sensitive indicator of bud condition, probably because terpene levels are the result of biological integration of many more factors than air temperature alone. Degree days are useful, however, for scaling both changes in terpene composition (Nault, 2003) and bud suitability to budworms under different temperature regimes. For example, expressing changes in bud suitability on a degree-day scale removes much of the year-to-year variation at the site level (Figure 1A vs. B). Similarly, the degree-day scale reduces variation associated with elevation in any single year. However, interesting differences, remain at the site level. Note that within each region (Figure 1B–D and E–I, Okanagan and Nicola Valleys, respectively), there appears to be a negative correlation between degree days required for buds to become suitable and site elevation. In the Okanagan Valley, buds are mostly

unsuitable for budworms until accumulated degree days ≥ 200 in the low elevation site (Figure 1D), whereas almost all buds are suitable at the corresponding number of degree days at the higher elevation (Figure 1B). Similarly, in the Nicola Valley, buds do not become suitable for budworms until degree days ≥ 220 at 750 m (Figure 1I), but are ~50% suitable by that same thermal point at 1350 m (Figure 1E). There are other apparent site-related interactions that reduce the accuracy of using degree days to discriminate groups. Compare, for example, Figure 1B and G, both at 1050 m, but in different regions. Whereas expressing suitability in terms of degree days removes much of the year-to-year variation in site 8 (Figure 1B), there remains considerable year-to-year variation in site 3 (Figure 1G). Despite these complex site-related differences in the relationship between temperature and bud phenology, terpene composition predicted bud suitability to western spruce budworm very well in all sites (compare open vs. closed symbols in Figure 1). The fact that terpene composition tracks these site-to-site differences illustrates the value of this tool in detecting patterns that may be relevant in explaining spatial variation in defoliation caused by the western spruce budworm.

DISCUSSION

The suitability of current-year Douglas-fir buds to western spruce budworm following spring emergence depends upon the state of development of the buds (Shepherd, 1992). The phenology of this suitability varies by site and by date largely as a function of individual tree responses to ambient temperature. Discriminant functions based on percent terpene composition of buds can be used to indicate their biological suitability to budworm both at the tree and site levels. Using foliar chemistry as an indicator of bud suitability is an objective and repeatable method of describing plant phenology. We found that terpene profile was a consistent means of distinguishing buds that were suitable or unsuitable for penetration by the western spruce budworm in spite of considerable variation in site-specific and annual variation in ambient temperature and levels of previous defoliation. Thus, persistent differences in the response of particular trees or in specific site conditions and the effect of these differences on suitability to western spruce budworm can be investigated. Results can be used to provide real-time information to augment hazard-rating systems that are typically based on historical patterns as well as to identify individual trees with desirable phenological characteristics associated with reduced susceptibility to defoliation by the western spruce budworm.

Experimental studies exploring the possible role of terpenes as constitutive or induced plant defenses in the Douglas-fir/western spruce budworm system

have focused on the midseason phenological stages when shoots were elongating and budworms were feeding actively (e.g., Cates and Zou, 1990; Clancy et al., 1993). The results provided equivocal or little evidence of a role for terpenes as plant defenses (Clancy, 2002). The terpene profiles of Douglas-fir shoots during the later feeding stages of the western spruce budworm, however, are relatively stable compared to the dynamic shifts occurring in those profiles earlier in the season when buds are swelling and budworms are just attempting to establish feeding sites (Nault, 2003). Thus, although our results stress the use of terpenes as the indicators, not the causes, of foliage suitability, they may provide insight in the search for trees with lower susceptibility to western spruce budworm by revealing how variation in early-season rates of change in terpene chemistry are associated with host suitability.

Chen et al. (2003) showed the relative susceptibility of Douglas-fir to defoliation by the western spruce budworm was related to differences in the relative phenology of the tree's foliage and the budworm's development. Further, their experimental results indicate that relative susceptibility is determined by early-season phenology; once buds were penetrated by the insects, differences between susceptible and resistant trees was reduced or reversed. Our results show that these phenological differences among trees that determine host suitability for the western spruce budworm can be related directly to the terpene profile of individual trees early in the growing season. The relative phenology of bud development, and, therefore, susceptibility to western spruce budworm can be ascertained easily for individual trees, irrespective of their defoliation history—indeed, even in the absence of defoliation. This could provide an objective basis upon which to select desirable developmental characteristics of trees because both terpene characteristics and phenological development have a strong heritable component (von Rudloff and Rehfeldt, 1980; Chen et al., 2003).

The use of terpenes as indicators also has an application in population studies. Surveys of terpene profiles of trees within sites could identify the degree of homogeneity of particular phenological patterns in the host population. The degree of homogeneity in budburst phenology in combination with measures of population rates of change in budworms or severity of defoliation provides a direct link between weather, host–plant relationships, and outbreak dynamics. Similarly, known geographic differences in terpene profiles (von Rudloff and Rehfeldt, 1980) could help interpret large-scale patterns of outbreak behavior. We found systematic differences in the thermal requirements for buds to become suitable to budworm on trees at different elevations. If these differences reflect local adaptations by trees to ambient climate, then the relationship between insect outbreak behavior and weather may be mediated by the response of the host tree. The indicator provided by changes in the profile of terpenes makes this pathway tractable.

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VARIATION IN BIRCH (*Betula pendula*) SHOOT SECONDARY CHEMISTRY DUE TO GENOTYPE, ENVIRONMENT, AND ONTOGENY

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Abstract—Plant secondary chemistry is determined by both genetic and environmental factors, and large intraspecific variation in secondary chemistry has frequently been reported. The heritability of specific tree secondary metabolites is, however, mostly unknown. We tested the effect of plant genotype, environment, and ontogeny on the variation in shoot secondary chemistry of juvenile and mature European white birches (*Betula pendula*). Phenolic compounds and triterpenoids were analyzed in 30 naturally regenerated 20-year-old parental trees and micropropagated plantlets that originated from 14 of those same parental trees, planted at four growing sites. Most of the variation for phenolic compounds was explained by differences between parental trees, whereas triterpenoids had a high variation both between parental trees and within the canopy of individual tree. The effect of ontogeny was strong for some individual compounds. In mature trees, the amount of triterpenoids was less than 1 mg/g (DW), whereas the concentration in juvenile plantlets was up to 64 mg/g (DW). Clonal plantlets and parental trees were generally quite similar in their phenolic contents, but there were significant differences for all analyzed compounds among clones. Environment had no significant effect on the accumulation of some compounds,

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whereas for others, a significant environmental effect and/or significant genotype by environment interaction was found. These results suggest that birch shoot secondary chemistry is under strong genetic control and that the environmental effects depend on the studied chemical trait.

Key Words—Birch, clonal plantlets, clonal repeatability, condensed tannins, flavonoids, intraspecific variation, natural population, triterpenoids.

INTRODUCTION

Most plant secondary metabolites are thought to enhance the prospects for survival of the producer or its offspring through complex interactions with the environment (Waterman, 1992). The effect of environment on secondary chemistry has been considered to be of prime importance, as suggested by many ecological hypotheses such as the carbon nutrient balance hypothesis (see Bryant et al., 1983), the growth differentiation hypothesis (Herms and Mattson, 1992), and the protein competition model (Jones and Hartley, 1999). Although the role of environmental and genetic control of plant secondary chemistry is of major importance, few studies have concentrated on this question in forest trees. Hanover (1966) studied the heritability of five terpenes present in pine, and Orians et al. (1996) studied heritability of two phenolics in *Salix* (see also Hamilton et al., 2001). The reason for the paucity of studies on the heritability of tree secondary compounds might be the need for time-consuming field experiments and the complicated chemical analyses.

When birch species (*Betula* spp.), and even individuals within a species, have been compared, a high inter- and intraspecific variation in the types and concentrations of secondary compounds in leaves has been found (Keinänen et al., 1999 a,b; Laitinen et al., 2000, 2002; Salminen et al., 2002; Valkama et al., 2003). Similar results have also been reported in studies of bark chemistry, where different birch species have been compared (Taipale et al., 1994; Julkunen-Tiitto et al., 1996). Variations in the amounts of secondary compounds within birch species are known to be induced by both biotic and abiotic environmental factors such as defoliation, available nutrients, UV-light, temperature, etc. (Keinänen et al., 1999a; Tegelberg et al., 2001, 2002; Kuokkanen et al., 2001, 2003). However, in previous studies, the high phytochemical variation in naturally regenerated individual European white birch trees (Laitinen et al., 2000), and bark secondary chemistry among birch seedlings, were suggested to be mainly controlled by genotype (Tahvanainen et al., 1991). Accordingly, our previous study indicated large clonal differences among 1-yr-old greenhouse-grown plantlets, suggesting that intraspecific variation in bark phenolic chemistry could be explained, to a large extent, by genetic differences (Laitinen et al., 2004). However, the high variation in terpenoid

chemistry among clones and plantlets of the same clone suggests that for some genotypes, the environment might also modify the secondary chemistry and, thus, affect the resistance of birch against browsing of mammals (Laitinen et al., 2004).

During plant growth and ontogenetic development, secondary chemistry can change considerably. Generally, juvenile stages express stronger defenses than the mature stage (Bryant and Julkunen-Tiitto, 1995). At the birch population level, this would mean even more variation in secondary chemistry, which allows populations to adapt to different kinds of stresses. However, the causes of variation in chemistry within a natural population of *Betula pendula* are still scanty.

In the present study, we used samples from mature birch trees from our long-term study population (see Laitinen et al., 2000, 2002) and micropropagated clonal plantlets from the same trees. Clonal plantlets were grown in four experimental sites, to study the effect of genotype, environment, and ontogeny on the chemistry of *B. pendula*. The objectives were to: (1) examine variation in shoot secondary chemistry within a naturally regenerated population among individual trees and within a tree, (2) determine the degree to which genotype and environment affect birch shoot secondary chemistry, (3) estimate the heritability of secondary chemistry by studying clonal repeatability, (4) test how individual clones differ in their response to the environment, and (5) compare parental trees to clonal plantlets of the same trees in order to quantify the effect of ontogeny.

METHODS AND MATERIALS

Plant Material and Growing Conditions. A naturally regenerated birch (*B. pendula*) stand in Punkaharju (southeastern Finland; 61°48′N, 29°18′E) was selected for long-term studies on genetic variation within a natural population. From this stand, a random sample of 30 *B. pendula* trees were selected (for details, see Laitinen et al., 2000). The aim was to micropropagate 300 plantlets from each tree. In some cases, the micropropagation was not successful, which limited the number of clones in this study to 14. The identification numbers of the available trees and clones were 3, 4, 5, 6, 9, 15, 16, 17, 18, 19, 20, 24, 25, and 30. These are the same parent trees and clones that were also used in our previous studies (see Laitinen et al., 2000, 2002, 2004).

Parental trees were about 20-yr-old at the time of micropropagation. Small twigs were taken from the upper third of the trees, twice in June and once in August 1997, to get material for micropropagation. In the beginning of April 1998, micropropagated plantlets were transferred from laboratory cultures into 0.021 l plastic pots (EK 144) that contained perlite, unfertilized peat, and birch forest soil (2:2:1). Plantlets were grown in a greenhouse, where plantlets from

each clone were divided onto three tables in which the pots of all clones were placed randomly.

In May 1998, plantlets were transferred into 49 × 30 cm plastic containers (EK 28). Each container contained 28 pots of 0.28 l each, and each pot was filled with prefertilized commercial peat (VAPO, Finland). On the July 9, 1998, plantlets were transferred to an open-air exposure field in Punkaharju. Plantlets were watered as needed and fertilized once (April 22) with 0.1%, and six times (May 27, June 17 and 24, and July 1, 8, and 15) with 0.2% Kekkilä 9-Superex (N 19.4%, P 5.3%, K 20%) fertilizer. At the end of October 1998, winter-hardened plantlets were transferred to an unheated greenhouse where the roots were protected from freezing.

Plantlets for this study were planted in June 1999 into one of three growing sites in Punkaharju (Kuikanniitty 61°47′N, 29°21′E, 79 m above sea level, Putikko 61°43′N, 29°26′E 98 m above sea level, and Vaahersalo; 61°47′N, 29°17′E, 95 m above sea level) and one site in Parikkala (Parikkala 61°36′N, 29°36′E, 93 m above sea level), in southeastern Finland. The sites at Kuikanniitty and Vaahersalo were abandoned cultivated fields, whereas Parikkala and Putikko were *Myrtillus* forest-type areas. The soil type was defined as fine sandy till at all growing sites.

Plantations were established using a randomized complete block design. At each of the growing sites, there were six to nine blocks. In each block, four replicates (plantlets) of each clone were planted. A random selection of six blocks per site and one plantlet per clone per block (= six samples per clone per growing site) was made. A current year's growth from the first branch, from the top of clonal plantlets, and five branches of current year's growth from the upper third part of parental trees were collected on June 26, 2001. Branches were air-dried and stored at −20°C for chemical analysis.

Extraction of Compounds. Both phenolics and triterpenoids were analyzed from the same extract. For extraction, leaves were removed from study branches, and the current year's growth (≈ 10–15 cm) was cut into 2-mm pieces and weighed. Methanol (20 ml; 100%) was added, the sample was allowed to stand for 20 min on ice (≈ +4°C), and homogenized for 2 min using an Ultra-Turrax clipping homogenizer. The extraction was repeated ×2 with 15 ml of methanol and once more with 15 ml of diethyl ether for complete extraction of triterpenoids. The residue was washed with methanol. Extracts and washings were filtered and combined, and the solvents removed by vacuum evaporation. Each sample was redissolved in 10 ml of 100% methanol, and aliquots of 2 × 1, 1 × 2, and 1 × 3 ml were evaporated to dryness under nitrogen. Different aliquots were taken to ensure suitable concentrations for both HPLC-DAD and HPLC-MS runs. Dried samples were stored at −20°C for analysis by HPLC-DAD and HPLC-MS.

HPLC-DAD and HPLC-MS Analysis. Quantitative analysis of the low molecular weight phenolics (LMWP) in stem samples was done by HPLC using

gradient elution and diode array detection (DAD) as previously described by Laitinen et al. (2000). The wavelengths used for detection were 220, 270, 280, 320, and 360 nm. Identification of quercetin derivatives, 3,4′-dihydroxypropiophenone-3-β-D-glucopyranoside (DHPPG), caffeoyl quinic acids, cinnamic acid derivatives, and flavonoid aglycones, was based on comparison of HPLC retention times and spectral characteristics described in Keinänen and Julkunen-Tiitto (1998) and Julkunen-Tiitto et al. (1996). Secondary metabolites were quantified against commercial standards: salicin (Roth, Karlsruhe, Germany) for salidroside, DHPPG, rhododendrin, and platyphylloside; (+)-catechin hydrate (Aldrich Chemical Company, USA) for catechin derivatives; chlorogenic acid (Aldrich, Steinheim, Germany) for all phenolic acids, quercetin-3-*O*-glucoside (Extrasynthèse, Genay, France) for all quercetin derivatives; gallic acid (Aldrich) for gallotannins, and apigenin (Roth) for flavonoid aglycones. Before HPLC-DAD analysis, dried 1 ml phenolic samples were redissolved in 0.4 or 0.6 ml water/methanol (1:1), depending on sample concentration. Quantitative analysis of triterpenoid components was done by HPLC-MS. Isocratic elution was used with EtOH (94%): aqueous 1.5% tetrahydrofuran + 0.25% acetic acid (75:25, pH 5.4). HPLC/atmospheric pressure electrospray ionization (HPLC/API-ES) conditions were as follows: the column was a Hypersil RP C-18, 2-mm ID × 10 cm, the ES fragmentor voltage was 100 V, and the flow rate was 0.2 ml/min. Before HPLC-MS analysis, dried 1 ml terpenoid samples were redissolved in 5 ml clonal plantlets, and dried 3-ml samples were redissolved in 3 ml parental trees, 94% ethanol. Triterpenoid components were quantified by using purified papyriferic acid, which was kindly supplied by Prof. Paul Reichardt, University of Alaska, USA. The concentrations of pendulic acid were considered to be relative because papyriferic acid was used as a standard.

In our figures, phenolic acids are divided into two groups: neochlorogenic acid and chlorogenic acid, and six chlorogenic acid derivatives were grouped together as caffeoyl quinic acids, and three *p*-hydroxycinnamic acid-like compounds were grouped as total cinnamic acid derivatives. Catechin derivatives were combined as the total of four components [(+)-catechin and three catechin derivatives]. Total quercetin derivatives contained quercetin-3-galactoside, quercetin-3-glucuronide, quercetin-3-glucoside, quercetin-3-arabinoside, and four unknown quercetin derivatives. The flavonoid aglycone group contained several components, some of which could not be resolved in all samples by our method. Apigenin and one luteolin derivative were counted together, as was also done for chrysoeriol and one other luteolin derivative. In addition, a third luteolin derivative and two apigenin derivatives were included in the flavonoid aglycone group. Total low molecular weight phenolics (LMWP) include all of the analyzed phenolics, except condensed tannins. For triterpenoids, the papyriferic acid and four papyriferic acid derivatives were grouped as total papyriferic acid derivatives, and two pendulic acid derivatives were grouped to represent

the total of pendulic acid derivatives. Total triterpenoids include all analyzed triterpenoids.

Analysis of Condensed Tannins. Condensed tannins were analyzed from stem extract by an acid butanol assay as previously described in Porter et al. (1986). Quantification of tannins was based on purified *B. nana* leaf tannin.

Statistics. For evaluating chemical similarity in phenolic composition between parental trees within a population, a classification of parental trees to putative chemotypes was made by UPGMA clustering as previously described in Laitinen et al. (2000). Mean values of five samples per tree of all analyzed phenolic compounds were used for UPGMA-clustering analysis.

Due to nonnormality and heterogeneity of variances, the concentrations of the following compounds were transformed for statistical tests: a square-root ($x + 1$) transformation was used for parental tree concentrations of quercetin-3-glucuronide + quercetin-3-glucoside; a natural logarithm ($x + 1$) transformation of concentration was used for salidroside, chlorogenic acid, and quercetin-3-galactoside; a natural logarithm ($x + 0.5$) transformation of concentration was used for rhododendrin, quercetin-3-arabinoside, and quercetin derivative 3; and a natural logarithm ($x + 0.1$) transformation of concentration was used for platyphylloside, gallotannins, and all terpenoid compounds and compound groups. Data from micropropagated plantlets were treated by a square-root transformation for concentrations of quercetin-3-glucuronide + quercetin-3-glucoside. A natural logarithm of concentration + 1 transformation was used for papyriferic acid and total pendulic acids; a natural logarithm of concentration + 0.5 was used for DHPPG, catechin, chlorogenic acid, quercetin-3-galactoside, quercetin-3-arabinoside, and flavonoid aglycones, and a natural logarithm of concentration + 0.1 was used for gallotannins and platyphylloside.

Differences in the amount of chemical compounds or compound groups among parental trees, growing sites, and clones were tested by analysis of covariance (ANCOVA). For analysis of chemical data between clones and growing sites, the following model was used: site (S), clone (C), S × C, S × block (B), weight of the current year's growth was used as covariate. Growing site and clone were treated as fixed factors, and block was used as a random factor. Weight was used as a covariate to remove an additional factor that may give rise to variation, i.e., the wood-to-bark ratio, which varies with different sizes of branches. A sequential Bonferroni correction was used for evaluating the results of all ANCOVA tests for parental trees and for each growing site (Rice, 1989).

A variance component analysis was used to evaluate the effect of genotype on total chemical variation. For clonal plantlets, the observed phenotypic variance (V_P) can be partitioned into genotypic variance (V_G) and environmental variance (V_E), i.e., $V_P = V_G + V_E$. In the case of clonal material, plantlets within a clone have an equal genetic constitution, thus, the within clone, variance component is due to variation in the environment or an error in sampling and

measuring (Falconer, 1989). The between-clone component of variance is an estimate of V_G. The total genotypic variance component contains both the additive and nonadditive components, which cannot be separated by using clonal material. Thus, the extent to which individual phenotype is determined by genotype was expressed by the degree of genetic determination, i.e., heritability in a broad sense = V_G / V_P (Falconer, 1989). When studying clonal plantlets, there are environmental effects included in V_G, because some part of the environmental differences between parental plants may be transmitted to all of their clonal descendants. When the degree of genetic determination is estimated by using clones, the ratio V_G / V_P is an overestimate and, strictly speaking, it should be referred to as clonal repeatability as suggested by Falconer (1989, p. 128). Thus, the term clonal repeatability was also used in this study. The estimation of variance components was computed for all compounds and compound groups (varcomp procedure, REML), and the results are presented as variation between parental trees with 95% confidence limits (Searle, 1997) and as clonal repeatability ± SE (Falconer, 1989; Dickerson, 1969) for clonal plantlets. All figures are based on nontransformed data. All statistical analyses were made using SPSS 10.1.0, SPSS Inc., 1989–2000.

RESULTS

Chemical Variation among Parental Trees and within Individual Trees. There was significant variation among parental trees for all analyzed compounds and compound groups (univariate analysis of variance with sequential Bonferroni correction, $P < 0.001$) (Table 1 and Figures 1–5). The differences among parental trees accounted for most of the variation in chemical concentrations for phenolics, whereas for triterpenoid groups, pendulic acid derivatives, and total terpenoids, the variation within an individual parental tree was greater than that among parental trees (Table 1). For individual phenolic compounds, in many cases, the variation among trees was large. For example, in the case of (+)-catechin, the mean concentration of tree 25 was only 1.1 mg/g (DW), whereas several other trees contained more than 5 mg/g (DW) of (+)-catechin (Figure 3). With respect to chlorogenic acid, the differences among trees were more than 10-fold and nearly fivefold for salidroside (Figure 3).

Four distinct chemotypes were found among the 30 study trees by qualitative UPGMA clustering (Figure 1, groups 1–4). In the chemotype grouping (Figure 1), (+)-catechin and chlorogenic acid (compounds 7 and 8) were the main compounds in groups 1 and 2. One chlorogenic acid derivative (compound 6) was lacking from group 2; also, other chlorogenic acid derivatives (compounds 27 and 28) were lacking or only present in trace amounts. In group 3,

TABLE 1. RESULTS FOR ANALYZED COMPOUND GROUPS AND INDIVIDUAL COMPOUNDS: F-VALUES AND SIGNIFICANCE LEVELS WITH SEQUENTIAL BONFERRONI CORRECTION FOR TREE (PARENTAL TREES) BY ANALYSIS OF COVARIANCE, AND RESULTS OF VARIANCE COMPONENT ANALYSIS FOR VARIATION BETWEEN PARENTAL TREES AND BETWEEN CLONES

	Parental trees				Clonal plantlets	
Variable	*F*(tree)	*F*(weight)	Variation between trees	95% Confidence limits	Clonal repeatability	±SE
Compound groups						
Catechin derivatives	14.45***	1.87[a]	0.73	0.60, 0.84	0.127	0.07
Caffeoyl quinic acid derivatives	31.35***	0.19[a]	0.86	0.78, 0.92	0.600	0.25
Cinnamic acid derivatives	18.82***	1.12[a]	0.78	0.67, 0.87	0.721	0.29
Quercetin derivatives	11.21***	3.87[a]	0.67	0.53, 0.80	0.315	0.14
Flavonoid aglycones	9.31***	0.02[a]	0.62	0.47, 0.77	0.247	0.11
Gallotannins	7.53***	35.76***	0.55	0.38, 0.71	0.231	0.11
Condensed tannins	8.59***	17.24**	0.60	0.45, 0.75	0.246	0.11
LMWP	15.85***	1.63[a]	0.75	0.63, 0.85	0.121	0.06
Total terpenoids	4.83***	1.57[a]	0.29	0.13, 0.48	0.445	0.20
Individual phenolic compounds						
(+)-Catechin	14.35***	11.71*	0.73	0.60, 0.84	0.121	0.07
Chlorogenic acid	62.75***	4.06[a]	0.88	0.80, 0.93	0.603	0.25
Quercetin-3-galactoside	10.83***	3.95[a]	0.65	0.51, 0.79	0.503	0.21
Quercetin-3-glucoside + quercetin-3-glucuronide	31.06***	4.42[a]	0.84	0.75, 0.91	0.749	0.30
Quercetin-3-arabinoside	11.20***	8.92[a]	0.66	0.52, 0.80	0.332	0.14
Quercetin derivative 3	18.68***	0.15[a]	0.78	0.67, 0.88	0.496	0.20
DHPPG	[b]	[b]	[b]	[b]	0.739	0.30
Rhododendrin	7.17***	15.81**	0.47	0.30, 0.65	0.352	0.16
Salidroside	19.27***	0.63[a]	0.76	0.64, 0.86	0.243	0.12
Platyphylloside	23.58***	9.71*	0.71	0.57, 0.83	0.190	0.09
Individual triterpenoids						
Papyriferic acid	12.06***	2.07[a]	0.68	0.54, 0.81	0.475	0.20
Papyriferic acid derivatives	12.06***	2.07[a]	0.68	0.54, 0.81	0.443	0.19
Pendulic acid derivatives	4.08***	2.09[a]	0.27	0.12, 0.47	0.357	0.17

Notes: Sample weight was used as a covariate. Results of variance component analysis for variation between parental trees and between clones are presented as a proportion of total variation with 95% confidence limits for parental trees and as a clonal repeatability = V_G / V_P ± SE for clonal plantlets. Total variation (parental trees) = variation between trees + error (variation within a tree). Total variation (V_P) (clonal plantlets) = variation between clones (V_G) + variation due to clone × site and site × replicate interaction + error (variation within a clone).

[a] NS: Not significant.

[b] DHPPG was only found in tree 17.

*$P < 0.05$.

**$P < 0.01$.

***$P < 0.001$.

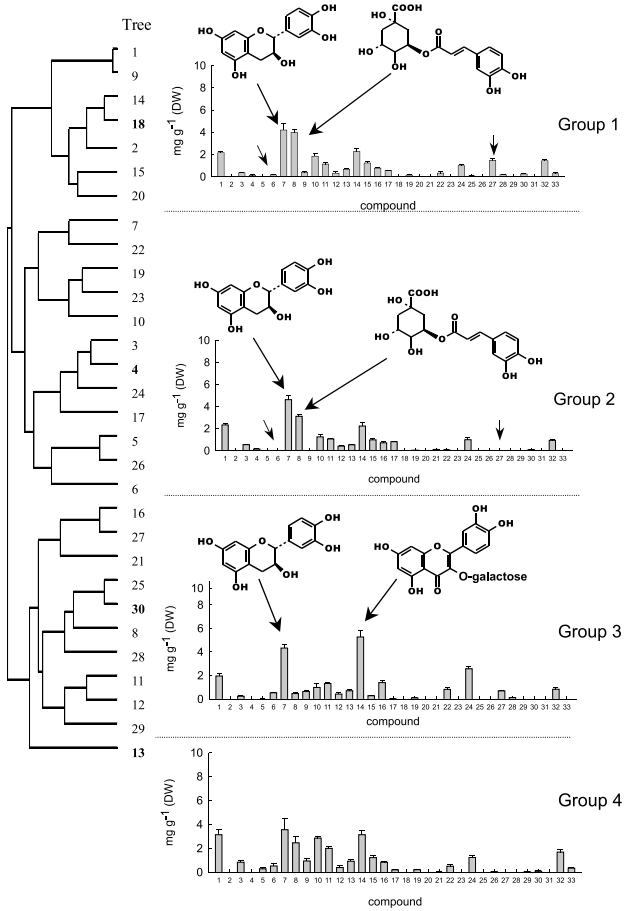


FIG. 1. UPGMA dendrogram for the chemical similarity in parental trees. Trees with an example chemical profile are marked with bold numbers. The chemical structures of main compounds used for defining different chemotypes are shown: for groups 1 and 2, (+)-catechin and chlorogenic acid; for group 3, (+)-catechin and quercetin-3-galactoside. Small arrows indicate the other compounds, which aided to define different chemotypes. Individual compounds are listed in order of retention times to illustrate the HPLC profile. Analyzed compounds that were used in UPGMA clustering are: 1 = salidroside; 2 = DHPPG; 3 = neochlorogenic acid; 4, 5, and 9 = *p*-OH-cinnamic acid derivatives; 6, 17, 21, 25, 27, and 28 = chlorogenic acid derivatives; 7 = (+)-catechin; 8 = chlorogenic acid; 10 = rhododendrin; 11, 12, and 13 = catechin derivatives; 14 = quercetin-3-galactoside; 15 = quercetin-3-glucoside + quercetin-3-glucuronide; 16 = quercetin-3-arabinoside; 18, 20, 23, and 24 = quercetin derivatives; 19 = pentagalloylglucose; 22 = platyphylloside; 26 and 29 = gallotannin derivatives; 30 = apigenin + luteolin derivative; 31 = chrysoeriol + luteolin derivative; 32 and 33 = apigenin derivatives.

the (+)-catechin and quercetin-3-galactoside were the main compounds. Tree 13 was separated from all other trees. It had no clear main compounds; that is, salidroside, (+)-catechin, chlorogenic acid, rhododendrin, catechin derivative 1, and quercetin-3-galactoside (compounds 1, 7, 8, 10, 11, and 14 in Figure 1) were all found in moderate concentrations. Minor compounds, such as p-OH-cinnamic acid derivatives, catechin derivatives, and platyphylloside, were present in all chemotype groups.

Clonal Variation in Chemistry: Effects of Genotype and Environment. There were significant differences among clones for all analyzed compounds and compound groups (Figures 2–5). For compound groups such as cinnamic acid derivatives, total quercetin derivatives, flavonoid aglycones, and gallotannins (Figure 2), and for individual compounds such as quercetin derivatives, DHPPG, and platyphylloside (Figures 3 and 4), there were significant differences only among clones; that is, there was no detected environmental effect in these cases. Especially high variation among clones for individual compounds was found for quercetin-3-glucoside + quercetin-3-glucuronide (these compounds could not be separated by our analytical method); the concentrations varied from 0.1 mg/g (DW) in clone 25 to 1.1 mg/g (DW) in clone 19. For DHPPG, the concentrations varied from 0 mg/g (DW) in clone 3 to 2.1 mg/g (DW) in clone 5 (Figures 3 and 4).

When the degree of genetic determination for the birch shoot secondary chemistry was calculated by the variance component analysis for compound groups, the highest values for clonal repeatability were found for caffeoyl quinic acids and cinnamic acid derivatives (Table 1). The genetic control of birch shoot secondary chemistry differed greatly among individual compounds or compound groups, as shown by high variation in repeatability values. For individual compounds, the clonal repeatability was highest (over 0.5) for chlorogenic acid, quercetin-3-glucoside, quercetin-3-glucoside + quercetin-3-glucuronide, and DHPPG (Table 1).

Significant environmental effects on some of the analyzed chemical groups were detected for the clonal plantlets, namely, for catechin derivatives, caffeoyl quinic acids, condensed tannins, and LMWP (Figure 2), and for total pendulic acid derivatives and total triterpenoids (Figure 5). For individual compounds, significant differences among growing sites were found for (+)-catechin, chlorogenic acid, rhododendrin, and salidroside (Figures 3 and 6a). For example, the mean concentration for (+)-catechin varied from 1.2 mg/g (DW) in Kuikanniitty to 2.7 mg/g (DW) in Vaahersalo (Figure 3). The minimum and maximum mean concentrations for chlorogenic acid were 0.83 mg/g (DW) in Kuikanniitty and 1.26 mg/g (DW) in Parikkala, respectively. Similar results were obtained for rhododendrin.

There was significant site × clone interaction for all individual terpenoid compounds and for total terpenoids (Figure 5), as well as for two of the phenolic

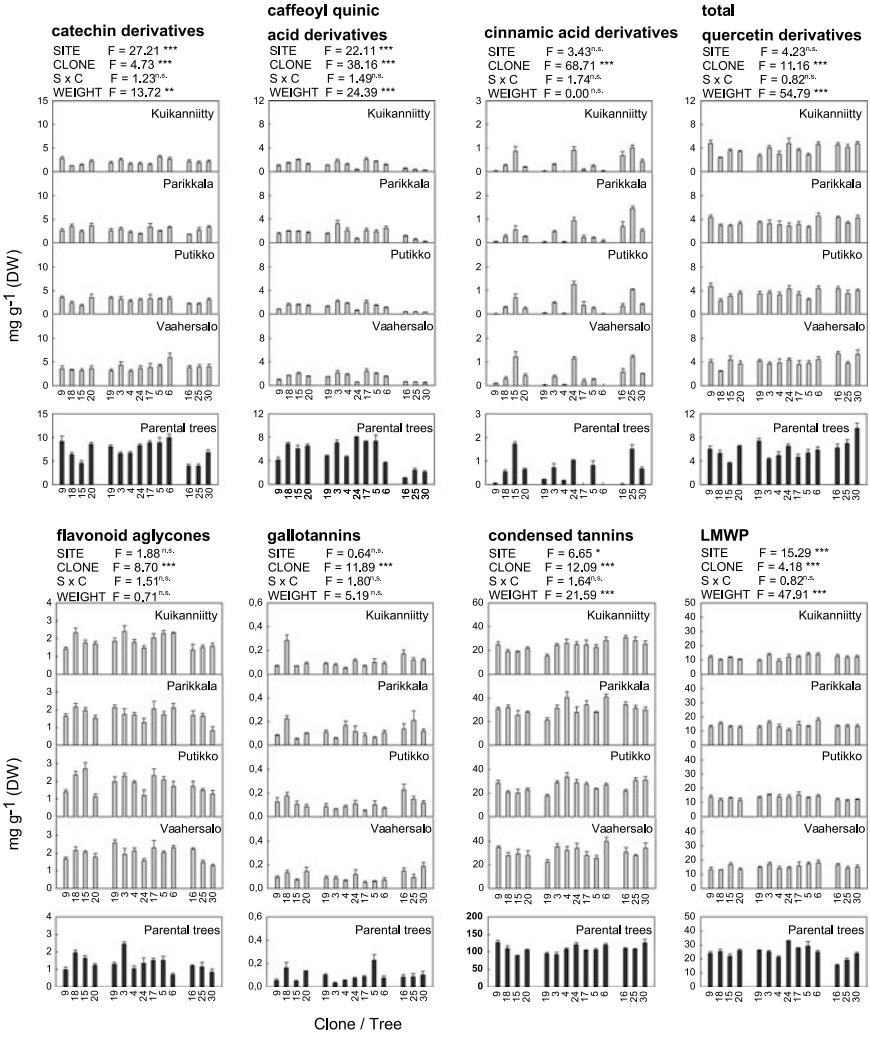


FIG. 2. Concentrations per parental tree or clone for analyzed compound groups. For parental trees, an average ± SE of five stem samples was used, and for individual clones, an average ± SE of six stem samples was counted for each growing site. Results are expressed as milligrams per gram of dry weight (DW) of plant material. Trees and clones are grouped by chemotype (see UPGMA clustering in Figure 1).

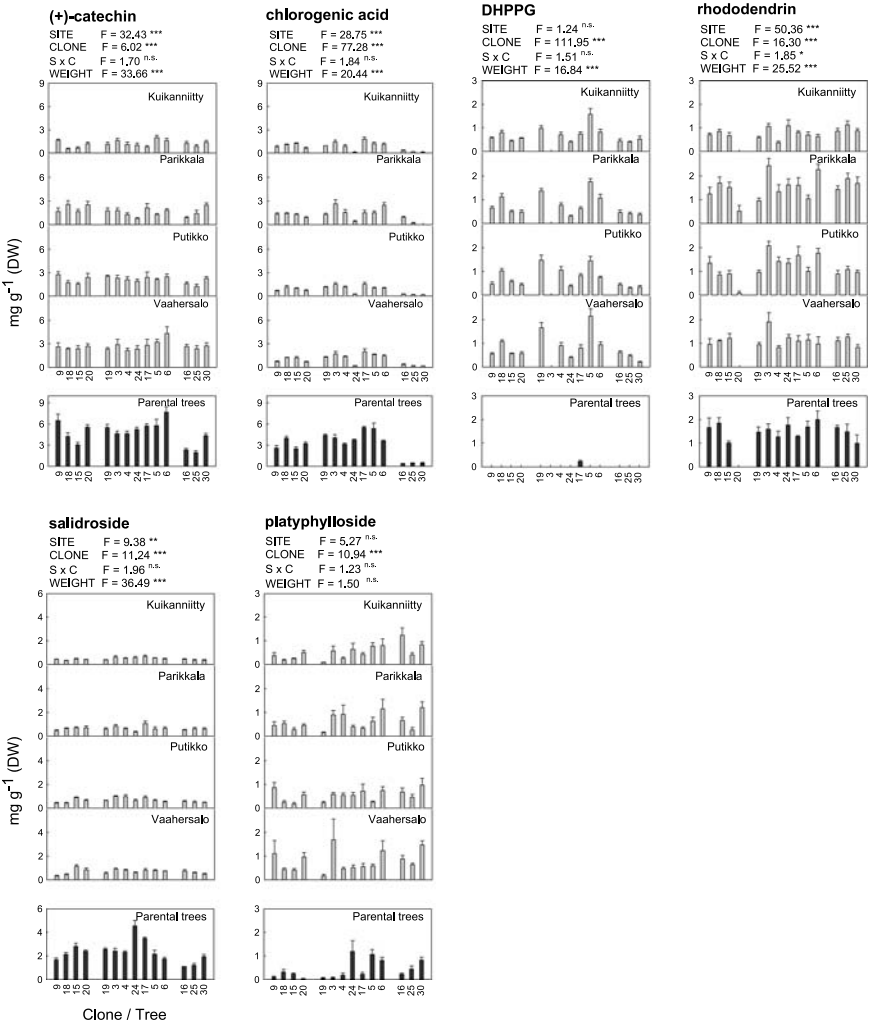


FIG. 3. Concentrations per parental tree or clone for main phenolic compounds: (+)-catechin, chlorogenic acid, DHPPG, rhododendrin, salidroside, and platyphylloside. For parental trees, an average ± SE of five stem samples was used, and for individual clones, an average ± SE of six stem samples was counted for each growing site. Results are expressed as millgrams per gram of dry weight (DW) of plant material. Trees and clones are grouped by chemotype (see UPGMA clustering in Figure 1).

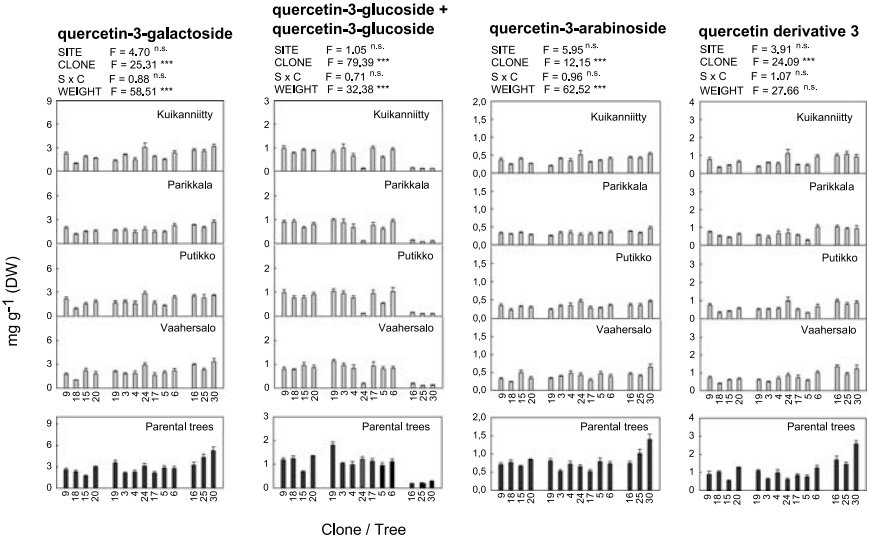


FIG. 4. Concentrations per parental tree or clone for main phenolic compounds: main quercetin derivatives. For parental trees, an average ± SE of five stem samples was used, and for individual clones, an average ± SE of six stem samples was counted for each growing site. Results are expressed as milligrams per gram of dry weight (DW) of plant material. Trees and clones are grouped by chemotype (see UPGMA clustering in Figure 1).

compounds, rhododendrin, and salidroside (Figure 3). This indicates that the response of individual clones to differences in growing environment differed among clones; that is, the environmental sensitivity was dependent on individual plant genotype (Figure 6b).

Comparison between Parental Trees and Clonal Plantlets. In both developmental stages of birches, the main phenolic compound group was condensed tannins (Figure 2). The greatest difference in chemical quality between parental trees and clonal plantlets was found in triterpenoids (Figure 5). The main phenolic compounds in the shoots of parental trees and in the shoots of young plantlets were salidroside, (+)-catechin chlorogenic acid, and quercetin-3-galactoside (Figures 1–4). For phenolics in general, the chemical profile was similar in parental trees and in their micropropagated plantlets. Two triterpenoid compounds, papyriferic acid, and one pendulic acid derivative were found in samples from parental trees (Figure 5), whereas in clonal plantlets, papyriferic acid and four papyriferic acid derivatives were found. In addition, two pendulic acid derivatives were found only in young shoots of micropropagated plantlets.

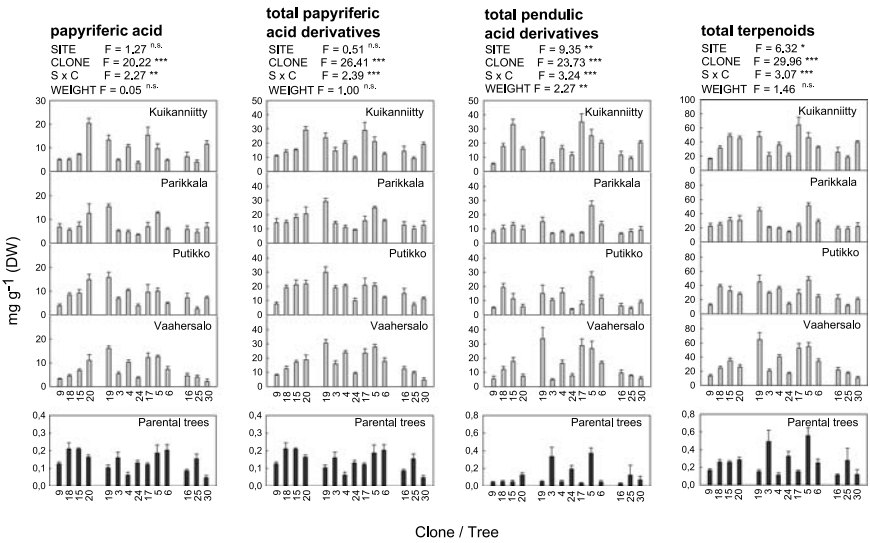
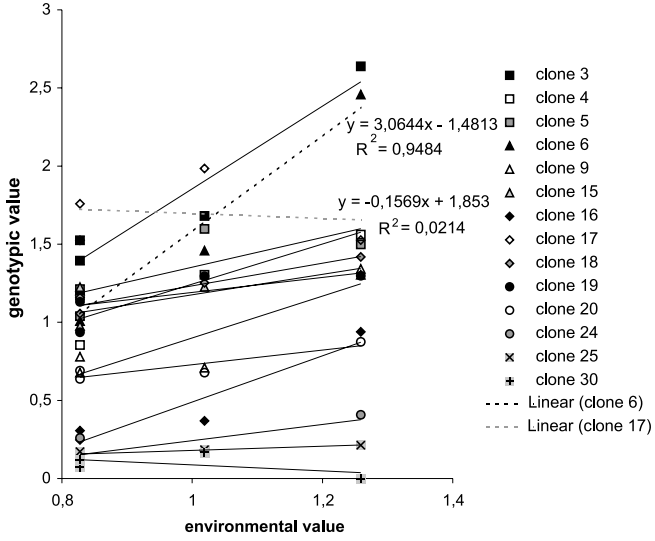


FIG. 5. Concentrations per parental tree or clone for papyriferic acid and triterpenoid groups. For parental trees, an average ± SE of five stem samples was used, and for individual clones, an average ± SE of six stem samples was counted for each growing site. Results are expressed as milligrams per gram of dry weight (DW) of plant material. Trees and clones are grouped by chemotype (see UPGMA clustering in Figure 1).

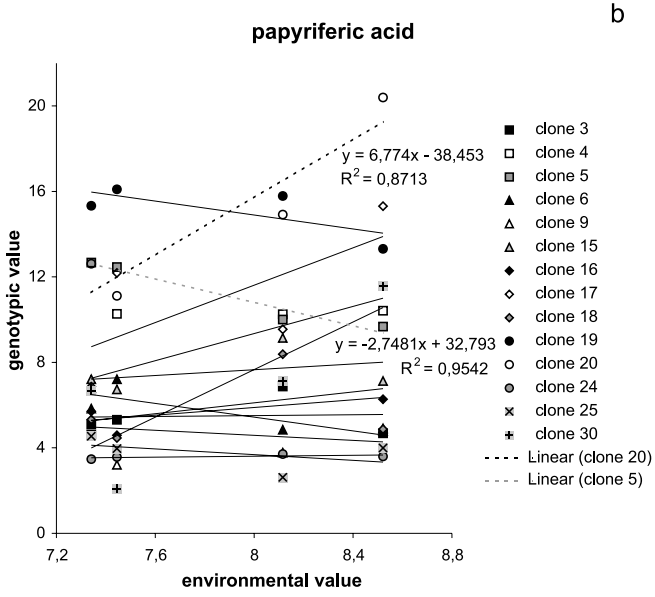
Quantitatively, there were great differences in secondary chemistry between parental trees and clonal plantlets. This difference can be seen when the results from phenolic compound groups are compared. The amount of condensed tannins in parental trees was twice the amount found in clonal plantlets (Figure 2). Also, the concentrations of catechin derivatives and caffeoyl quinic acid derivatives were clearly higher in parental trees. For

FIG. 6. Environmental sensitivities of individual clones. Environmental values are expressed as an average of all analyzed samples within a particular growing site, and genotypic values are expressed as an average of samples from one clone in a particular growing site. The value of each genotype is plotted against the environmental mean of all genotypes to determine differences in the environmental sensitivities of different genotypes. a Environmental values are 0.83 mg g^{-1} (DW) for Kuikanniitty (KU) and Putikko (PU), 1.02 mg g^{-1} (DW) for Vaahersalo (VA), and 1.26 mg g^{-1} (DW) for Parikkala (PA). b environmental values are 7.34 mg g^{-1} (DW) for PA, 7.44 mg g^{-1} (DW) for VA, 8.12 mg g^{-1} (DW) for PU, and 8.52 mg g^{-1} (DW) for KU. Linear regressions are presented as lines for all clones. The equation and R^2 values are presented for those clones differing mostly from each other.

a


chlorogenic acid
genotypic value
environmental value
y = 3,0644x - 1,4813
R2 = 0,9484
y = -0,1569x + 1,853
R2 = 0,0214
clone 3
clone 4
clone 5
clone 6
clone 9
clone 15
clone 16
clone 17
clone 18
clone 19
clone 20
clone 24
clone 25
clone 30
Linear (clone 6)
Linear (clone 17)

b


papyriferic acid
genotypic value
environmental value
y = 6,774x - 38,453
R2 = 0,8713
y = -2,7481x + 32,793
R2 = 0,9542
clone 3
clone 4
clone 5
clone 6
clone 9
clone 15
clone 16
clone 17
clone 18
clone 19
clone 20
clone 24
clone 25
clone 30
Linear (clone 20)
Linear (clone 5)

triterpenoid concentrations, the difference between parents and clonal plantlets was even more pronounced (Figure 5). All parental trees had less than 1 mg/g (DW) of terpenoids in their current year growth. In clonal plantlets, the amounts of terpenoids varied from 10.2 mg/g (DW) (clone 30 in Vaahersalo) to 64.6 mg/g (DW) (clone 19 in Vaahersalo) (Figure 5).

When individual phenolic compounds such as (+)-catechin, chlorogenic acid, and salidroside were analyzed, the concentrations were clearly higher in samples from parental trees than in samples from clonal plantlets. On the contrary, DHPPG was only found in low concentration in samples from one of the parental trees (tree 17), whereas 0.2–2.1 mg/g (DW) was found in all clones except clone no. 3 (Figure 3).

DISCUSSION

Between parental trees, a high variation in birch shoot secondary chemistry was found. Variation within an individual tree and between branches at the same level of tree canopy was low for most of the studied compounds and compound groups (Table 1). This indicates a high population level variation in the secondary chemistry of the European white birch, a result that is in accordance with our previous results where leaf chemistry from these same parental trees was investigated (Laitinen et al., 2002). UPGMA clustering of shoot chemistry (Figure 1) gave different chemotype groups than grouping on the basis of leaf chemistry (see Laitinen et al., 2000). This is apparently due to qualitative differences between leaf and shoot chemistry's.

The main phenolic compounds were the same in both mature trees and young clonal plantlets of the same trees; that is, the chemical profiles were similar. This indicates genetic control of these secondary compounds in European white birch. Generally, the concentrations of defensive compounds in deciduous trees are thought to be higher during the juvenile stage (Bryant and Julkunen-Tiitto, 1995), which was also the case in our present study—especially for triterpenoids. The amounts of total terpenoids for some clones were almost 100 times greater in clonal plantlets than in parental trees (Figure 5). This is in accordance with a previous study on Alaska paper birch (*Betula resinifera*), where Reichardt et al. (1984) found 25 times greater amounts of papyriferic acid in juvenile stems than in mature twigs. Contrary to terpenoids, the concentrations of some phenolic compound groups and individual compounds, e.g., catechin derivatives, caffeoyl quinic acids, condensed tannins, (+)-catechin, and salidroside, were higher in parental trees (Figures 3 and 4). The reason for this difference may be related to the different roles for those compounds. Catechins play a role as important structural elements of condensed tannins (Strack, 1997; Seigler, 1998),

whereas triterpenoids are effective defenses against hares particularly in the juvenile developmental stages of white birches (e.g., Reichardt et al., 1984). Ontogenic differences make it difficult to predict the quantity of triterpenoids in clonal plantlets by only using results gained from parental trees, whereas a higher similarity between clonal plantlets and parental trees occurs for many studied phenolic compounds.

Previous studies have documented significant differences in the concentrations of foliar phenolic compounds, e.g., among clones of *Salix* sp. (Nichols-Orians et al., 1993; Hakulinen et al., 1995; Hakulinen, 1998) and *B. pendula* (Keinänen et al., 1999a). In this study, a statistically significant variation in shoot chemistry was also found among clones (Figures 2–5), which agrees with our previous study that used greenhouse-grown *B. pendula* clonal plantlets (Laitinen et al., 2004). Apparently, the higher environmental variation at the four growing sites in the present study increased the chemical variation within clones and may partly explain the slight differences in the pattern of chemical variation between these studies. Regardless of the differences, the results in these two studies are similar in cases where clonal repeatability (i.e., heritability in a broad sense) was found to be high. This indicates that genetic control of the accumulation of many birch shoot secondary compounds, such as chlorogenic acid, quercetin-3-glucoside + quercetin-3-glucuronide, and DHPPG, is strong (Table 1).

In clonal repeatability, the additive and nonadditive component of genetic variation cannot be separated (Falconer, 1989). However, it is used especially when the possible gain obtainable by vegetative production of trees is considered (van Buijtenen, 1992) and also because of the huge plant material needed for reliable estimates of narrow sense heritability (see King et al., 1997). Differences in heritability among different compounds may be due to both the origin and/or different bioactivities of the compounds (Orians et al., 1996). Intense selection for adaptive traits may lead to low heritability values. Thus, high heritabilities for compounds with high bioactivity may suggest a short period of selection time (Falconer, 1989; Orians et al., 1996).

The especially low heritability (i.e., clonal repeatability) in our study for (+)-catechin and catechin derivatives may be due to their role as important precursors for condensed tannin synthesis (e.g., Strack, 1997). Condensed tannins occur in ferns, gymnosperms, and angiospermous plants (e.g., Seigler, 1998), which indicates that they are of rather ancient origin. Some condensed tannins are also known to have a high degree of bioactivity (e.g., Waterman, 1988; Hagerman et al., 1998; Kraus et al., 2003); thus, they can be assumed to have been under selective pressures for a long time. Therefore, the low heritability found in this study for condensed tannins might be due to a strong, long-lasting selection that decreased heritability during generations of time. Some other phenolic compounds or compound groups had high heritabil-

ities that could indicate either low selection pressures or their more recent origin.

Many triterpenoids are characteristic especially for rapidly evolving (e.g., Dugle, 1966) birch species (e.g., Reichardt, 1981; Vainiotalo et al., 1991; Taipale et al., 1993; Julkunen-Tiitto et al., 1996). Triterpenoids are defensive compounds against mammalian herbivory (Reichardt et al., 1984; Risenhoover et al., 1985; Rousi et al., 1991; Laitinen et al., 2004). In spite of their ecological importance (i.e., high bioactivity), the heritabilities of triterpenoids were high. Therefore, we postulate that these compounds have a rather recent evolutionary origin. To further study the possibilities for the selection of genotypes, the additive genetic variation for these traits should be studied.

The profound effect of environment on plant secondary chemistry was seen in our study as statistically significant differences between growing sites for the accumulation of 45% of the studied phenolic compound groups and individual compounds, e.g., catechin derivatives, condensed tannins, (+)-catechin, chlorogenic acid, rhododendrin, and salidroside (Figures 2 and 3). The site with the highest or lowest concentrations differed, depending on the compound studied, indicating that the immediate environment affected different compounds or compound groups differently. For rhododendrin, salidroside, and terpenoids, both the effect of growing site and the genotype by growing site interaction were statistically significant (Figures 3 and 5), suggesting that different clones may respond differently to environmental variation; that is, genotypes are specialized (Via, 1984) in their production of such compounds in different environments (e.g., Figure 6b). These results agree with previous studies that have shown that in *B. pendula* leaves, there are significant genotype × environment (different fertilization, defoliation, and ozone treatments) interactions for some of the phenolic compounds (Keinänen et al., 1999a; Yamaji et al., 2003). Also, the secondary chemistry of some willow clones is more sensitive to environmental factors than other willow clones (Hakulinen et al., 1995; Veteli et al., 2002). It seems that there is a clear genetic component in the determination of secondary chemistry profiles in European white birches, and the sensitivity of chemical accumulation to environmental factors is highly dependent on the studied compound and tree genotype.

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EFFECTS OF MECHANICAL WOUNDING ON ESSENTIAL OIL COMPOSITION AND EMISSION OF VOLATILES FROM *Minthostachys mollis*

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Abstract—Plant tissues may show chemical changes following damage. This possibility was analyzed for *Minthostachys mollis*, a Lamiaceae native to Central Argentina with medicinal and aromatic uses in the region. Effects of mechanical damage on its two dominant monoterpenes, pulegone and menthone, were analyzed by perforating *M. mollis* leaves and then assessing essential oil composition at 24, 48, and 120 hr; emission of volatiles was also measured 24 and 48 hr after wounding. Mechanical damage resulted in an increase of pulegone and menthone concentration in *M. mollis* essential oil during the first 24 hr. These changes did not occur in the adjacent undamaged leaves, suggesting a lack of systemic response. Postwounding changes in the volatiles released from *M. mollis* damaged leaves were also detected, most noticeably showing an increase in the emission of pulegone. Inducible chemical changes in aromatic plants might be common and widespread, affecting the specific compounds on which commercial exploitation is based.

Key Words—Phytochemical induction, aromatic plants, Lamiaceae, mechanical wounding, monoterpenes, pulegone, menthone, *Minthostachys mollis.*

INTRODUCTION

Plants respond to injuries or wounds from biotic or abiotic stresses by deploying various biochemical defense mechanisms (Karban and Baldwin, 1997). These

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defenses can be either direct, making the plant more resistant to further herbivory (e.g., toxic secondary metabolites, digestibility reducers) or indirect (e.g., volatiles released after attack). The latter facilitate "top-down" control of herbivore populations by allowing pathogens, predators, and parasitoids to distinguish between infested and noninfested plants, thus aiding them in host or prey location (Baldwin and Preston, 1999).

The distinction between both types of defenses is not clear-cut, since some direct defenses, particularly those that slow herbivore growth by reducing their digestive efficiency, may need the third trophic level to be effective (Jongsma and Bolter, 1997; De Leo et al., 1998). Hence, for some direct defenses, we should expect coordinated induction of both defense types (Baldwin and Preston, 1999).

Among the best studied examples of plant secondary metabolites with defensive functions, monoterpenes (the C10 members of the terpenoid family of natural products) occupy a prominent place. These colorless, lipophilic, volatile substances represent the major constituent in plant essential oils and have been implicated as defenses against a variety of insects and pathogens (Langenheim, 1994; Phillips et al., 1995; Isman, 2000; Ciccia et al., 2000; Bekele and Hassanali, 2001; Harrewijn et al., 2001).

Induced chemical changes could also be important from a different point of view, when aromatic or medicinal plants are considered. In this case, the change *per se* would matter, particularly if the specific compounds giving economic value to the plant, for fragrance, flavor, or pharmaceutical industries, are affected (Valladares et al., 2002).

In the present paper, we analyze changes, induced by mechanical damage, in the essential oil composition and emission of volatiles from *Minthostachys mollis* (Kunth.) Griseb., a Lamiaceae native to Central Argentina with medicinal and aromatic uses in the region. Damage-induced changes in essential oil composition and in the volatiles emitted have rarely been considered simultaneously (e.g., Zabaras and Wyllie, 2001).

We have dealt with monoterpenoids, since the most important compounds of *M. mollis* belong to this group (Valladares et al., 2002) and were given previous records of increased production of such compounds following herbivory in various plant species (Karban and Baldwin, 1997; Tumlinson et al., 1999; Valladares et al., 2002). Temporal and spatial (translocation) variations in the plant response have been included in the analyses.

METHODS AND MATERIALS

Plants. Healthy, pest-free, 6-month-old *M. mollis* plants, were grown in a glasshouse without supplementary lighting. In each plant, a 5-cm-diam hole was

punched in the middle of the lamina of five leaves (of approximately the same age and size, but from different nodes), thus removing 30–40% of the leaf area.

Damaged leaves were cut off after either 24, 48, or 120 hr. The undamaged leaves adjacent to wounded ones were collected at the same time and kept separately. Five leaves similar in size and age to the damaged ones from an undamaged plant were used as controls. All leaves were frozen until the chemical analysis was carried out. Ten different plants (replications) were used for each treatment.

Essential Oil Extraction. After weighing, the plant material was extracted by hydrodistillation in a micro-Clevenger-like apparatus for 40 min. The volatile fraction was collected in dichloromethane. Internal standard was added (12 μg of tymol in 2 μl dichloromethane). Essential oils of *M. mollis* contain 50 different compounds, with two monoterpenes accounting for about 80% of their volume: pulegone and menthone (Valladares et al., 2002). These two compounds were used for the present study. The monoterpenes were quantified with respect to thymol. The FID response factors for each compound generate an equivalent area with a negligible error (<5%).

Collection of Plant Volatiles. The volatile collection system consisted of a vacuum pump that created a constant air flow (300 ml/min) through a polyethylene terephthalate (PET) chamber (1500 ml in volume) containing a plant; the chamber was closed at one end with a cap predrilled to fit exactly the collection trap. At the other end, a cap, with a hole through which the plant stem passed, separated the bottom of the chamber from the plant pot ground. Air exited the chamber through a reusable glass collection trap packet with 30 mg Super Q absorbent (80–100 mesh)(Alltech), which was rinsed (5–10 ml dichloromethane) prior to each volatile collection to remove impurities. Volatiles were collected for 2 hr. Immediately after, the compounds were eluted from the absorbent traps with 200 μl dichloromethane and internal standard was added (12 μg of tymol in 2 μl dichloromethane). Collected volatiles were analyzed by gas chromatography (GC) as described below. Once the volatiles were collected, the plant was cut and weighed.

For each plant, volatiles were collected 24, 48, and 96 hr after damage. Volatiles were also collected from undamaged control plants. All plants were of the same age and size, and collections done taken under similar conditions of light, temperature (20–24°C), and relative humidity (~70%). Collections made without plants, from an empty chamber, established that the background of monoterpenes present was negligible. Each treatment was repeated at least five times.

Chemical Analyses. Analyses were accomplished by using a Perkin-Elmer Q-700 gas chromatograph equipped with a CBP-1 capillary column (30 m $\times$ 0.25 mm) and a mass-selective detector. Analytical conditions: injector and detector temperatures 250 and 270°C, respectively; oven temperature pro-

grammed from 60°C (3 min) to 240°C at 4°/min; carrier gas helium at a constant flow of 0.9 ml/min; and source 70 ev. Oil components were identified by a combination of mass spectral and retention time data, which were compared both with those of authentic compounds and with those published in Zygadlo et al. (1996). GC analyses were performed with a Shimadzu GC-RIA gas chromatograph, fitted with a 30 m × 0.25 mm fused silica capillary column coated with a Supelcowax 10. The GC operating conditions were: oven temperature programmed from 60°C (3 min) to 240°C at 4°/min; injector and detector temperatures 250°C; detector FID; carrier gas nitrogen at a constant flow of 0.9 ml/min.

Volatile Emission Rate. An estimation of the amount of monoterpenes emitted in relation to their content in the plant tissues was obtained by calculating, for each treatment, the quotient between the concentration values of menthone or pulegone in the headspace and their respective concentration in the essential oil (headspace/essential oil).

Statistical Analyses. Differences in menthone and pulegone content between treatments were tested for statistical significance using Mann–Whitney test, since data were not normally distributed.

RESULTS

Oil Concentration. Menthone and pulegone concentration in the essential oil of wounded leaves (Figure 1) increased six and four times, respectively, in comparison to control plants ($P < 0.05$, $U_{\text{menthone}} = 239.0$, $U_{\text{pulegone}} = 373.0$),

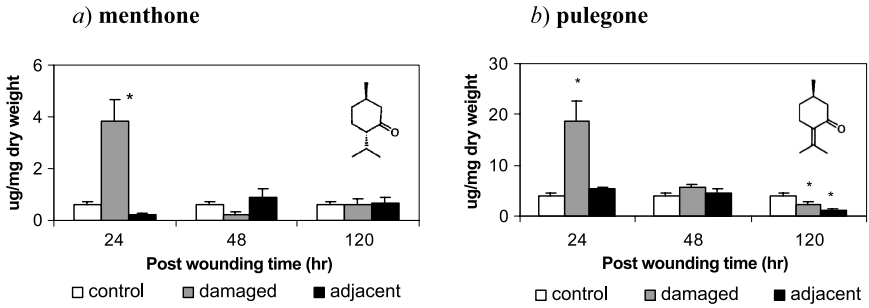

FIG. 1. Changes in oil concentration (means ± SE) of *a* menthone and *b* pulegone from *Minthostachys mollis* leaves as a result of mechanical damage. *Values significantly different from controls ($P < 0.05$, Mann–Whitney test).

TABLE 1. VARIATIONS IN THE MAIN VOLATILES RELEASED FROM *Minthostachys mollis* PLANTS AS A RESULT OF MECHANICAL DAMAGE

Compound	Control	24-hr Postwounding	48-hr Postwounding
Menthone	1.13 ± 0.14	0.85 ± 0.39	0.88 ± 0.37
Pulegone	0.21 ± 0.03	0.85 ± 0.27*	0.23 ± 0.07

Values are expressed in nanograms per milligram fresh weight (means ± SE).
*Values significantly different from controls ($P < 0.05$, Mann–Whitney test).

during the first 24 postwounding hours. After that time, concentration of both compounds returned to its prewounding level.

Undamaged leaves adjacent to wounded leaves did not differ significantly from controls regarding pulegone and menthone concentration after the first 24 hr ($P > 0.05$, $U_{\text{menthone}} = 48.0$, $U_{\text{pulegona}} = 241.5$), despite the dramatic increase observed in their opposite (damaged) leaves (Figure 1). However, and in coincidence with the damaged leaves, after 120 hr, their concentration of pulegone decreased below the level of control leaves ($P < 0.05$, $U = 56.0$).

Plant Volatiles. Headspace experiments showed a change in concentration of the compounds emitted by damaged plants compared with undamaged ones. Emission of menthone was similar in both damaged and undamaged plants ($P > 0.05$, $U_{24\ \text{hr}} = 46$, $U_{48\ \text{hr}} = 73.5$) and did not change over time (Table 1). Instead, emission of pulegone increased dramatically upon wounding ($P < 0.05$, $U_{24\ \text{hr}} = 105$) and then decreased back to prewounding levels ($P > 0.05$, $U_{48\ \text{hr}} = 73.5$) (Table 1).

Emission Rate of Volatiles. The emission rate of pulegone with regard to its concentration in the essential oil did not show important changes following

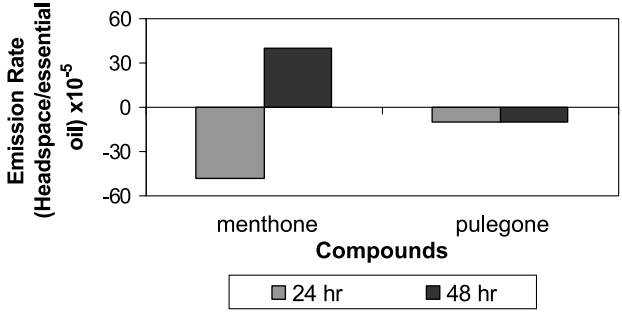

FIG. 2. Volatile emission rates of menthone and pulegone from *Minthostachys mollis* plants, 24 and 48 hr after being mechanically wounded, presented as differences from the corresponding rates in undamaged plants.

mechanical wounding, whereas the emission rate of menthone was noticeably lower at 24 hr and higher at 48 hr than that of control plants (Figure 2).

DISCUSSION

Oil Concentration. M. mollis plants responded to mechanical damage by dramatically increasing, after 24 hr, the concentration of the two most abundant monoterpenes (menthone and pulegone) in their essential oil. Zabaras and Wyllie (2001) found a comparable behavior in another aromatic plant, *Ocimum minimum*, whose main compound linalool increased 24 hr after suffering mechanical wounding.

In the present work, leaves adjacent to damaged ones remained unaltered in their chemical composition at least regarding menthone and pulegone concentration. In other systems, the defensive reaction has been recorded not only at or near the site of damage, but also throughout the plant, as a result of signaling molecules enabling communication among different plant tissues (Mc Auslane et al., 1997; Constabel and Ryan, 1998; Baldwin and Preston, 1999; Gatehouse, 2002). An elicitor may be required for a systemic response to be induced (Tumlinson et al., 1999).

In *M. mollis*, most of the essential oil transformations after injury occurred during the first 24 postwounding hours, after which the damaged leaves started to return to their prewounding metabolic state. From an ecological perspective, rapid chemical changes may be interpreted as an attempt to minimize subsequent predation (Clausen et al., 1991).

Monoterpenes are known to be induced by herbivore feeding in *M. mollis* (Valladares et al., 2002) and in other plant species (e.g., Mc Auslane et al., 1997; Paré and Tumlinson, 1999). Therefore, their role during the wounding response is likely to be involved in protecting damaged leaves from further attack. This function could be attributed mainly to pulegone, considering that its toxic (Fournet et al., 1996; Franzios et al., 1997; Ellis and Baxendale, 1997; Lamiri et al., 2001; Harrewijn et al., 2001), growth-inhibiting (Hummelbrunner and Isman, 2001), repellent, and oviposition-deterring (Harrewijn et al., 1994) properties have been demonstrated on various insect species. However, menthone has also showed insecticidal (Lee et al., 2001) and genotoxic activities (Franzios et al., 1997).

Plant Volatiles. In agreement with other studies (e.g., Turlings et al., 1990; Paré and Tumlinson, 1997), our results show that artificial damage can induce the release of volatile terpenoids. Although both major constituents of *M. mollis* essential oil increased after wounding, only the emission of pulegone changed. Zabaras et al. (2002) also found a dramatic increase of volatile emission in

Melaleuca alternifolia leaves after mechanical damage, but in that case, the compounds involved remained unchanged in the essential oil.

The increase in pulegone emission rate 24 hr after damage may be interpreted as an attempt to minimize subsequent predation (Harrewijn et al., 2001) or as a potential cue for natural enemies of herbivores (De Moraes et al., 1998).

Emission Rate of Volatiles. The amount of monoterpenes emitted as volatiles represented only a small fraction of the total monoterpenes present in the plant. Extrapolation over a typical 6-month growing plant suggests that <1% of total monoterpenes would be released to the atmosphere. A low rate of volatilization is consistent with observations in other Lamiaceae (e.g., Tyson et al., 1974; Gershenzon et al., 2000).

Even in undamaged plants, the relative concentration of various monoterpenes emitted from *M. mollis* was quite different from that found in the essential oil within the plant. Emitted monoterpenes contained higher proportions of menthone (60.2% in the headspace vs. 10% in the oil) and lower amounts of pulegone (11.1 vs. 85%). Studies on other plant species have also shown that the composition of emitted monoterpenes may differ from their presence in the plant oil (Werker, 1993; Guillet et al., 1998; Gershenzon et al., 2000). Membranes of storage compartments might be selectively more permeable to particular monoterpenes, or the emitted monoterpenes may be associated with entirely different secretory compartments than the stored compounds (Gershenzon et al., 2000).

It is likely that changes in both oil concentration and headspace serve important ecological roles during the leaf postwounding period. Moreover, from a commercial point of view, the effects of wounding on leaf oil composition could also have an important impact.

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BROWSE SELECTION IN RESPONSE TO SIMULATED SEASONAL CHANGES IN DIET QUALITY THROUGH POSTINGESTIVE EFFECTS

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Abstract—Browse species undergo seasonal changes in nutritional value and secondary plant compound concentrations. The capacity of herbivores to monitor such change through postingestive effects and to modify their food choice appropriately was investigated. Twenty-four goats were offered a different conifer species on four successive learning days per 7-d period for six periods. During conifer consumption on learning days, animals received either a positive or a negative digestive stimulus to simulate the nutritional rewards and toxic consequences of browse consumption. For each animal, a different postingestive stimulus treatment was associated with each conifer species. The treatments consisted of an increasing positive stimulus, a decreasing positive stimulus, an increasing negative stimulus, or a decreasing negative stimulus. The levels of the stimuli were adjusted in 20% increments in successive periods (from 0 to 100% for the increasing treatments and from 100 to 0% in the decreasing treatments) to simulate seasonal changes in browse characteristics. Diet preference was measured on d 5 of each period. Animals adjusted their diet choice in response to the changing intensity of the negative stimulus, but not the positive stimulus. Animals avoided foods associated with the negative stimulus to a greater extent when the stimulus was increasing each period compared to when it was decreasing. The results suggest that herbivores are adept at monitoring and responding to temporal change in secondary compound concentrations through assessing their changing postingestive effects and adjusting their food choice accordingly.

Key Words—Diet choice, mammalian herbivore, nutritional value, season, secondary plant compound.

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INTRODUCTION

Browsing herbivores have a major influence on the composition of vegetation communities through their selective feeding behavior. They generally have access to a large range of plants and plant parts from which to construct a diet (Dejong et al., 1995; Ramirez et al., 1997), and an understanding of the mechanisms that underly their diet-choice decisions is important for better prediction of the impact of herbivores on vegetation composition. Factors that may influence whether or not a food item is consumed include the rate at which it can be harvested, the extent to which it contributes to gut fill, the rate at which it gives up its nutrients for absorption, and the likelihood that its ingestion will lead to toxic effects (Belovsky, 1981; Belovsky and Schmitz, 1994). The balance of these and other factors will determine optimal diet composition from a given array of food options.

However, the vegetation resource is not static: seasonal variation in food supply and quality is a feature of most ungulate foraging environments. The seasonal nature of plant growth and senescence leads to a continually changing food resource from which ungulate herbivores must construct their diet. The food resource for ungulate herbivores is inherently dynamic: nutrient yields from particular plant species may undergo systematic seasonal changes (Cederlund and Nystrom, 1981; Cooper et al., 1988; Owen-Smith, 1994). Similarly, secondary compound concentrations in plants and plant parts change with season and with the phenology of the plant (Jalal et al., 1982; Palo et al., 1985; Brooks et al., 1987). The optimum diet is not static in composition, but changes with the changing nature of the food resource, and this is reflected in major seasonal differences in the diet composition of ungulate herbivores (Cederlund and Nystrom, 1981; Watson and Owen-Smith, 2000).

Although a substantial component of this change is related to seasonal changes in food availability, adjustments of diet selection in response to the changing nutrient yield and toxic properties of particular food items are also likely to be important. One means by which such change in food quality might be detected is through changes in the postingestive consequences that follow consumption of particular food items. Recent research suggests that mammalian herbivores can monitor the positive and negative physiological consequences of consuming particular foods, associate such consequences with food flavors or other sensory characteristics, and adjust their diet preference accordingly (reviewed in Provenza et al., 2003). Changes in postingestive effects are not necessarily closely coupled to changing flavor or other preingestive cues; herbivores may learn to associate particular flavors with particular postingestive effects, but the flavors themselves may be unrelated to those effects (Lawler et al., 1999). There is some evidence for flexibility in the associations that herbivores make between food flavors and postingestive effects,

which would be necessary given the dynamic nature of natural browse diets (Launchbaugh et al., 1993). However, the extent to which ungulate herbivores monitor systematic changes in postingestive consequences and adjust their preference has not been studied.

In the work reported here, we set out to assess how effectively herbivores can monitor systematic changes in the positive and negative consequences of consuming particular food items and adjust their diet selection accordingly. We tested this by offering goats a series of conifer species, and by associating different postingestive stimuli with each. We made systematic changes in the strength of administered stimuli over time to simulate seasonal changes in nutritive value and toxicity, and monitored temporal change in diet choice.

METHODS AND MATERIALS

Experimental Design. Twenty-four male, juvenile Scottish Cashmere goats were used. The animals were 8 mo old at the start of the experiment and had not previously been exposed to conifer foliage. The experiment consisted of six periods lasting 7 d each; each period consisted of four consecutive learning days, followed by a preference test day, then by two rest days. On the four learning days, goats were offered a cut branch of a different conifer species each day. During learning days, goats were allowed to consume from their allotted conifer branch for 4 hr between 0900 and 1300 hr, and they were dosed at the end of each hour during conifer consumption with either an aversive stimulus or a nutrient stimulus according to their allotted treatments. In this way, a different postingestive consequence was associated with each of the four feed types. On d 5 of each period, all four conifer species were offered simultaneously without their associated postingestive stimuli to measure preference. Two rest days followed before the start of the next period. The conifer species used were *Picea sitchensis* (Sitka spruce), *Pseudotsuga menziesii* (Douglas fir), *Picea abies* (Norway spruce), and *Pinus sylvestris* (Scots pine).

Treatments and Their Allocation to Animals and Species. The strength of the imposed postingestive consequences of consuming conifer material was varied systematically over the six periods, according to treatment, to simulate seasonal changes in nutrient rewards or secondary compound concentrations. Four treatments were applied as follows: Inc_Pos, increasing dose rate of a positive stimulus; Dec_Pos, decreasing dose rate of a positive stimulus; Inc_Neg, increasing dose rate of a negative stimulus; and Dec_Neg, decreasing dose rate of a negative stimulus.

The positive stimulus, simulating nutrient release, was sodium propionate at a maximum dose rate of 90 mg/kg DM consumed. Sodium propionate is a short-chain fatty acid and one of the major end products of carbohydrate digestion in ruminants. Ruminants utilize short-chain fatty acids as a source of energy (McDonald et al., 2002). The negative stimulus was lithium chloride at a maximum dose rate of 20 mg/kg DM consumed. Lithium chloride stimulates the emetic system of vertebrates causing sensations of nausea without long-lasting toxic effects. Lithium chloride has been widely used as a model-aversive agent to simulate the aversive effects of plant secondary metabolites (Gustavson, 1977). The rates of administration of positive and negative stimuli used in this experiment have been shown to be effective in eliciting conditioned preferences and aversions in previous experiments (Du Toit et al., 1991; Villalba and Provenza, 1996; Duncan and Young, 2002).

Treatments were associated with conifer species and allocated to animals as illustrated in Table 1. A number of characteristics of the design contributed to its balance. Within each period, each animal received each conifer species and each treatment. Each order of treatment and each order of conifer species appeared exactly once in the design. Each combination of conifer species and treatment (e.g., Douglas fir, Inc_Neg) occurred exactly six times in the design. Each combination of species and treatment occurred exactly twice with each other possible combination (e.g., Douglas fir, Inc_Neg with Sitka Spruce, Dec_Pos). The design was such that once conifer species had been allocated to treatments and learning days for an animal, that animal would experience the same postingestive treatment on consumption of a particular conifer species for the same day each week throughout the experiment. Dose rates of stimuli were altered systematically with period and were either increased or decreased by 20% of the maximum dose rate in successive periods according to treatment as shown in Table 1. For example, goat 1 was offered Douglas fir on d 1 of each wk, and this was associated with the Inc_Neg treatment. This animal would, therefore, experience an increasingly aversive stimulus for Douglas fir as the experiment progressed.

Preference tests were used to assess the ability of each goat to monitor the changing postingestive effects of the different food sources. Preference tests consisted of 20-min tests during which each goat was offered all four conifer species simultaneously. Absolute intake of each conifer species during preference tests was measured by weighing material before and after offering it.

Feeding and Dosing Procedure. Cut branches of Sitka spruce, Douglas fir, Norway spruce, and Scots pine were offered to goats for 4 hr/learning day from 0900 to 1300 hr. At the start of each 4-hr feeding period and at the end of each hour within feeding period, branches were weighed with a spring balance accurately to within 5 g, and animals were dosed according to the consumption of foliage during the previous hour. LiCl pellets and sodium propionate pellets

TABLE 1. SUBSET OF THE EXPERIMENTAL DESIGN (SHOWING FOUR OUT OF A POSSIBLE 24 ANIMALS AND THREE OUT OF A POSSIBLE SIX PERIODS) ILLUSTRATING ALLOCATION OF POSTINGESTIVE STIMULI AND SPECIES TO ANIMALS

		Goat 1		Goat 2		Goat 3		Goat 4	
		Treatment	Species	Treatment	Species	Treatment	Species	Treatment	Species
Period 1	Day 1	Inc_Neg (0% LiCl)	Fir	Inc_Pos (0% VFA)	Sitka	Dec_Neg (100% LiCl)	Sitka	Inc_Neg (0% LiCl)	Fir
	Day 2	Dec_Neg (100% LiCl)	Norway	Inc_Neg (0% LiCl)	Norway	Dec_Pos (100% VFA)	Norway	Dec_Pos (100% VFA)	Sitka
	Day 3	Inc_Pos (0% VFA)	Sitka	Dec_Pos (100% VFA)	Fir	Inc_Pos (0% VFA)	Pine	Inc_Pos (0% VFA)	Norway
	Day 4	Dec_Pos (100% VFA)	Pine	Dec_Neg (100% LiCl)	Pine	Inc_Neg (0% LiCl)	Fir	Dec_Neg (100% LiCl)	Pine
	Day 5	Preference test		Preference test		Preference test		Preference test	
	Day 6	Rest		Rest		Rest		Rest	
	Day 7	Rest		Rest		Rest		Rest	
Period 2	Day 1	Inc_Neg (20% LiCl)	Fir	Inc_Pos (20% VFA)	Sitka	Dec_Neg (80% LiCl)	Sitka	Inc_Neg (20% LiCl)	Fir
	Day 2	Dec_Neg (80% LiCl)	Norway	Inc_Neg (20% LiCl)	Norway	Dec_Pos (80% VFA)	Norway	Dec_Pos (80% VFA)	Sitka
	Day 3	Inc_Pos (20% VFA)	Sitka	Dec_Pos (80% VFA)	Fir	Inc_Pos (20% VFA)	Pine	Inc_Pos (20% VFA)	Norway
	Day 4	Dec_Pos (80% VFA)	Pine	Dec_Neg (80% LiCl)	Pine	Inc_Neg (20% LiCl)	Fir	Dec_Neg (80% LiCl)	Pine
	Day 5	Preference test		Preference test		Preference test		Preference test	
	Day 6	Rest		Rest		Rest		Rest	
	Day 7	Rest		Rest		Rest		Rest	
Period 3	Day 1	Inc_Neg (40% LiCl)	Fir	Inc_Pos (40% VFA)	Sitka	Dec_Neg (60% LiCl)	Sitka	Inc_Neg (40% LiCl)	Fir
	Day 2	Dec_Neg (60% LiCl)	Norway	Inc_Neg (40% LiCl)	Norway	Dec_Pos (60% VFA)	Norway	Dec_Pos (60% VFA)	Sitka
	Day 3	Inc_Pos (40% VFA)	Sitka	Dec_Pos (60% VFA)	Fir	Inc_Pos (40% VFA)	Pine	Inc_Pos (40% VFA)	Norway
	Day 4	Dec_Pos (60% VFA)	Pine	Dec_Neg (60% LiCl)	Pine	Inc_Neg (40% LiCl)	Fir	Dec_Neg (60% LiCl)	Pine
	Day 5	Preference test		Preference test		Preference test		Preference test	
	Day 6	Rest		Rest		Rest		Rest	
	Day 7	Rest		Rest		Rest		Rest	

Treatments imposed were Inc_pos, increasing dose rate of a positive stimulus; Dec_pos, decreasing dose rate of a positive stimulus; Inc_neg, increasing dose rate of a negative stimulus; and Dec_neg, decreasing dose rate of a negative stimulus. The positive stimulus, simulating nutrient release, was sodium propionate (VFA) at a maximum dose rate of 90 mg/kg DM consumed. The negative stimulus was lithium chloride (LiCl) at a maximum dose rate of 20 mg/kg DM consumed. Conifer species offered were Sitka spruce (*Picea sitchensis*), Douglas fir (*Pseudotsuga menziesii*), Norway spruce (*Picea abies*), and Scots pine (*Pinus sylvestris*) denoted Sitka, Fir, Norway, and Pine, respectively.

were prepared in advance, in a range of sizes, by using tissue paper and water-soluble glue to facilitate their rapid release in the rumen. A background diet of dried grass pellets (Vitagrass Farms, Grange-Over-Sands, UK; 18% CP) was fed daily to ensure that the animals were in a controlled nutritional state. All animals were fed-dried grass pellets at a level calculated to ensure that total food intake satisfied their energy requirements for maintenance (Agricultural Research Council, 1980). The background diet was fed at 1500 hr each day starting 14 d in advance of the conifer feeding phase, and continued throughout the experiment. Animals were weighed each week to verify that liveweights remained stable as confirmation that the nutritional state of the animals was constant throughout the experiment.

Conifer branches were cut in a commercial forestry plantation (Drumelzie Wood, Auchenblae, Kincardineshire, UK) each week. Monoterpene composition of conifers varies between species (Von Rudloff, 1975) and among individuals within species (Duncan et al., 1994). Because of the known influence of variation in terpene concentrations on food preference (Duncan et al., 1994), steps were taken to reduce variation in preference, which could have been caused by variation in terpene concentrations: within-day variation was minimized by using a single tree of each conifer species for each learning day. Systematic between-day variation was minimized by measuring foliar monoterpene concentrations of each tree in advance of the experiment and by distributing trees among periods and days on the basis of their terpene concentrations in order to avoid systematic temporal trends in background preferences. Samples of the conifer branches fed to goats were taken weekly during the experiment and were analyzed for monoterpenes by a modification of the method of Sjödin et al. (1996). Foliage was also analyzed for neutral detergent fiber (NDF) (Van Soest, 1963), acid detergent fiber (ADF), and acid-indigestible lignin (Van Soest and Wine, 1967). Crude protein concentrations were calculated as 6.25 $\times$ nitrogen concentrations determined by elemental analysis.

Statistical Analyses. Summaries of the proportions selected during preference tests were formed by calculating means of appropriate values to derive simple graphical presentations of the results that did not depend on the fitted model.

Formal analyses were performed on the relative amounts of the four species eaten by using log-ratio analysis (Aebischer, 1993) as in our previous work (Ginane et al., in press) to avoid the many difficulties otherwise associated with analysis of compositional data. For each preference test, we calculated the logarithms of the ratios of the amounts eaten of Douglas fir, Norway spruce, and Scots pine (the numerator species) to the amount eaten of Sitka spruce (the denominator species), although the results are not dependent on which species was used in the denominator (Aitchison, 1986). The calculated log ratios were analyzed by fitting linear mixed models using the method of residual maximum

likelihood (Patterson and Thompson, 1971) to handle the complex correlation structures in these data.

We selected the random effects in our analysis by taking a maximal fixed-effect model, then adding random terms and observing changes in the residual deviance. First, the three log ratios from each preference test were correlated because they are formed with respect to the same denominator: our analysis allowed for this by treating the three log ratios as coming from a multivariate normal distribution. Second, correlations exist in the data because of the repeated use of the same animals: we allowed for this in the analysis by introducing an additional error term for individual animal-based preferences. Thus, the structure of the analysis is described by the model: $y_{ijk} = \mu_{ijk} + e_{ik} + f_{ijk}$, where for animal i in period j, y_{ijk} is the observed log ratio for species k with respect to Sitka spruce, with corresponding mean, μ_{ijk}, and error terms, e_{ik} and f_{ijk}, at the animal and preference test levels, respectively. These error terms have a mean of zero and variances and correlations specified by the (estimated) covariance matrices $V = \text{cov}(e_{i1}, e_{i2}, e_{i3})'$ and $W = \text{cov}(f_{ij1}, f_{ij2}, f_{ij3})'$. Third, this error structure is itself augmented to allow for a lag-1 autoregressive correlation, c, in the trial-level error term.

We modeled the mean, μ_{ijk}, as being dependent on the interaction of species and period, together with additive effects of learning day, the applied treatments, and their interactions. Species, period, and learning day were always taken as categorical, whereas applied treatments were either treated as categorical or as linear contrasts. To respect the skew-symmetric nature of log ratios, namely, $\log(z_1/z_2) = -\log(z_2/z_1)$, contrasts were formed by treating positively the usual contrast value associated with the species in the numerator and treating negatively the corresponding value for Sitka spruce (which always formed the denominator). Categorical effects other than species were fitted by using dummy variables, formed in the same manner as for linear contrasts, with entries 1 for factor levels that were associated with the numerator species, -1 for the denominator species, or 0 for factor levels absent from the observations used to form a log ratio. Covariates for terpene concentrations on learning and preference test days were constructed in a similar manner as the difference between log-terpene concentrations on numerator species and the corresponding value for Sitka spruce.

The effects of principal interest were those due to treatment level, estimated in the animal by species stratum, and dosage within treatment, estimated in the animal by species by week stratum. As there were a large number of residual degrees of freedom in both strata, tests of the significance for treatment effects were estimated by referring Wald statistics to X^2 distributions with degrees of freedom determined by the treatment effects being tested. All models were fitted by using the REML directives of the statistical program Genstat for Windows release 6.1 (VSN International, Oxford). Where a species was not eaten during

the preference test, the observed zeros were replaced, in forming the log ratio, by one half of the smallest nonzero amount eaten in any of the trials used in the analysis.

Preliminary analysis suggested that a relatively simple fixed-effect model should be used for the presentation of results. This contained the interaction between species and period, the interaction between learning day and species, together with the interaction between treatments and dosage. The terpene covariate for preference tests was not required because it was absorbed within the interaction between species and period, while the terpene covariate for learning days was dropped because there was no evidence supporting its inclusion when fitted after tree species.

To demonstrate the effects of the Pos-treatments on preference, we fitted a model ignoring Neg-treatments, considering either the two Pos-series (Inc_pos and Dec_pos) as categorical or as summarized by two intercepts and two linear contrasts. Conversely, to demonstrate the effects of the Neg-treatments, we fitted a model ignoring Pos-treatments, considering either the two Neg-series (Inc_neg and Dec_neg) as categorical or as summarized by two intercepts and two linear contrasts.

RESULTS

General. Foliage consumption during preference tests in the early periods of the experiment was relatively low as animals gained familiarity with the novel browse species (Figure 1). Scots pine was initially favored by the goats,

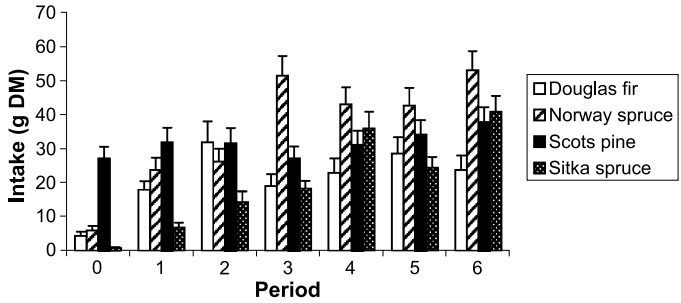

FIG. 1. Development of preference for conifer species over periods. Values represent mean proportions of total intake during preference tests. Error bars denote standard errors of means (N = 24). The conifer species used were *Picea sitchensis* (Sitka spruce), *Pseudotsuga menziesii* (Douglas fir), *Picea abies* (Norway spruce), and *Pinus sylvestris* (Scots pine).

TABLE 2. CHEMICAL COMPOSITION OF CONIFER FOLIAGE[a,b] OFFERED TO GOATS

	Douglas fir	Norway spruce	Scots pine	Sitka spruce
Nitrogen	1.25 (0.021)[c]	1.05 (0.028)	1.36 (0.034)	1.01 (0.048)
Lignin	21.9 (0.57)	19.2 (0.62)	16.8 (0.33)	22.2 (0.29)
Acid detergent fiber	37.9 (0.75)	41.3 (0.97)	42.0 (0.37)	43.4 (0.40)
Neutral detergent fiber	44.9 (0.57)	50.4 (0.95)	53.6 (0.45)	51.3 (0.52)
Total terpenes[d] (mg/kg DM)	10.80 (0.799)	0.30 (0.047)	5.52 (1.264)	0.48 (0.232)

[a] Values are averages of foliage samples pooled by period.
[b] Units are percent DM unless otherwise stated.
[c] Values in parentheses are standard errors of means.
[d] For terpene data, values from samples collected on preference test days are presented.

and the amount of pine consumed during preference tests remained relatively stable over the course of the experiment. Norway spruce initially formed only a small component of the food consumed during preference tests, but by period 3, it was the most preferred species and remained so for the rest of the experiment. Intake of Douglas fir during preference tests followed a similar pattern to that observed for Scots pine, while consumption of Sitka spruce increased steadily throughout the experiment. The chemical composition of the conifer species were relatively similar (Table 2), and there was no evidence that nutrient composition varied systematically with time (data not shown). Results of fiber analyses need to be interpreted with some caution since studies have shown that in browse species, reported fiber fractions are contaminated with phenolic residues and are likely to be significant overestimates of the true fiber composition of the material analyzed (Makkar et al., 1995). Terpene concentrations varied markedly according to species. The balanced design employed in the current study, however, meant that between-species variation in nutritive value or terpene concentration was spread across treatments, and that the main effects of our applied treatments could be viewed in isolation from background, between-species differences in preference that could have arisen from variation in the chemical composition of the conifers. Many of the preference tests in period 1 included tree species that were uneaten by the goats. In view of the difficulty this causes the log-ratio analysis and the fact that the goats were evidently becoming accustomed to novel forages, we only analyzed data from periods 2 to 6 (which contained a total of 10 observed zero values). Live weights of animals remained stable throughout the experiment (period 1: mean 22.8 kg, SD 2.99; period 6: mean 22.3 kg, SD 3.07). Between-week coefficients of variation for individual goats were always less than 9%.

Treatment Effects. There was strong evidence to suggest that goats preferred species allocated Pos-treatments to those allocated Neg-treatments ($X_1^2 = 39.0$, $P < 0.001$) (Figure 2). Although there was also strong evidence of a

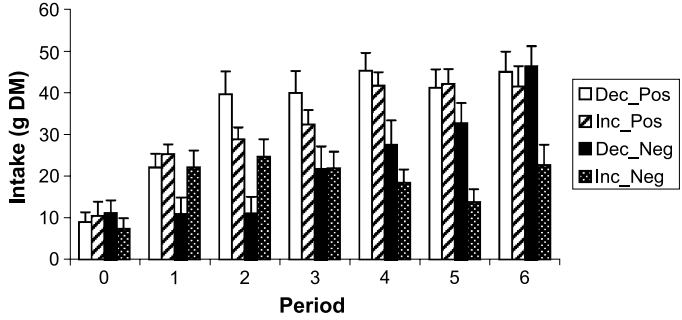

FIG. 2. Effect of associated postingestive consequences during learning on the relative preference for conifer species during preference tests. For treatment of Dec_Neg, a declining dose of lithium chloride was applied, while for Inc_Neg, an increasing dose of lithium chloride was applied. For Dec_Pos, a declining dose of sodium propionate was applied, while for Inc_Pos, an increasing dose of sodium propionate was applied. Values represent mean proportions of total intake. Error bars denote standard errors of means ($N = 24$).

difference in mean preference depending on whether Neg was increasing or decreasing ($X_1^2 = 15.2$, $P < 0.001$), the same was not true of the two Pos-treatments ($X_1^2 = 0.44$, $P = 0.51$). The linear contrast for dose level was highly significant for the Neg-treatments ($X_1^2 = 36.0$, $P < 0.001$) (Figure 3a), but did not appear to differ between the increasing series and the decreasing series ($X_1^2 = 1.15$, $P = 0.28$). The linear contrast for dose level was not significant for the Pos-treatments ($X_1^2 = 0.07$, $P = 0.8$) (Figure 3b).

The effects of the Neg-treatments relative to the average of the Pos-treatments and the Pos-treatments relative to the average of the Neg-treatments are shown in Figure 3. These reinforce the formal tests in showing for the Neg-treatments, linear regressions with mean levels, but not slopes, depending on whether dose rate was increasing or decreasing. Conversely, for the Pos-treatments, they show the absence of evidence for an effect of the dose rate.

Species Preferences. The overall effect of species on preference expressed as log ratios was highly significant ($P < 0.001$), indicating an underlying preference for Norway spruce over the course of the experiment (Figure 1). Mean log ratios for Norway spruce, Scots pine, and Douglas fir relative to Sitka spruce were 0.55, 0.13, and −0.18, respectively (SE 0.23). Species preferences changed with period ($P < 0.01$), indicating that these changed as the experiment progressed, although the balanced design allowed treatment effects to be assessed against this background of changing species preferences.

Effect of Learning Day. There was strong evidence that a goat's preference for a tree species was affected by the gap between the day of the preference test and the learning day on which that goat was given that tree species. The main

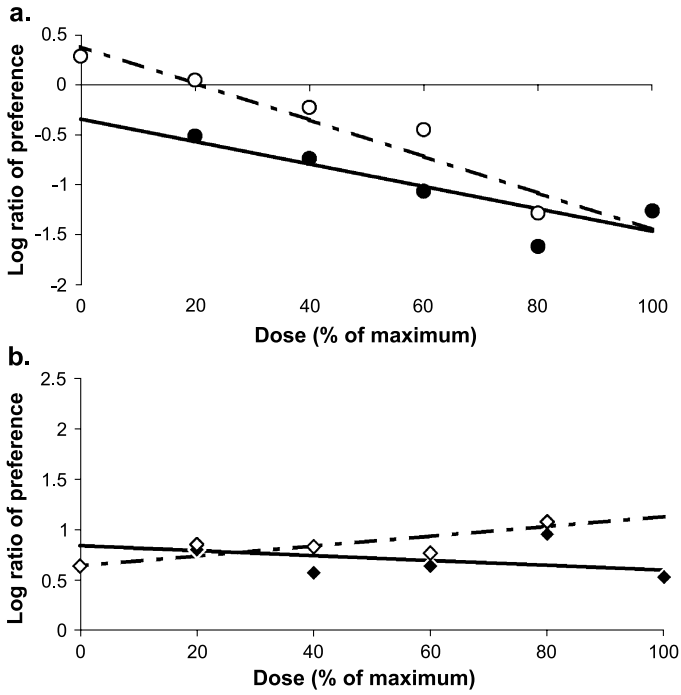

FIG. 3. Relationship between dose levels of a the negative stimulus and b the positive stimulus on parameter estimates for log ratio of preference. Data are expressed on the analysis (log-ratio) scale; log ratio of preference refers to the logarithm of the ratio of the amount of conifer eaten relative to the amount of Sitka spruce eaten during the same week. Points refer to categorical effects. Lines refer to linear contrasts. Separate points and lines are presented for the increasing (closed symbols, solid lines) and decreasing (open symbols, broken lines) series of each stimulus.

effect for day was highly significant ($P < 0.001$) with goats on preference test days tending to avoid the species given most recently although there was also evidence for an interaction with species ($P = 0.007$). Again, the balanced nature of the design meant that treatment effects could be assessed effectively despite the significant effect of learning day on preference.

DISCUSSION

The plant cues that herbivores use in coming to food selection decisions are not well understood. The animal may assess food quality by using general proximate cues such as color, taste, and texture (Bazely, 1990). However, these

cues are not necessarily a reliable indicator of nutritive value or toxicity. Recently, the role of postingestive cues as an alternative mechanism for learning, by herbivores, about the quality and toxicity of their food plants has gained credence (Provenza, 1995). The initial ideas arose from the psychological literature relating to conditioned learning mechanisms (Garcia et al., 1966). The idea that long-delay learning played a role in the development of food selection patterns among large herbivores soon appeared in the ecological literature (Zahorik and Houpt, 1977; Westoby, 1978). Many tests of the ability of large herbivores to associate postingestive effects with food flavors have since been reported, initially for the negative postingestive effects associated with toxicity (Provenza et al., 1990), but also, more recently, for the positive effects that follow nutrient ingestion (Villalba et al., 1999). The majority of these tests have relied on simple, two-way choice scenarios between flavored articificial foods, with animals given a series of learning opportunities over time. Some doubt remains as to the importance of learning, based on postingestive cues, to large herbivores foraging in botanically complex environments (Duncan and Young, 2002). This is because free-ranging herbivores encounter multiple food options simultaneously, making direct association of flavors and postingestive effects problematic. Furthermore, in natural situations, the consequences of ingesting particular plants changes with time since the nutritive value and secondary chemistry of plants change seasonally and with plant phenology (Jalal et al., 1982; Cooper et al., 1988). The current study was set up to explore the flexibility of the diet-learning mechanism when systematic simulated changes in nutrient rewards and toxic consequences were associated with a series of model browse foods. Natural browse species were used in preference to the flavored artificial foods used in many previous studies in order to more closely mimic the natural situation. Four different conifer species were used to allow each animal to associate each treatment with a particular species. The allocation of treatments to species was balanced across animals to allow the main effects of the postingestive treatments to be assessed using global means across species. The design of the experiment thus allowed the effects of the applied postingestive treatments to be assessed without the confounding effects of changing background preference for the different tree species.

The results of the experiment confirm the ability of goats to attribute different postingestive consequences to each of a series of foods and adjust their diet selection appropriately. Goats appeared more sensitive to negative than to positive postingestive effects. This may partly relate to the greater risks associated with making poor choices when toxic foods are involved than when nutrient-rich foods are offered. However, since the positive and negative stimuli had different currencies, we cannot draw firm conclusions about their relative effects in the current experiment. The success with which goats can associate positive postingestive effects with particular foods seems to decline as the

complexity of the diet increases. In our early work with three foods, one of which was associated with a fixed rate of sodium propionate administration, goats quickly learned which food was associated with positive postingestive effects (Duncan and Young, 2002). In later work (Ginane et al., in press) and in the current experiment with more complex learning scenarios, diet choice was not strongly influenced by association with the stimulus providing nutrient rewards, namely, sodium propionate. Similar conflicting results following use of sodium propionate as a positive conditioning stimulus have been found by others (Villalba and Provenza, 1996, 1997), and further work to investigate the preference response of ruminants to foods associated with sodium propionate and other short-chain fatty acids is required. By contrast, LiCl is a reliable negative conditioning stimulus, and this may explain its widespread use in conditioned aversion studies. Studies using natural negative stimuli found in plants have been reported (reviewed in Provenza, 1995) although welfare concerns have probably limited experimentation in this area. LiCl cannot be regarded as an adequately representative model of *all* plant secondary compounds in aversion studies, but given that it acts via the emetic system (responsible for sensations of nausea), it is likely to be a good model for plant secondary compounds that have some negative action at the level of the digestive tract.

The ability of animals to learn about temporally varying food resources through postingestive effects has been studied before. When lambs were offered flavored foods associated with a dose of LiCl that varied randomly on different days, the amount of food consumed was based on the maximum dose of toxin they had ingested, suggesting that animals were adopting a risk minimization strategy (Launchbaugh et al., 1993). In a further study, when the toxicity and energy rewards of test foods were changed unpredictably every 3 to 6 d, sheep quickly regulated their intake of the foods in line with expectations (Wang and Provenza, 1997). However, in this case, toxins and nutrients were incorporated directly into the feeds so changes in preference may not have been mediated only through associations with postingestive effects; animals may have adjusted preference based on the direct taste of the nutrients and toxins added to the test feeds.

In our own work, the learning scenario was more complex with four species being offered rather than two. Also, the changing quality and toxicity of food plants were simulated by oral administration of the postingestive stimuli thus removing the possible involvement of preingestive cues in mediating preference. Furthermore, systematic changes in quality and toxicity were used to simulate progressive seasonal changes. This allowed assessment of how the direction of change influenced the animals' ability to sense changes to their food plants and adjust their diet accordingly. In the case of negative postingestive effects, goats were able to adjust preference in line with changing toxicity, both when the strength of the toxic stimulus was increasing with time and when it

was decreasing. Animals appeared more sensitive to increasing toxicity than to decreasing toxicity; the relationship between the strength of the negative stimulus and preference differed, depending on whether toxicity was increasing or decreasing, although this was largely associated with a lower overall preference when toxicity was increasing (intercepts differed, but slopes did not). The greater caution that goats exhibited for foods whose toxicity was increasing with time may represent a strategy for minimizing risk: foods which are increasing in toxicity represent a greater risk than those whose toxicity is declining and hence require a more cautious approach.

We can say little about the relative sensitivity of goats to the direction of temporal change in nutritive value since no overall relationship between the positive postingestive stimulus we used and preference was evident in our data. The use of postingestive consequences by ruminant herbivores in assessing nutritive value may be less than for assessing toxicity since preingestive cues may be a more reliable indicator of nutritive value than of toxicity. Seasonal changes in digestibility, for example, might be relatively easily assessed by animals using characteristics such as texture and toughness so that postingestive mechanisms might be less important.

Seasonal changes in diet composition are often reported in the literature (Ramirez et al., 1997; Watson and Owen-Smith, 2000), but it is often difficult to disentangle the multiple influences which contribute to observed preference. For example, protein and condensed tannin concentrations in browse species on a South African savanna both vary seasonally, making it difficult to attribute changing foliage acceptability to any one factor (Cooper et al., 1988). In our study, we artificially restricted the change in simulated toxicity or quality to one characteristic per plant species offered, and we varied only the postingestive consequences of food consumption, keeping the sensory properties of the test foods constant. By using this approach, we have shown that goats can monitor changes in postingestive consequences simulating toxicity even when multiple food choices are available. Goats appeared less sensitive to simulated change in nutritive value, and this may be partly related to the complexity of the food-choice scenario.

In summary, our study has demonstrated the ability of ungulate herbivores consuming browse material to monitor systematic changes in the toxic consequences of consuming particular food plants and to adjust their food selection accordingly. Further work with a range of positive and negative postingestive stimuli is needed to assess the generality of this phenomenon.

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FEEDING RESPONSE TO HOST AND NONHOST COMPOUNDS BY MALES AND FEMALES OF THE SPRUCE BARK BEETLE *Ips typographus* IN A TUNNELING MICROASSAY

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Abstract—Research on host selection by bark and wood boring insects has concentrated on flight orientation behavior. Less is known of the factors that govern the steps successive to host landing. Here, we discuss chemical factors involved in host acceptance by bark beetles and a new microassay. Adult males and females of *Ips typographus* were offered an artificial diet treated with various concentrations of different plant-derived compounds (host terpenes and nonhost compounds) in a no-choice mode. Beetles were tested individually in a glass tube for 4 hr, and the length of feeding was measured and compared to a control (diet with only solvent). The first effect was diet rejection, especially when nonhost compounds were tested at high concentrations. Most compounds reduced feeding, in proportion to concentration. Females fed more readily than males after addition of both host and nonhost compounds. Diet removal was significantly affected by all the tested factors (sex, compound, dose) as well as by their interactions. With increased concentrations, males were more responsive than females to antifeedants, as all compounds (except juglone) showed clear sex differences of diet consumption. 3-Octanol, 1-hexanol, and a Green Leaf Volatile (GLV)-blend (three C6 alcohols) showed the strongest antifeedant effects, which started at a low dose (0.1%) and had a low Effective Dose 50 (ED50, 0.3–1%). In contrast, host monoterpenes, limonene and α-pinene, inhibited feeding at high

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doses (10–30%) only, with ED50 > 10%. The highest Antifeedant Indexes were shown by verbenone, carvone, and 1-hexanol (AFI = 0.90–1.00). Both host and nonhost compounds inhibited feeding at some concentration. No significant stimulation of feeding by any host compound at concentrations reported in the literature as optimal were found, with the possible exception of α-pinene at low concentrations in females.

Key Words—Host acceptance, host selection, tunneling, feeding, antifeedant, nonhost volatiles, NHV, monoterpene, assay, coleoptera, scolytidae, AFI, ED50, effective threshold.

INTRODUCTION

Host selection by bark beetles (Coleoptera: Scolytidae) is mainly individual and is governed by long-range attractive signals such as host volatiles (Wood, 1982; Schlyter and Birgersson, 1999). After a searching period, bark beetles land on a host tree and begin feeding activity (Paynter et al., 1990). If the chosen tree is found unsuitable, the flight is resumed and the process is repeated (Byers, 1995; Wallin and Raffa, 2002). For many xylophagous insects, avoidance of nonhost tree species is due either to lack of nutritional compounds or to the detection of potentially toxic secondary metabolites (Agelopoulos et al., 1999). Decisions regarding oviposition are often discerning and include both host species' recognition and the assessment of the host's defensive reactions (Safranyik et al., 1975). Thus, the chemical composition of the medium is the last threshold to be overcome before colonization.

During host selection, both attractive and repellent signals may be active. The former includes pheromones and kairomones, which act in a behavioral sequence (Wood, 1982; Raffa et al., 1993; Borden, 1997; Schlyter and Birgersson, 1999). The latter includes verbenone and angiosperm volatiles—such as nonhost volatiles (NHV)—which inhibit attraction to pheromones and kairomones in conifer-inhabiting Scolytidae (Borden, 1997; Zhang et al., 2000; Zhang and Schlyter, 2004). The use of NHV protects the potential host tree from being attacked, because the beetle does not recognize the substrate as suitable for reproduction.

The secondary plant metabolites important in insect host selection include mainly alkaloids, flavonoids, and terpenes (Frazier and Chyb, 1995), although quinones and phenols may also be important. Terpenes can either be repellent or attractive according to concentration or insect species. They can first increase then inhibit feeding activity once they have reached a toxic concentration (Reddemann and Schopf, 1996). Field bioassays indicate that different concentrations of bark monoterpenes (α-pinene and limonene) have different effects on the colonization rate of *Ips typographus* (L.) (Reddemann and Schopf, 1996).

Uninfested trees contained lower quantities of monoterpenes compared to colonized trees. Trunks colonized by *I. typographus* were characterized by a concentration of α-pinene ranging between 0.08 and 0.35 nmol mg^{-1} of fresh weight (Reddemann and Schopf, 1996). Similar results have also been obtained for American bark beetle species such as *I. grandicollis* (Eichhoff) for which α-pinene was not only attractive but also enhanced response to its pheromone (Erbilgin and Raffa, 2000). Consistent attraction to α-pinene is shown by the red turpentine beetle, *Dendroctonus valens* LeConte (Erbilgin and Raffa, 2000), although (−) α-pinene inhibits response to (+) α-pinene. Little is known about feeding deterrents in tree-inhabiting Coleoptera (Byers, 1995). Ascher et al. (1975) found that females of *Scolytus rugulosus* (Müller) [= *mediterraneus* (Eggers)] were deterred from feeding on peach twigs that had been dipped in hexa-methylditin. The number of *I. pini* (Say) entering a phloem-based medium decreases with increased concentrations of many monoterpenes (Wallin and Raffa, 2000). Evaluation of antifeedants (e.g., carvone) against the large pine weevil, *Hylobius abietis* L., has shown antifeedant effects of nonhost bark compounds in both sexes (Klepzig and Schlyter, 1999). A higher sensitivity to repellents, however, must be expected in the host-selecting sex. When responding to prelanding signals, males of *I. typographus* are more sensitive to verbenone (Schlyter et al., 1989) and NHV (Zhang and Schlyter, 2004), whereas females of *S. rugulosus* are more susceptible to hexa-methylditin than males (Ascher et al., 1975). In Europe, the spruce bark beetle *I. typographus* is a major mortality factor in mature spruce forests (*Picea abies* Karsten) (Christiansen and Bakke, 1988). Recent research on control strategies for the protection of conifers has focused on the use of antiattractive semiochemicals such as NHV from nonhost trees, mainly angiosperms (Zhang and Schlyter, 2004).

The aim of the present study was to develop a protocol for testing the feeding performances of bark beetles on an artificial diet, as well as the evaluation of antifeedant effectiveness of host and nonhost compounds on *I. typographus*.

METHODS AND MATERIALS

Insect Breeding and Handling. Adults of *I. typographus* were removed daily from breeding cages kept in climatic rooms (25±1°C, RH = 70%). Specimens having emerged from colonized spruce logs (10-cm diameter, 30 cm long) were sexed (Schlyter and Cederholm, 1981) and starved at room temperature for 24 hr before being tested.

Pilot Studies on Artificial Diet and Tunnels. Preliminary assays were performed to find the best experimental protocol. Tests were carried out using narrow strips of fresh bark, cut from spruce logs and inserted into artificial

tunnels consisting of transparent glass tubes (3-mm diameter, 30 mm long). Feeding activity could not be easily quantified, and bark characteristics (thickness, moistness, age, and amount of cork) were extremely variable. Later, an artificial agar-based diet modified from Šimsek and Führer (1993) (composed of 87.6% water, 2% cellulose, 2.6% glucose, 4.3% starch, and 3.5% agar) was tested. Cellulose was obtained from dry spruce sawdust, whereas starch was added as reground maize flour. After a few minutes heating, the diet was poured into Petri dishes to a thickness of 13 mm; the diet was allowed to cool down for few minutes and was transferred by pressing the previously employed glass tubes into the cold diet (Figure 1), where it was left to dry. A direct estimate of the extent of adult host acceptance and feeding could be deduced from the length of diet removed. Because tunneling and diet removal does not necessarily mean feeding, during preliminary analyses several adults were dissected in order to detect ingested diet. The gut of all these previously starved insects contained diet particles.

Feeding Tests. Compounds used were grouped into two sets: those from nonhost trees, such as juglone, 3-octanol, 1-hexanol, and a blend of GLV, and those host compounds normally present in low (verbenone and carvone) or high (limonene and α-pinene) quantities in healthy host tissues (*P. abies*), (Table 1). Different concentrations of each compound, diluted in ethyl acetate, were tested

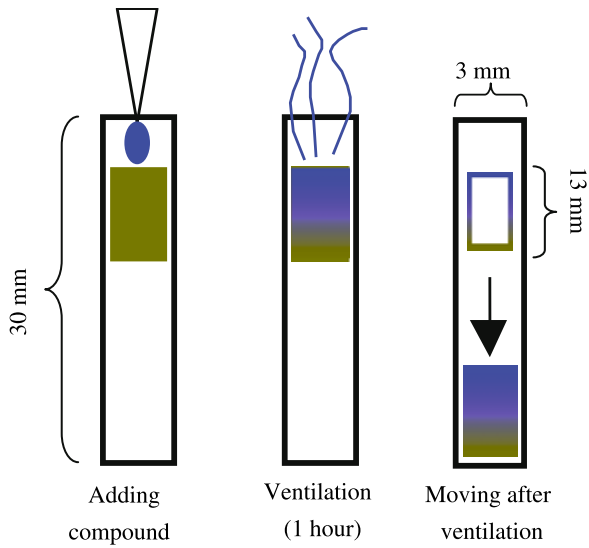


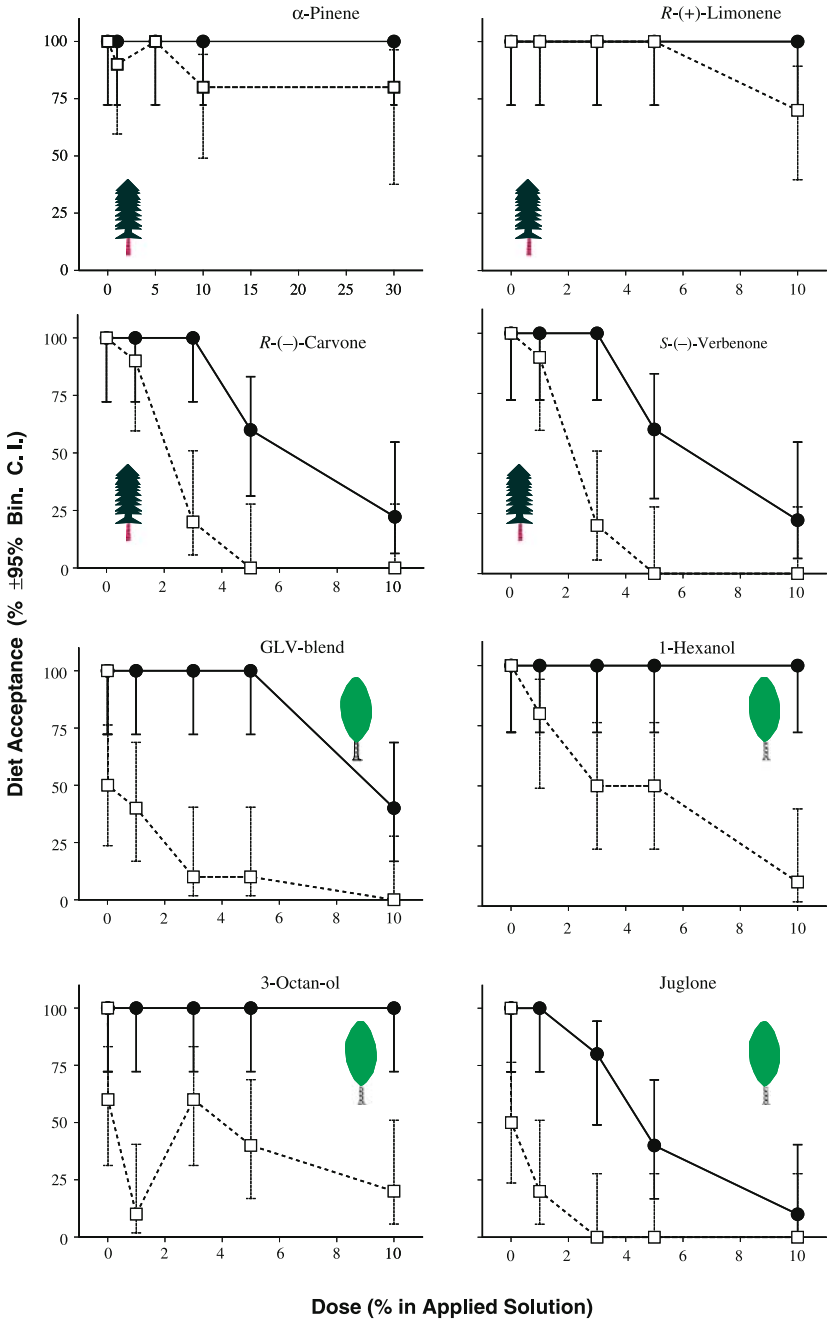
FIG. 1. Experimental protocol of adding treatment solutions to artificial diet in the glass tube "galleries".

TABLE 1. CHEMICAL AND COMMERCIAL PROPERTIES OF THE TESTED COMPOUNDS

Compound	Origin	Tested concentrations (% of applied solution)	Purity (% by GC)	Supplier
(±)-α-Pinene (low concentration)	Host	0.01–0.05–0.1–0.5	97.4	Acros, USA
(±)-α-Pinene (high concentration)	Host	1–5–10–30	97.4	Acros, USA
S-(−)-Verbenone	Host	0.1–1–3–5–10	75.2	Bedukian, USA
R-(+)-Limonene	Host	1–3–5–10	95.5	Aldrich, USA
R-(−)-Carvone	Host	1–3–5–10	99.7	Aldrich, USA
Juglone (5-Hydroxy-*p*-naphtoquinone)	Nonhost	0.1–1–3–5–10	ca 97	Aldrich, USA
(±)-3-Octanol	Nonhost	0.1–1–3–5–10	96.4	Acros, USA
1-Hexanol	Nonhost	0.1–1–3–5–10	96.4	Aldrich, USA
GLV-blend (1:1:1 of 1-hexanol, (*Z*)-3 Hexen-1-ol, and (*E*)-2-Hexen-1-ol)	Nonhost	0.1–1–3–5–10	86.8 (30:29:27)	Aldrich, USA

on adults of *I. typographus* (Table 1). Each concentration was tested individually on 20 adults (10 males and 10 females). A 10-µl solution of each compound (Table 1) or the solvent (blank) was added separately to the tube. The latter was kept open for 1 hr at 21°C for ventilation, thus allowing the solvent to completely evaporate (Figure 1). Subsequently, one beetle was inserted into each tube, closed with a plastic cap, and was allowed to feed on the diet for 4 hr under illumination. Previous tests have shown that 4 hr are sufficient for insects to tunnel through a diet. Each beetle was used only once. The amount of diet consumed was measured (mm) by using a graduated stereoscope. For each compound, 20 adults (10 males and 10 females) feeding in tubes containing only diet (solvent blank) were used as control.

Statistical Analysis. Data were analyzed by ANOVA to find differences between sexes, compounds, and their concentrations. Homogeneity of variance was tested using Cochran's test, and when necessary, data were log-transformed [$X' = \log(x + 1)$] or arcsin-transformed ($X' = \arcsin\sqrt{P_x}$) to obtain homogeneous variances. Wherever significant differences occurred, Tukey's honestly significant difference (HSD) multiple comparison test was applied for mean separation (Zar, 1984). Differences at $P \leq 0.05$ were considered significant for ANOVA and for the Effective Threshold (ET) concentration, at which the amount of eaten diet becomes statistically lower than the control. For each compound and concentration, an Antifeedant Index (AFI) varying between −1 (attraction) and 1 (repulsion) (Klepzig and Schlyter, 1999) was calculated as follows: $\text{AFI} = (C - T)/(C + T)$, where T = amount of diet consumed in the


α-Pinene
R-(+)-Limonene
R-(–)-Carvone
S-(–)-Verbenone
GLV-blend
1-Hexanol
3-Octan-ol
Juglone
Diet Acceptance (% ±95% Bin. C. I.)
Dose (% in Applied Solution)

tested treatment and C = amount of diet consumed in the control. In order to compare the effect of active compounds, Effective Dose 50 (ED50) was calculated. The ED50 concentration was obtained from the linear regression of AFI against the concentration.

RESULTS

Most tested chemicals decreased the feeding activity of adults of *I. typographus*, with an effect that was concentration-dependent. Sex was a factor both in diet acceptance and in feeding.

Diet Acceptance. Nonhost compounds had a strong effect on diet acceptance, showing a high level of rejection. At high concentrations, a variable percentage of specimens did not feed at all (Figure 2), whereas in the controls (solvent blanks), all insects feeding. Independently from compound and concentration, diet acceptance was always higher in females than in males (Figure 2). In several cases, males showed a concentration dependence trend similar to that of females, although male rejection started at lower concentrations compared to females. Host compounds, such as α-pinene and limonene, always showed a high feeding frequency (Figure 2), and reduction in feeding started only at very high doses (30% and 10%, respectively).

Diet Consumption. Diet removal was affected by all three of the considered factors: sex, dose, and compound (Table 2). Interactions among these factors were significant, indicating differences in concentration–response slopes due to both sex and compound.

Sex. Sex was the most important factor affecting consumption (Table 2). Males were more susceptible to antifeedants than females, and all compounds showed clear statistical differences between sexes, with the highest feeding responses found in females (Figure 3). Different compounds and doses had different effects on feeding responses of males and females (Table 2 and Figure 3). ED50 was always significantly higher in females, indicating that both host and nonhost compounds had a greater effect on male feeding (Table 3). Feeding performed on control (concentration = 0) showed no consistent difference between sexes (Figures 2 and 3).

FIG. 2. Diet acceptance frequency in the sexes, estimated as the proportion of insects showing >0 mm removal of diet (feeding) in glass tubes after 4 h. Binomial 95% Confidence Intervals. Females: —●— and males: - - -□- - -. Tree symbols, dark or pale, indicates compound origin from gymnosperm host or angiosperm non-hosts, respectively. Only *x*- axis values common to all tested compounds are plotted (i.e., values like 0.1% are not plotted).

TABLE 2. ANOVA OF FACTORS COMPOUND, DOSE, AND SEX AND THEIR INTERACTIONS IN THEIR EFFECT ON THE LENGTH OF DIET REMOVED IN TUNNEL FEEDING BY *Ips typographus*

Factor	*Df* effect	*Df* error	*F* value	*P* level[a]
Compound	7	732	136.06	*
Dose	4	732	286.82	*
Sex	1	732	685.63	*
Compound × dose	28	732	14.05	*
Compound × sex	7	732	8.69	*
Dose × sex	4	732	47.11	*
Compound × dose × sex	28	732	5.86	*

[a] The ANOVA was applied on $\log(x + 1)$ transformed data in order to approach normal distributions. The homogeneity of variances was tested using Cochran's test (Zar, 1984).
* $P < 0.001$.

Dose. Concentration was the second most important factor affecting diet removal (Table 2). In general, all compounds showed an antifeedant effect that increased with dose, although this was always stronger in males (Figure 3). In some cases [e.g., (−)-carvone, (−)-verbenone, and GLV-blend], differences between sexes disappeared at high concentrations, as the effects were strong enough to reduce even female feeding activity (Figures 2 and 3). Compounds and dose showed a significant interaction (Table 2), thus establishing that different compounds are active at different concentrations (Figure 3). Furthermore, *I. typographus* showed a variable sensitivity to compounds, which became effective (ET) at different concentrations (Table 3). For males, the ET was reached at the lowest dose (0.1%) of many compounds.

Compound. Host compounds always had higher ET and ED50 values than nonhost compounds, indicating lower antifeedant activity. Host monoterpenes, such as α-pinene and limonene, showed low activity as antifeedants; α-pinene at low doses (<1%) acted as a weak stimulus for females (Figure 3), whereas limonene affected diet consumption in females at the highest concentrations only. Oxygenated host monoterpenes (carvone and verbenone) showed similar trends (Figure 3), both having a strong antifeedant effect with a relatively high AFI (Table 3), which depended upon concentration (Figure 4). Nonhost compounds were the more active (Table 3 and Figure 2), with the strongest effect by 3-octanol, which showed both the lowest ED50 value (1.2%) and the highest mean AFI (0.6). Moreover, feeding inhibition caused by nonhost compounds increased rapidly and in a nonlinear way with increasing concentrations, especially for males (Figure 3).

Relative Feeding (AFI). In general, with the exception of α-pinene at low concentrations, mean AFI was always positive, indicating overall feeding

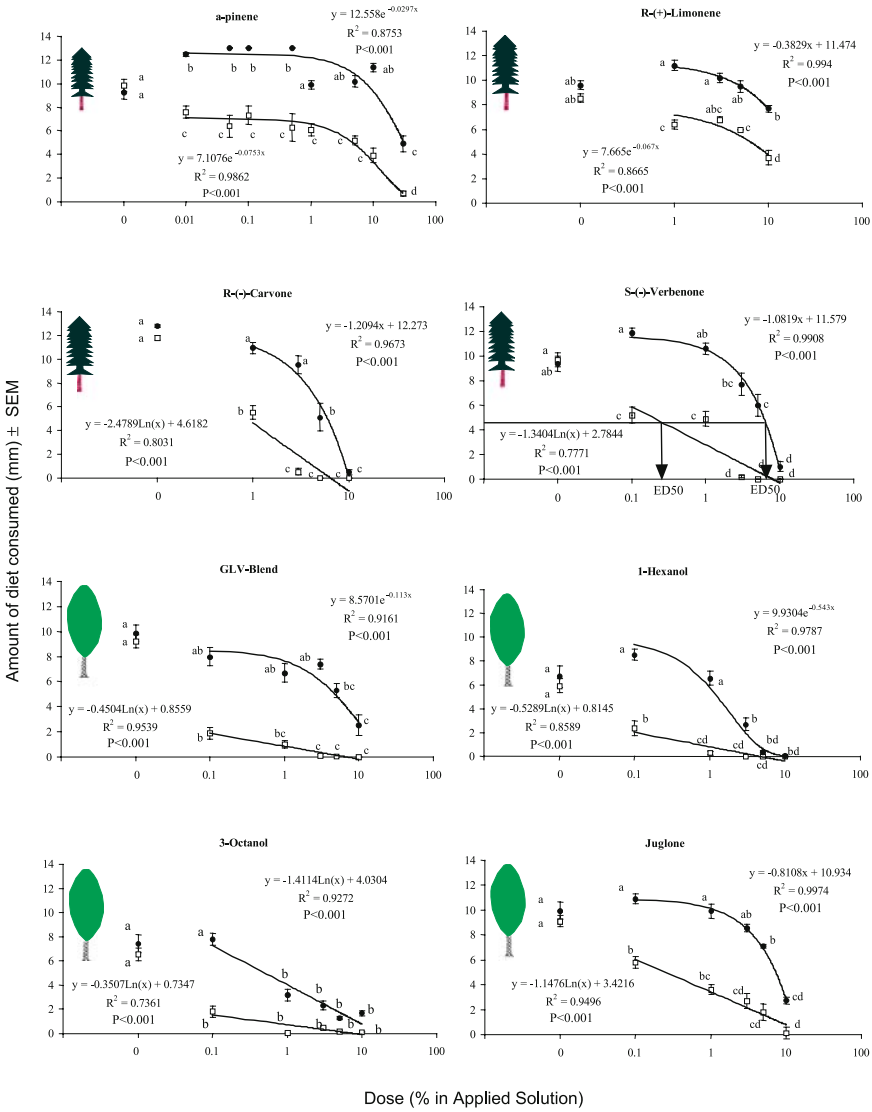


FIG. 3. Linear regressions of *Ips typographus* feeding activity for each tested compound. Females: ●, males: □. ED50: Effective Dose halving the feeding performances of the insects (indicated only for Verbenone as an example, see Table 3). For overall means per compound and sex, see Table 2. Data points with the same letter are not different by ANOVA on log(x + 1) followed by the Tukey's honestly significant difference post hoc test at α = 0.05.

TABLE 3. EFFECTIVE THRESHOLD (ET), EFFECTIVE DOSE 50 (ED50), AND ANTIFEEDANT INDEX (AFI) VALUES CALCULATED PER EACH TESTED COMPOUND

Compound	ET[a] males	ET females	ED50[b] males	ED50[b] females	AFI[c]
α-Pinene (low conc.)	–	–	–	–	−0.002
α-Pinene (high conc.)	1	30	6.4	34.7	0.21
S-(−)-Verbenone	0.1	10	0.2	6.4	0.39
R-(+)-Limonene	5	–	8.7	17.4	0.09
R-(−)-Carvone	1	5	0.6	4.8	0.56
Juglone	0.1	5	0.4	7.4	0.31
3-Octanol	0.1	1	0.001	1.2	0.61
1-Hexanol	0.1	3	0.02	2.2	0.59
GLV-blend	0.1	5	0.0002	4.9	0.50

[a] Effective Threshold: compound concentrations at which the feeding performances of *Ips typographus* become significantly lower than control ($P \leq 0.05$) by ANOVA test on $\log(x + 1)$ applied to diet removal.
[b] Effective Dose 50: compound concentrations (%) at which the feeding performances of *Ips typographus* become half than control (Figure 2).
[c] Mean of all concentrations of the Antifeedant Index calculated as $(T - C)/(T + C)$ (Klepzig and Schlyter, 1999).

inhibition (Table 3). AFI increased with compound concentration (Figure 4). High concentrations of carvone, verbenone, and 1-hexanol gave the highest AFI values (≈ 1), meaning total inhibition of feeding. In contrast, α-pinene and limonene showed low antifeedant indexes even at high concentrations. The moderately negative AFI values reported for α-pinene at several low concentrations (Figure 4) indicate a weak stimulating effect on feeding, especially females (Figure 3). At high concentrations (10% and 30%), most compounds showed strong antifeedant effects (AFI ≥ 0.5), whereas at low concentrations (1–3%), strong effects were obtained only for 1-hexanol, GLV-blend, and 3-octanol.

DISCUSSION

Several studies have demonstrated that conifer phloem contains extractable compounds that elicit feeding behavior in bark beetles. *Ips paraconfusus* Lanier recognizes susceptible ponderosa pines after having entered the phloem (Elkinton et al., 1981). Outer bark extracts of the host pine species stimulate feeding behavior in both the southern pine beetle, *Dendroctonus frontalis* Zimmermann (Thomas et al., 1981), and the mountain pine beetle, *D. ponderosa* Hopkins (Raffa and Berryman, 1982). Feeding stimulants have been identified also for bark beetles attacking angiosperms such as *Scolytus*

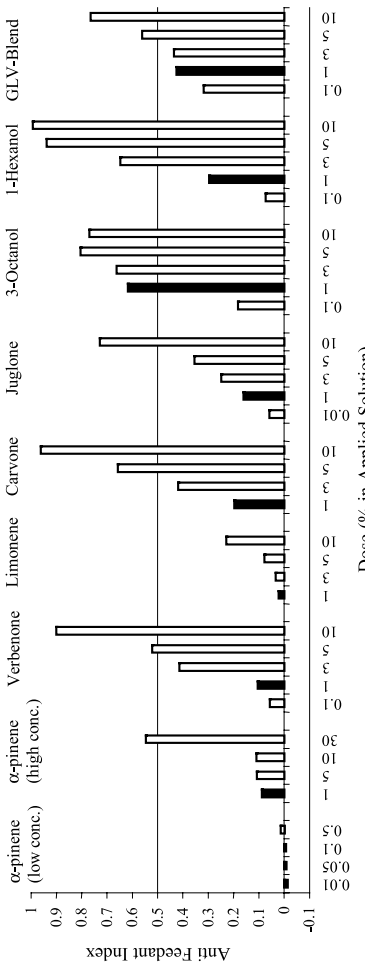


FIG. 4. Antifeedant Index calculated for each compound and concentration (black columns correspond to the 1% concentration), sexes joined.

multistriatus (Marsham) and *S. rugulosus* (Doskotch et al., 1970; Levy et al., 1974). Literature data suggest that phloem metabolites are mainly responsible for feeding stimulation (Bedard, 1966; Elkinton et al., 1981; Byers, 1995). McNee et al. (2003) reported that several chemicals extracted from ponderosa pine phloem, such as stilbenes, ferulic acid gluciside, and sugars, neither stimulated nor reduced male feeding activity in *I. paraconfusus*. Nevertheless, few studies have tested the possible antifeedant effect of host extracts on bark beetles. In the present study, an antifeedant effect, strongly dependent on sex, concentration, and origin (host or nonhost) of the compound, was established.

No significant differences between sexes in antennal responses to NHV have been recorded previously (Zhang and Schlyter, 2004), although a higher antennal sensitivity (lower response threshold) would be expected for the host-selecting sex (the males in the case of *I. typographus*). In this respect, Dickens (1981) reported that the male antennae of *I. typographus* were 10 times more sensitive to α-pinene than females. Rudinsky et al. (1971) reported that α-pinene and limonene attracted *I. typographus* adults in a ratio that favored males. High concentrations of verbenone and NHV skew the sex ratio of *I. typographus* trap samples towards females (Schlyter et al., 1989; Zhang and Schlyter, 2004), being more repellent to males. It was thought that the verbenone released by tunneling males could counteract the effect of the aggregation pheromone and shift the attack to uninfested neighboring trees (Schlyter et al., 1989). Comparing male and female feeding responses, controls did not generally show significant differences between sexes. However, when exposed to nonhost compounds, male feeding was inhibited at relatively low doses, ranging between 1 and 3%. Nevertheless, high doses of verbenone, carvone, and 1-hexanol decrease female feeding responses as well.

Terpenoids play a fundamental role in host acceptance by conifer-inhabiting bark beetles (Byers, 1995). Limonene, α-pinene, and β-pinene are involved in preexisting and induced defenses against bark beetles (Baier et al., 1999). Compared to many other insects, conifer bark beetles are relatively immune to toxic terpenes, although they can still be lethal at high doses (Everaerts et al., 1988). Some experiments suggest that some monoterpenes are still sufficiently toxic to bark beetles as to significantly influence their ecology. Studies by Sturgeon (1979) of the *P. ponderosa*/*D. brevicomis* LeConte association suggest that tree resistance is linked to higher limonene contents. In a study similar to ours, Wallin and Raffa (2000) found that the number of *I. pini* that entered a phloem-based medium decreased with increased concentrations of most monoterpenes. In particular, the total length of tunnels excavated in the medium decreased with increasing concentrations of α-pinene and limonene (Wallin and Raffa, 2002). Regarding host-tree status, high monoterpene concentrations correspond to trees that have begun to respond to an attack, whereas lower concentrations likely represent constitutive phloem

from unattacked trees (Erbilgin and Raffa, 2000). Long exposure to α-pinene- and limonene-saturated vapors can also be lethal for *I. typographus* adults (Everaerts et al., 1988). Smelyanets and Vasechko (1973), studying the chemotaxis of *I. typographus* to terpenoids, found α-pinene to be repellent at concentrations higher than 3%, whereas limonene was attractive only at concentrations of 0.2–0.6% and repellent above 3–4%. Verbenone, which in many bark beetle species is usually released by tunneling males as a repellent for colonizing adults, shows a concentration-dependent repellence pattern (Schlyter et al., 1989). No previous studies concerning the antifeedant characteristics of verbenone has been conducted. We observed a strong repellent effect at high concentrations, especially to males.

In general, compounds from nonhost trees have strong antifeedant effects. Green leaf volatiles (GLVs) are aliphatic 6-carbon primary alcohols, aldehydes, and acetates found in broad-leaved trees (Visser, 1986). In our experiment, the GLV-blend showed an effective threshold starting at low concentrations. Moreover, *I. typographus* antennae strongly respond to 1-hexanol (Zhang and Schlyter, 2004). Similar responses have also been found in *D. ponderosae*, *Tomicus piniperda* (L.), *T. minor* (Hartig), *I. duplicatus* (Sahlberg), and *I. sexdentatus* (Boerner) (Zhang and Schlyter, 2004). In our experiment, males of *I. typographus* were strongly affected by hexanol and GLV, indicating a possible sex-specific effect of one or more of these compounds. Among the tested nonhost chemicals, 3-octanol is an 8-carbon alcohol extracted from the bark of European birch species (*Betula pendula* and *B. pubescens*) and aspen (*Populus tremula*) (Zhang et al., 2000). This alcohol showed the strongest effect on the feeding responses of *I. typographus*, being active at low concentrations (0.1%). Finally, juglone is a quinone derivative (5-hydroxy-1,4-naphthoquinone) already known to inhibit the feeding activity of *Periplaneta americana* (L.) and *S. multistriatus* by reacting with aminoacids, especially cysteine (Ferkovich and Dale, 1971). In our study, however, juglone only showed a moderate antifeedant effect. Following starvation, the need for water induces beetles to ingest nonhost or neutral diet, thus masking low antifeedant properties of some compounds. As we found some antifeedant activity in all compounds, the optimal starvation period before test might be longer than that applied in our study. Raffa (1988) hypothesized that phloem colonization does not continue in the presence of repellents, but progresses in the absence of stimulants. This would indicate repellents as the key factor influencing tree colonization. Our results support that hypothesis.

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DO NAÏVE RUMINANTS DEGRADE ALKALOIDS IN THE RUMEN?

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Abstract—Three different methods for the culture of rumen microorganisms (Hungate's technique, the Hohenheim *in vitro* gas production method, and the semicontinuous rumen simulation technique) were employed to study the influence of various alkaloids (sparteine, lupanine, cytisine, atropine, quinidine, lobeline, harmaline, arecoline, nicotine, caffeine, pilocarpine, gramine, senecionine, and monocrotaline) on rumen microorganisms. Rumen microorganisms from naïve ruminants (sheep, cattle) that had not been exposed to the alkaloids before were generally not able to degrade most of the alkaloids. Only the alkaloids pilocarpine, gramine, and monocrotaline appeared to be degradable. Rumen microorganisms from a sheep preconditioned to lupin alkaloids tolerated lupanine much better than nonadapted microorganisms, but no degradation occurred. The findings indicate that the main site of detoxification in naïve ruminants is not the rumen but more likely the liver and kidneys as in nonruminants.

Key Words—Rumen, ruminants, detoxification, alkaloids, alkaloid degradation, adaptation.

INTRODUCTION

Herbivores are often confronted with toxins in their diet and have evolved various mechanisms during evolution to overcome plant defenses (Harborne, 1993; Wink, 1993). Whereas some poisons are detoxified or degraded in the digestive tract, many others are metabolized by enzymes primarily after absorption. In vertebrates, the liver is the first postabsorptive organ and thus, responsible for the detoxification of xenobiotics. In most animals, the inducible

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microsomal mixed function oxidase (MFO) system, including the cytochrome P-450, is the major metabolizing enzyme system (Freeland and Janzen, 1974; Smith, 1992). These enzymes are substrate nonspecific and are predominantly concentrated in the liver and kidney of vertebrates. Some mammalian herbivores avoid toxicity of plant secondary metabolites by inactivating them before they reach the digestive tract. Deer are known to produce a salivary protein that binds tannins, thus allowing the animals to feed on tannin-rich plants (Robbins et al., 1987). The digestive tract itself is another potential site of detoxification. Ruminants and other foregut fermenters, for example, have evolved complex stomachs with a fermentation chamber, e.g., the rumen, where food particles are degraded by a diverse bacterial and protozoal community. The main benefits of a foregut fermentation system are the microbial degradation of cellulose and the microbial *de novo* biosynthesis of proteins. Nevertheless, many authors suggest that microbial detoxification of xenobiotics has played an important role in the evolution of the foregut fermentation system (Freeland and Janzen, 1974; Van Soest, 1994).

Plants containing alkaloids are widespread in the plant kingdom, and due to their toxicity (review: Wink 1993, 2000), many of them represent an important farming problem and are responsible for extensive poisoning of both humans and livestock. In the western USA, severe livestock losses result from the poisoning by tansy ragwort, *Senecio jacobaea*, which contains pyrrolizidine alkaloids (Huxtable, 1979; Wachenheim et al., 1992a,b). Larkspur (*Delphinium* sp.) is responsible for more cattle death than any other poisonous plant in the USA (Cheeke, 1998), and the plants known as locoweed, *Astragalus* sp. and *Oxytropis* sp., which contain swainsonine and other indolizidine alkaloids, are responsible for the neurological syndrome known as "locoism" in mammals (Molyneux and James, 1982). The lupin alkaloids anagyrine and ammodendrine are present in numerous lupin species in the USA and are of particular importance because of their teratogenic effect in cattle, causing skeletal deformities referred to as "crooked calf disease" (Cheeke and Kelly, 1989). In Afghanistan, over 1600 people were poisoned in 1974 by consuming wheat contaminated with seeds of *Heliotropium*, which contain high amounts of pyrrolizidine alkaloids (Mohabbat et al., 1976). *Heliotropium europaeum* and *Echium plantagineum*, both containing pyrrolizidine alkaloids, are responsible for poisoning of poultry (Pass et al., 1979), cattle (Harper et al., 1985), sheep (Seaman, 1985), horses (Giesecke, 1986), and pigs (Jones et al., 1981) in Australia. Often ruminants are the target of poisoning. This is somewhat surprising since ruminants have several potential detoxification systems (rumen, liver). We were interested in studying the role and efficiency of the rumen in detoxifying dietary toxins, especially alkaloids.

According to the position of the enlarged chamber in the digestive tract, herbivores are divided into two groups. In hindgut fermenters, the microbial

action occurs after the stomach and the small intestine and takes place in the cecum or large intestine (e.g., rabbits and horses), which is anatomically similar to the organs of carnivores and omnivores but much enlarged. In foregut fermenters, the chamber precedes the stomach with its pepsin digestion and the small intestine and is either a separate organ, e.g., the crop in the hoatzin (*Opisthocomus hoazin*), the only avian with foregut digestion, or part of a complex stomach not found in carnivores or omnivores, e.g., the rumen in ruminants (Hungate, 1988; Van Soest, 1994). Other ruminant-like digestive systems without a rumen have been found in a wide variety of other herbivores: the hoatzin, *Colobus* and *Macaca* monkeys, and camelids, and many of them are regarded as "functional ruminants" (Van Soest, 1994).

Cellulose, hemicellulose, pectins, fructans, starches, and other polysaccharides are hydrolyzed by rumen microbes to monomeric and dimeric sugars, which are further fermented to acetic, butyric, and propionic acids, methane, and carbon dioxide (Bryant, 1977; Van Soest, 1994). The gases are released by the animal and are essentially waste products, but the acids are absorbed through the rumen wall into the bloodstream and finally converted into sugars and lipids required for energy and tissue building. Proteins are hydrolyzed to amino acids and peptides, which are then deaminated to ammonia and fatty acids. The latter are further metabolized, whereas most of the bulk of ammonia is absorbed through the rumen wall to be converted into urea (Stewart et al., 1997).

The rumen ecosystem is composed of a large number of different species of bacteria, protozoa, and fungi. Rumen bacteria are predominantly strict anaerobes, although a few facultative anaerobes exist (Van Soest, 1994). Strict anaerobic bacteria are present in the rumen in numbers of about 10^{10} ml^{-1} and, together with the rumen Archaea (methanogens), are considered as the true rumen bacteria (Stewart et al., 1997). Methanogens form methane from H_2, CO_2, or formate (Hungate et al., 1970). The bacteria account for about half of the total biomass in a normal rumen but are responsible for most of the metabolic work (Van Soest, 1994; Stewart et al., 1997). There is little information concerning geographical and interspecific differences in the composition of rumen bacterial communities. Most studies in wild ruminants have been limited to microscopic observations on morphology and the Gram stain. According to Yokohama and Johnson (1988), the major species of bacteria are ubiquitous in ruminants, and there is no evidence of host specificity. *Synergistes jonesii*, a bacterium capable of detoxifying the hydrolysis products of nonprotein amino acids, such as mimosine, 3-hydroxy-4(1*H*)-pyridone (3,4-DHP), and 2,3-dihydroxypyridine (2,3-DHP), is the only well documented case of geographical differences in rumen bacterial community composition (Jones and Megarrity, 1983, 1986; Allison et al., 1990; Hammond, 1995). *S. jonesii* was isolated from the rumen of Hawaiian goats and was absent in Australian ruminants.

Protozoa are the most conspicuous organisms in the rumen. Unlike bacteria, they are not essential for the survival of the host ruminant. The most obvious and most important protozoa in the rumen are the ciliates, of which two different groups are present, both in the subclass *Trichostomatia*, the holotrich protozoa that belong to the order *Vestibuliferida*, and the entodiniomorphs that belong to the order *Entodiniomorphida* (Williams and Coleman, 1992). The latter are well adapted to the rumen environment and utilize particulate rather than soluble food materials. In contrast, the holotrichs can use mostly soluble food materials and are more aerotolerant. Most protozoa, if not all, have bacteria in vesicles in their cytoplasm, and there is evidence that the species present reflect those in the surrounding medium and include methanogens (Finlay et al., 1994; Lloyd et al., 1994). Population densities of protozoa in the rumen under normal conditions are on the order of 10^4–10^6 ml^{-1} (Williams and Coleman, 1992) and form a large proportion of the rumen microbial biomass (20–40% of net microbial nitrogen); yet their output may be minimal because of high retention and slow turnovers (Williams and Coleman, 1992; Van Soest, 1994).

The presence of anaerobic fungi in the rumen has been known since the 1970s (Orpin and Joblin, 1997). Before that, swimming zoospores were mistaken for flagellated protozoan. The contribution of fungi to the microbial biomass may be small, and their major function in the rumen may be in causing or facilitating cell wall disappearance (Van Soest, 1994).

The benefits derived from symbiosis with rumen microbes have long been recognized and include predominantly the transformation of dietary constituents into nutrients needed for growth, milk production, and energy. Because of the observation that ruminants are often more resistant to plant toxins and other xenobiotics than nonruminants, several authors have proposed that the degradation of toxic compounds present in the diet of the ruminant is conducted by the rumen microorganisms (Carlson and Breeze, 1984; Dawson et al., 1997; Cheeke, 1998; Weimer, 1998). Similarly, other authors proposed that these detoxification activities might have been a driving force during the evolution of the foregut fermentation digestive system (Freeland and Janzen, 1974; Van Soest, 1994). Sometimes, degradation activities are related to adaptive changes in the rumen microorganism populations, which result in an acquired resistance to specific toxins (Duncan et al., 2000; Duncan and Milne, 1992; Blythe and Craig, 1994; Dawson et al., 1997; Newbold et al., 1997; Odenyo et al., 1997).

In this investigation, we examined the hypothesis that rumen microbes are involved in detoxification processes in ruminants (Freeland and Janzen, 1974; Van Soest, 1994). For this purpose, the degradation of a set of 14 alkaloids (sparteine, lupanine, cytisine, atropine, quinidine, lobeline, harmaline, arecoline, nicotine, caffeine, pilocarpine, gramine, senecionine, and monocrotaline), as one of the most prominent groups of toxins, by rumen microorganisms was analyzed *in vitro*. Three more widely used methods in the study of rumen processes were

employed: batch cultures using Hungate's technique, the Hohenheim *in vitro* gas production method (HFT, "Hohenheimer Futterwert Test"), and the semi-continuous rumen simulation technique (RUSITEC).

METHODS AND MATERIALS

Alkaloids. Except for lupanine and senecionine, all alkaloids were purchased from Sigma (Munich, Germany) or Roth (Karlsruhe, Germany). Senecionine was a gift from R. Molyneux (USDA). Lupanine was isolated and purified from seeds of bitter lupins (*Lupinus angustifolius*). Alkaloids were dissolved in PBS buffer. In order to improve the solubility of some alkaloids, the pH of the stock solution was acidified with HCl. The effect of the pH of the alkaloid solutions on the respective assays was tested using the same volume of test solution without alkaloid but at the same pH.

Isolation and Quantification of Alkaloids. Samples were dissolved in 20 ml, 0.5 M HCl and incubated overnight under agitation and in darkness. In order to detect the potential presence of N-oxides, 100 mg of zinc powder were added to the corresponding samples, and these were stirred for another 3 hr under continuous agitation. The solution was made alkaline with 6 M NaOH (pH 12). Samples were extracted using a solid–liquid phase system with Isolute (ICT) as solid and CH_2Cl_2 as liquid phase. The dichloromethane extracts were collected in round-bottom flasks and evaporated under vacuum (890 mbar) at 40°C. Alkaloids were recovered from the flask with 2–3 ml CH_2Cl_2 and transferred to a vial. The solvent was evaporated under N_2 flow or overnight in a hood and protected from light. Dried alkaloid samples were stored at −20°C until GLC analysis.

Two Carlo Erba gas chromatographs were used for the GLC analysis. The first gas chromatograph (series 5100) was equipped with a DB-1 capillary column (J&W, 15 m long and 0.25 mm inner diam) and the second GLC (series 6000) with an OV-1 column (Ohio Valley, 15 m long and 0.25 mm inner diam). Both GLCs were equipped with flame ionization detectors (FID). The split was 1:5 and 1:10, respectively. Helium was used as carrier gas. The temperature of the injector and the detector were 250 and 300°C, respectively. A 1-µl aliquot of each sample was injected into the gas chromatograph for analysis. The oven temperature program used for each alkaloid was 150°C for 3 min, 150–250°C at 15°C/min, and 250–300°C at 25°C/min for lupanine, sparteine, atropine, quinidine, harmaline, senecionine, monocrotaline, and pilocarpine, and 80°C for 2 min, 80–150°C at 10°C/min, and 150–300°C at 20°C/min for arecoline, caffeine, nicotine, gramine, and lobeline. Alkaloids were quantified by using external standards consisting of a solution in methanol.

Alkaloids were identified by GLC-MS using authentic alkaloids as reference. For GLC-MS, an OV-1 fused silica capillary column (30 m × 0.25 mm) was used coupled to a quadrupole Finnigan Mat 4515 mass spectrometer. EI-MS were recorded at 40 eV and evaluated with the INCOS data system. The conditions are as follows: carrier gas He; splitless injection; temperature 250°C; oven temperature program—initial temperature 120°C, 3 min isothermal, 120–300°C, 8°C/min.

Rumen Microbial Culture Techniques. Rumen microbes are sensitive to their environment, and successful cultures must satisfy both environmental and nutritional requirements. In *in vitro* cultures, all products accumulate in the system; thus, it is necessary that the maximum levels of acidic products remain below the limiting levels of osmotic pressure and buffer capacity, especially in closed systems. For this reason, *in vitro* cultures must be diluted by about an order of magnitude with respect to both feed and organisms to avoid hyperacidity and high osmotic pressures (Van Soest, 1994).

Diluting the substrate in the culture medium increases the susceptibility of fermentation to traces of oxygen. Therefore special precautions must be taken to protect dilute concentrations of rumen microorganisms from oxygen contamination. The methanogens and pure cultures are especially sensitive to traces of oxygen (Van Soest, 1994), but batch cultures in small volumes, such as the Hungate tubes and the syringes in the "Hohenheim *in vitro* gas production method" (HFT), also require an oxygen-free atmosphere (Hungate, 1969; Van Soest, 1994). Some anaerobes and the methanogens cannot initiate growth at redox potentials greater than -0.33 V, which corresponds to 10^{-75} of the concentration of oxygen in the atmosphere or 2.5×10^{-80} mmol O_2/l (Hungate, 1969). Normally, most of the oxygen is removed from the medium by boiling followed by gassing out with CO_2, and any residual oxygen is removed by adding cysteine. Commercial sources of CO_2 usually contain traces of oxygen and need to be purified by passing the gas through heated copper turnings (Hungate, 1969).

Hohenheim In Vitro Gas Production Method or HFT. The assay consisted of rumen batch cultures kept in glass syringes and incubated for up to 2 d in a water bath. The method was based on Menke et al. (1979). To determine the fate of lupanine and sparteine incubated *in vitro* with rumen liquor and their effect on fermentation parameters, 500-mg dry matter of hay has incubated in triplicate in 100-ml graduate glass syringes (Fortuna, Germany) containing 40 ml of the *in vitro* medium with bovine or ovine rumen liquor. Syringe pistons were lubricated with Vaseline to facilitate their movement allowing space for the increasing gas volume in the syringe. Two control sets were prepared: control 1 containing no hay and control 2 containing no rumen liquor. Alkaloids were added to the treatment and control syringes at a concentration of 1 mM. Three syringes were used as blank and were incubated without alkaloids. All

syringes were incubated under anaerobic conditions at 37°C for 36 hr and shaken manually every hour for the first 6 hr and every 3 hr for the rest of the incubation period in order to avoid the accumulation of hay on the superficies of the medium. Three syringes were collected every 6 hr for the treatment group and every 12 hr for each control group. Blank syringes were incubated for 36 hr. The content of each syringe was immediately acidified to pH 3 with 0.5 ml, 7 M HCl in order to stop any microbial reaction and then stored at −20°C for later analyses. These assays were performed at the Institute for Animal Production in the Tropics and Subtropics, University of Hohenheim.

Preparation of Culture Medium for HFT. Rumen inoculum was collected from a cow and two sheep fitted with permanent rumen fistulas, kept at the University of Hohenheim (Germany), and fed on a roughage-based diet. Rumen contents were obtained through the fistulas and immediately strained through sterile cheesecloth into insulated prewarmed thermoses, avoiding formation of air bubbles. Thermoses were transported to the lab within 15 min, where the experiments were started immediately.

Culture medium was prepared under anaerobic conditions. Constantly bubbling the medium with CO_2 (Messer Griesheim GmbH 4.5), previously passed through a column of heated cooper (350°C) in order to remove any oxygen traces (Hungate, 1969), provided acceptable anaerobic condition in the medium. Rumen liquor was added to the warm culture medium (37°C) under constant agitation and gas bubbling. The composition of the culture medium was as outlined by Menke et al. (1979).

Adaptation of Rumen Microorganisms to Alkaloids Present in the Diet. To investigate the possible induction of lupanine and sparteine degradation metabolism in rumen bacteria, two merino sheep were fed during 4 wk on a diet containing increasing amounts of seeds of alkaloid-rich *L. angustifolius* (lupanine was the major, 13-hydroxylupanine and angustifoline were additional alkaloids). During the first week, the sheep (40-kg weight) obtained 2.5 mg/kg alkaloids; the dose was increased via 5, 10, and 20 mg/kg during weeks 2, 3, and 4, respectively.

Rumen Batch Cultures using Hungate's Technique. Alkaloids were studied *in vitro* following the method described in Hungate (1969). Hungate tubes with butyl rubber stoppers (Bellco Glass) were filled with 5-ml culture medium 2 (Hobson, 1969), medium RA-1, or medium RA-2 under anaerobic conditions, and inoculated with 100-µl diluted fresh rumen liquor. An aliquot of concentrated alkaloid solution was injected into each tube in order to reach a final concentration of 1 and 10 mM. Rumen liquor was obtained as explained before and diluted 1:9 in medium 2. Controls without bacteria (blanks) or alkaloids were performed. All cultures were incubated at 37°C. Three tubes from each treatment and from the control group without rumen inoculum were collected after 0, 2, and 7 d in order to measure the alkaloid contents. An aliquot (100 µl)

of each 7 d-old culture was inoculated in fresh medium under the same conditions. The alkaloid content of the refreshed cultures was measured 0, 2, and 7 d later. An aliquot of 200 μl, 6 M HCl was added to each collected tube to stop any bacterial activity. Samples were then stored at −20°C.

Culture solution was boiled and bubbled with CO_2 (Messer Griesheim GmbH 4.5) under constant agitation with a magnet until the pink color of the resazurine disappeared (10–15 min). Resazurine is used to indicate oxidation–reduction potentials above −0.042 that are known to inhibit the growth of most ruminal strict anaerobic bacteria (Hungate, 1969). The culture medium was allowed to cool to about 60°C under CO_2 flow using a cold-water bath before cysteine was added as a reducing agent. Culture medium was distributed with a glass pipette into Hungate tubes, which were gassed for 30 sec with CO_2. Gassing was maintained for 30 additional sec after the medium was inoculated. The tubes were hermetically closed with the butyl rubber stoppers and autoclaved for 15 min at 121°C. Afterwards, culture tubes were stored at room temperature and darkness until used. Tubes in which the medium had turned to a reddish color were discarded. Purified rumen fluid was obtained by autoclaving (121°C, 15 min) and centrifuging rumen liquor at 4000 rpm for 30 min.

Rumen Simulation Technique. Since the rumen is a form of open and continuous culture, many researchers have attempted to establish *in vitro* continuous cultures of mixed rumen microorganisms with a volume of more or less whole rumen contents as the starting "inoculum." The most important of these methods is the RUSITEC (Czerkawski and Breckenridge, 1977, 1979a,b; Cheng and McAllister, 1997), allowing the culture of bacteria, protozoa, and fungi that inhabit the different physiological compartments of rumen for an almost indefinite period of time (Van Soest, 1994).

The RUSITEC technique is a semicontinuous rumen fermentation system consisting of four to six reaction vessels (fermenters). Fermentation patterns and processes observed in a RUSITEC closely resemble the fermentation patterns and processes observed in the rumen *in vivo*. The control fermenters of an already going 30-d-long assay were used in this study during the last 4 d. Fermenters were kept in a steady-state fermentation phase during at least 20 d before the assays. Control fermenters were then inoculated with alkaloids and treated as explained below. Values from the five previous days (before alkaloid addition) were used as control values.

A RUSITEC assay is started by placing in each fermenter 80 g of solid rumen digesta in one nylon bag (1-mm mesh), and the experimental diet (12-g dry matter of hay, and 3.4-g calf food concentrate pellets) in a second nylon bag. Both bags are inserted (the one with rumen content above) in a perforated cage and this cage into the reaction vessel with 500-ml fresh collected rumen liquor, 200-ml RUSITEC buffer (Czerkawski and Breckenridge, 1977), and

100-ml deionized water. The fermenters are placed in a water bath at 39°C, and the perforated cages are fixed to a motor that slides them with 6- to 7-cm amplitude at about 8–9 cycles/min. The fermenters are connected to a buffer reservoir allowing a buffer flow rate of 280–290 μl/min and then filled with CO_2 (Messer Griesheim GmbH 4.5). Buffer overflow is collected in a 2 l Erlenmeyer (filled with 40 ml, 7 M HCl), which is also connected to a collection gas bag (Linde, Art. No. 037660006).

After 24 hr incubation, the buffer flow is stopped, the gas bag changed, the overflow collected, and the solid inoculum bag removed and replaced with a new bag of experimental diet. The fermenter is filled again with CO_2 and connected again to a new Erlenmeyer and a new gas bag. The buffer flow is restarted. Subsequently, the older food bag is replaced with a new one each day, and the needed samples are collected to measure the fermentation parameters of interest. The content of the food bag is washed with RUSITEC buffer and pressed to squeeze out excess liquid, which is returned to the fermenter.

The fermentation parameters decrease during the first days but reach a steady state after 5–7 d, which can be maintained indefinitely with a balanced diet and correct handling of the fermenter. The steady state must be reached before an experiment can be started. Normally, two fermenters are used as control in an experiment, feeding them with the same diet and maintaining them in the steady state. In these experiments, the rumen liquor and the solid inoculum were collected from a fistulated cow 3 hr after the morning feeding.

Fate of Alkaloids in Rumen Simulation Technique. A concentrated alkaloid buffer solution was inoculated into each fermenter to a final alkaloid concentration of 2 mM during the daily change of the food bag. Alkaloid stock solutions were as concentrated as possible in order to use the smallest possible aliquot, but in such a way that every fermenter received the same volume of stock solution. Because the RUSITEC is a semiopen system, the alkaloid concentration in each fermenter constantly decreased even without alkaloids being degraded. The concentration decrease can be predicted with the equation $C_2 = (1-(\mathrm{fr}/\mathrm{Vt})^t)C_1$, where C_1 and C_2 are the initial and final concentrations, respectively, fr is the buffer flow rate, Vt is the total volume in the fermenter, and t is time. To measure the actual alkaloid concentration in each fermenter, 40 ml of fermenter content were taken at 0, 6, 12, 24, 48, and 72 hr. Every sample was divided into two 20-ml aliquots and stored at −20°C until they were processed for GLC analyses. Any microbial activity was stopped in each sample by adding 0.5 ml, 6 M HCl.

Determination of Fermentation Parameters

pH Determination. The pH value of a freshly taken aliquot (10 ml) of the fermenter content was measured using a calibrated pH meter (WTW, Type pH-91).

NH_3 Determination. The same aliquots, used for pH measurements, were immediately used to determine ammonia concentration. A calibrated ion-sensitive electrode (Orion 9515) and an ion analyzer (Orion 920-EA) were used. Samples were first treated with 100 µl, 2 M NaOH in order to change all ammonium ions in solution to free ammonia because the electrode can measure only the latter.

Volumetric Determination of Total Gas Production. The volume of gas produced during 24 hr in each fermenter and collected in hermetic gas bags (Linde, Art. No. 037660006) was determined by using the principle of water displacement. Three 1-l calibrated cylinders were filled with water and placed upside down in a bowl with water. The content of each gas bag was transferred with a plastic tube into the cylinders. The amount of water displaced corresponds to the amount of gas produced in the respective fermenter. During this procedure, a sample of gas was collected for the gas composition analysis.

Chromatographic Analysis of the Gas Composition. A sample was taken with a 1-ml syringe (Hamilton, TLL 1001) from the gas bag to determine the composition of the gas mixture. Each sample was collected immediately before GLC analysis. Samples (1 ml each) were injected manually into a gas chromatograph (Shimadzu, GC-8A). The impulses produced by the thermal conductivity detector (TCD) were processed on an integrator (Shimadzu, CR-3A). The GC was calibrated every day with a special gas mixture (Messer Griesheim). A correction factor for each gas was calculated by the integrator from the mean value of two consecutively injected calibration samples (1 ml each).

RESULTS AND DISCUSSION

Influence of Alkaloids on Gas Production in Bacterial Cultures from Rumen. The "Hohenheimer Futterwert Test" (HFT) and the Hungate anaerobic technique were used in a first approach to follow the fate of alkaloids in the rumen ecosystem that mainly targets the bacterial members. The total gas production in the rumen correlates linearly to the production of short-chain fatty acids (Van Soest, 1994; Blümmel et al., 1997b). Its reduction is indicative of a negative effect on ruminal fermentation processes. Since rumen microbes, especially bacteria, hydrolyze plant polysaccharides to monomeric or dimeric sugars, which are further fermented to acetic, propionic, and butyric acids, methane, and carbon dioxide, any effect on the total gas production in the rumen can be interpreted as an effect on the rumen microbial community.

The quinolizidine alkaloids lupanine and sparteine, which exhibit antimicrobial activities at higher concentrations (Wink, 1984), were selected for the first pilot experiments. At a physiological concentration of 1 mM, neither lupa-

nine nor sparteine had a significant effect on *in vitro* gas production (HFT) of microbial cultures derived from rumen liquor of cattle (Table 1, column c). Both alkaloids slightly decreased gas production when rumen liquor from a sheep fed on a roughage-based diet was used (Table 1, column a). The known antibacterial properties of lupin alkaloids (Wink, 1984) could be responsible for the negative effect of both alkaloids on the *in vitro* microbial fermentation of sheep.

Using rumen liquor from a sheep fed for 1 mo on a diet containing up to 3.2% of lupin seeds (containing lupanine as main alkaloid and no sparteine), we observed that lupanine increased *in vitro* gas production whereas sparteine had a negative effect (Table 1, column b). The effect of sparteine on *in vitro* gas production is four times stronger than the effect of lupanine. An even more pronounced adaptation of the bacterial community to lupanine was observed after feeding the sheep increasing amounts of lupin seeds (diet II) for 1 mo: Rumen bacteria from a sheep fed on a diet without alkaloids (diet I) were not able to grow at an alkaloid concentration of 10 mM in contrast to bacteria from a sheep fed on diet II, which showed an even better growth at 10 mM than at 1 mM (Table 2). After 7 d of incubation in media containing alkaloids as a sole carbon source (RA-1 and RA-2), only the microbes from diet II were able to grow after reinoculation in fresh medium. This finding suggests an acquired resistance to the added lupanine (Table 2).

Feeding a ruminant on a diet containing plant toxins can modify the microbial composition of its rumen, favoring those microorganisms that can tolerate or even metabolize such toxins (Duncan and Milne, 1992; Blythe and Craig, 1994; Newbold et al., 1997; Odenyo et al., 1997; Duncan et al., 2000). This could also be the case in our experiment, thus favoring the growth of those microbes able to tolerate the presence of lupanine in the rumen. If the favored microbes play an important role in the fermentation processes, this is observed as an enhancement of total gas production (Table 1). The higher negative effect

TABLE 1. EFFECT OF LUPANINE AND SPARTEINE ON THE TOTAL GAS PRODUCTION *In vitro* IN HFT

	Gas production (ml/200 mg hay)		
	a	b	c
Control	42.8 ± 0.28n	42.9 ± 0.99n	49.9 ± 1.04n
Lupanine 1 mM	41.6 ± 0.53m	44.5 ± 0.23m	47.9 ± 1.14n
Sparteine 1 mM	41.7 ± 1.45m	36.9 ± 0.14o	48.7 ± 0.92n

Rumen liquor was obtained from a sheep fed on a roughage-based diet (a) from a sheep fed on an adaptation diet, including increasing amounts of lupin seeds (b), or from a cow fed on a roughage-based diet (c).
Values are given in means and standard error. Assays were done in triplicate. Means with different letters in a column differ at $P < 0.05$ (ANOVA).

TABLE 2. GROWTH OF RUMINAL BACTERIA IN BATCH CULTURES WITH DIFFERENT CONCENTRATIONS OF ALKALOIDS

		Lupanine		Sparteine	
Culture medium	Treatment	1 mM	10 mM	1 mM	10 mM
M2	a	+++	−	+++	−
	b	++	+++	++	+++
RA-1	a	n.d.	−	n.d.	−
	b	n.d.	**	n.d.	**
RA-2	a	n.d.	−	n.d.	−
	b	n.d.	**	n.d.	**

Bacterial inocula were obtained from a sheep fed on a diet without (a) or with (b) lupin seeds. −: Neither tolerance nor growth; ++: good growth; +++: very good growth; **: tolerance without growth; n.d.: not determined.

of sparteine after the adaptation feeding trial can be interpreted as a differential activity of both alkaloids on the rumen microbial community. Although both lupanine and sparteine have a similar molecular structure (differing only by a ketone group in position 2 of lupanine), both have different biological activities. Sparteine is more active on Na^+ channels and muscarinic acetylcholine receptors than lupanine, whereas lupanine is more active on nicotinic acetylcholine receptors (Wink, 1993, 2000; Schmeller et al., 1994). Sparteine is also more toxic to mice but less toxic to insects (Wink, 1992).

Fate of Lupanine and Sparteine in HFT. Lupanine and sparteine were incubated *in vitro* in glass syringes. Incubations were conducted in triplicate and by using rumen fluid from only one animal in each assay. Both lupanine and sparteine were stable in control incubations for 36 hr. No degradation of alkaloids was found during an incubation period of up to 36 hr in the assays with rumen liquor, neither from a cow nor from a sheep fed on a roughage-based diet. In addition, in experiments with the Hungate technique, no degradation of either alkaloid could be detected.

The recovery data were about 94% in the assays using rumen liquor from a sheep preconditioned for 4 wk on a roughage-based diet with increasing amounts of lupin (*L. angustifolius*) seeds (Table 3). Although these values were lower than those of the corresponding experiments and could indicate some degradation, the differences from controls were not statistically significant.

Fate of Various Alkaloids in Anaerobic Batch Cultures using Hungate's Technique. Rumen inocula from a "naïve" cow that had not been exposed to alkaloids showed good growth in Hungate tubes together with alkaloids of different structures and biological activities (1 mM each; sparteine, lupanine, atropine, quinidine, harmaline, cytisine, and senecionine). GLC analyses showed no degradation of alkaloids after 7 d of incubation. All tested alkaloids were also

TABLE 3. FATE OF LUPANINE AND SPARTEINE IN HFT

	Recovery of alkaloids		
Alkaloid	a	b	c
Lupanine	108.3 ± 2.5	94.5 ± 3.75	124.5 ± 17.6
Sparteine	124.5 ± 17.6	93.8 ± 7.93	97.8 ± 2.49

Rumen liquor was obtained from a sheep fed on a roughage-based diet (a), from a sheep fed on an adaptation diet including increasing amounts of lupin seeds (b), or from a cow fed on a roughage-based diet (c).
Alkaloids were determined by GLC. Values are given in means and standard error of the percent of alkaloids recovered after 36-hr incubation. Assays were done in triplicate. No statistical differences were found at $P < 0.05$ (*t*-student), compared to the initial concentrations.

stable for 7 d in control incubations without active rumen inoculum (Figure 1). It is remarkable that the pyrrolizidine alkaloid (PA) senecionine is not degraded; several authors have reported that PAs can be degraded by rumen microorganisms. We suggest that they had used rumen microorganisms from preconditioned ruminants that had been exposed to PAs before (Swick et al., 1983; Craig et al., 1986, 1992; Cheeke, 1988, 1998; Wachenheim et al., 1992a,b).

Rumen Simulation Technique. It could be argued that the negative results from our HFT and Hungate experiments are due to the fact that rumen protozoa

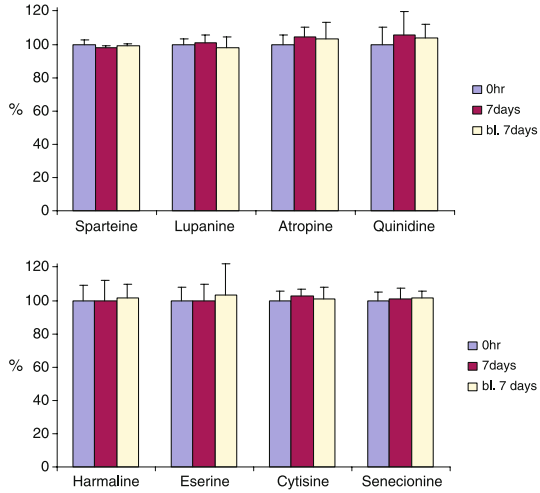

FIG. 1. Recovery of alkaloids after 7-d incubation in Hungate tubes inoculated with culture medium M2 and bovine rumen liquor. Values are given in mean and standard deviation. No significant differences were found between 0 hr and 7 d either for treatment or for blank (bl.) incubations at $P < 0.05$ (*t*-student).

and their associated bacterial consortia cannot be cultured by using Hungate's technique and that their culture in HFT is unstable. In addition, bacteria that live attached to food particles cannot be cultured using Hungate's technique or in HFT but in RUSITEC fermenters. In order to overcome the technical problems, we designed a second set of experiments with RUSITEC to study the fate of alkaloids in the rumen.

The following alkaloids of various structures and bioactivities (2 mM each of sparteine, lupanine, atropine, quinidine, lobeline, harmaline, arecoline, nicotine, caffeine, pilocarpine, gramine, and monocrotaline) were added on day 5 to the RUSITEC culture vessels. Several physiological parameters were determined during the run of the fermentation experiments, such as hydrogen ion and ammonia concentrations and gas and protein production.

Changes in hydrogen ion concentrations were analyzed continuously from the start of the cultures and 3 d after the alkaloids had been added to monitor any adverse effects. Hydrogen ion concentrations (pH values) varied between 6.56 and 6.99, being mostly between 6.7 and 6.9, both during the alkaloid-free control days (d 1–5) and the posttreatment days (d 6–8). These variations are within the normal pH variation found in previous RUSITEC experiments done in the Rumen Laboratory at the Veterinary School of Hannover (TiHo), indicating that alkaloids did not cause any drastic effect, thus confirming the findings from our Hungate experiments.

The concentration of ammonia in the fermenters showed a high variation already during the alkaloid-free days varying from 15 to 23 mmol/l but were within the range found in previous RUSITEC experiments. After addition of the alkaloids on d 5, a reduction of ammonia concentrations was observed in most fermenters (Figure 2). This effect was more pronounced for lobeline, harmaline, and atropine. Ammonia production in the rumen fluid is a consequence of amino acid and urea breakdown by both rumen bacteria and protozoa. Amino acids are deaminated to ammonia and fatty acids by most rumen protozoa (Allison, 1970) and a broad group of rumen bacteria, the latter being responsible for most of the ammonia production. Urea is broken down in the rumen only by bacteria (Sakurada et al., 1994). Considering this and the fact that weak antimicrobial activities seem to be exhibited by the applied alkaloids (Wink, 1993; Verpoorte, 1998), it is not surprising that the addition of alkaloids to the RUSITEC fermenters has a negative effect on fermentation parameters. The alkaloid concentration used was not high enough to have a more pronounced antimicrobial effect. The reduction of ammonia in the fermenters agrees with the results reported by Wiedmeier et al. (1987), who found that the ammonia concentration in cattle rumen decreased by 37% when the animals were fed on a diet containing the alkaloid pilocarpine at a dose of 4 mg/kg body weight.

Effect on Ruminal Gas Production. All fermenters showed a high variation in the daily total gas production. The amount of variation extends from 250 ml

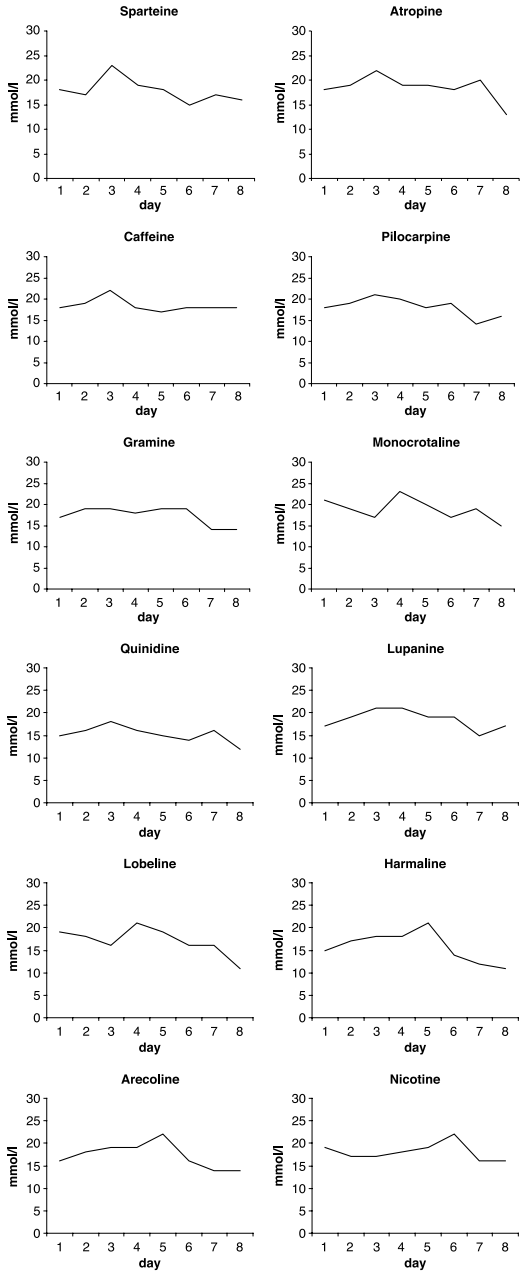

FIG. 2. Effect of alkaloids on ruminal NH_3 concentration measured in RUSITEC fermenters for 72 hr. Alkaloids were added on day 5 at a final concentration of 2 mM.

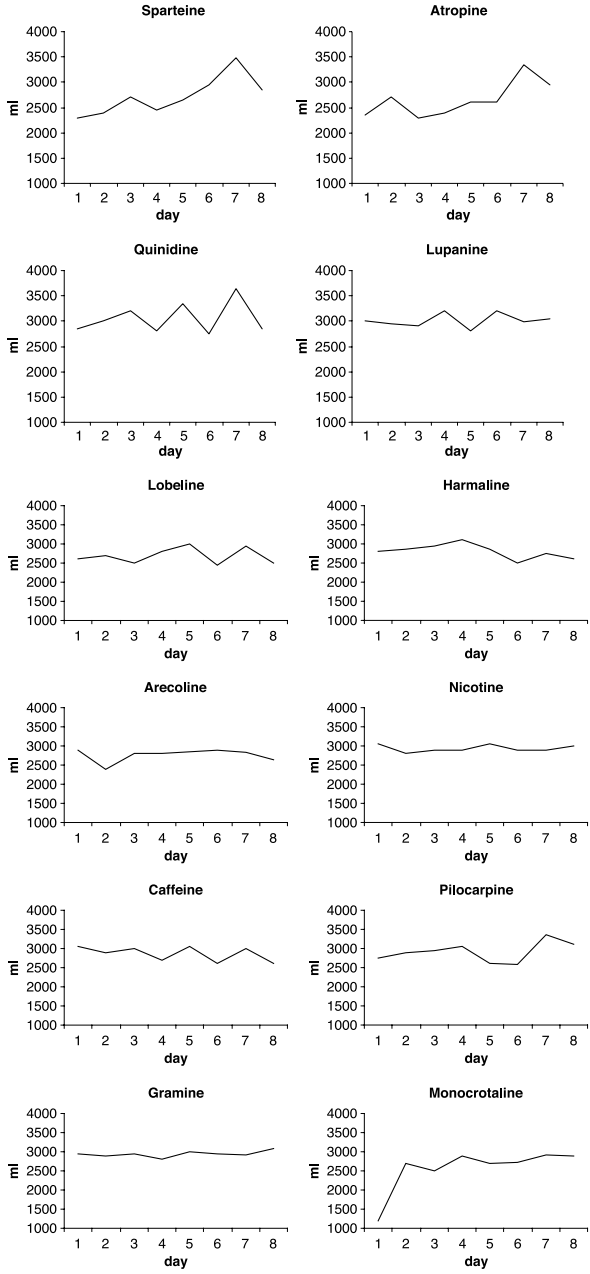

FIG. 3. Effect of alkaloids on ruminal total gas production measured in RUSITEC fermenters for 72 hr. Alkaloids were added on day 5 at a final concentration of 2 mM.

for arecoline and nicotine to 900 ml for quinidine (Figure 3). The extreme low value on the first day of the fermenter treated with monocrotaline is probably an artifact due to incorrect handling of the gas bag. However, a significant increase in total gas production is observed after the addition of sparteine, atropine, and pilocarpine (Figure 3). Harmaline, in contrast, has a negative effect on fermentation (it has pronounced antimicrobial properties; Wink, 2000), thus reducing total gas production.

A reduction of ammonia production in the fermenters was interpreted as a result of the antimicrobial activities of the employed alkaloids. This interpretation seems contradictory to the observation that some alkaloids also increased total gas production in the fermenters, which corresponds to an improvement of the microbial fermentation processes in the reaction vessels. Although total gas production correlates linearly to the production of short-chain fatty acids and consequently resembles the efficiency of the microbial fermentation, the latter is not only dependent on the microbial biomass, but also on the microbial fermentative capacities. It has been demonstrated that *in vitro* gas production can have an inverse relationship to microbial biomass production (Makkar et al., 1995a,b; Blümmel et al., 1997a,b). The addition of sparteine, atropine, and pilocarpine could favor those bacteria with the highest fermentative capacities. Similar results have been reported for caffeine (Campbell et al., 1976) and pilocarpine (Wiedmeier et al., 1987).

In contrast to total gas production, gas composition was almost constant for all fermenters during experiments (data not shown). Only in the fermenters treated with atropine, quinidine, and caffeine a slight increase in the proportion of CO_2 after addition of alkaloids was observed (data not shown), which suggests a slight improvement in the carbohydrate fermentation. In addition, quinidine appears to be the only tested alkaloid with a negative effect on the methanogen community with its concomitant reduction of methane production. Since total gas production was not affected by this alkaloid and since carbon dioxide, together with hydrogen, is the major precursor of methane in the rumen (Wolin et al., 1997), the observed increase of CO_2 can be interpreted as a direct effect of methane reduction.

Effect on Ruminal Protein Concentration. The addition of quinidine and caffeine (Figure 4) decreased the protein concentration in the fermenters by 43 and 47%, respectively. No significant effect was observed after addition of the other alkaloids. Since only bacterial protein was measured, a reduction of protein concentration resembles a decrease in the bacterial biomass and can correspond to antimicrobial activities of the alkaloids. Most alkaloids had a negative effect in at least one fermentation parameter, which can be interpreted as a result of antimicrobial activities (Verpoorte, 1998). Yet the bacterial biomass and most fermentation parameters were not disturbed; we can, therefore, assume that the microbes were alive and should have been able to degrade alkaloids.

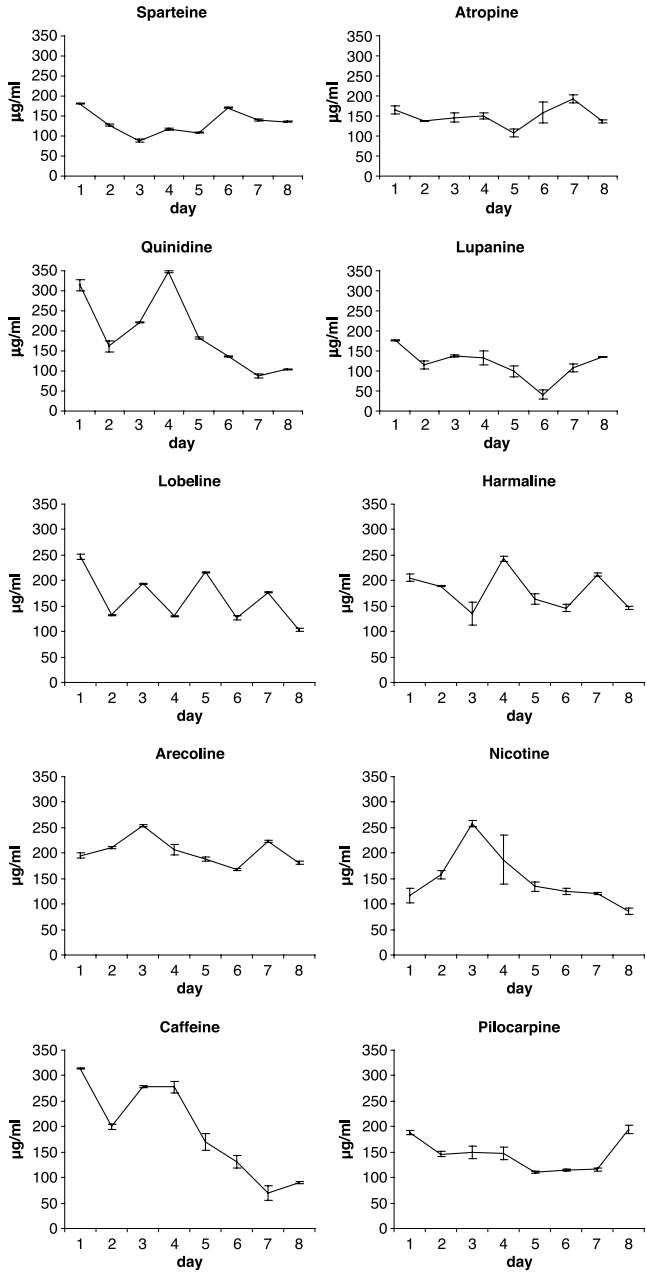

FIG. 4. Effect of alkaloids on ruminal protein concentration measured in RUSITEC fermenters for 72 hr. Alkaloids were added on day 5 at a final concentration of 2 mM.

Fate of Alkaloids in Rumen Simulation Technique Fermenter. All tested alkaloids were stable in control incubations that used sterilized rumen liquor to avoid possible degrading metabolism from rumen microorganisms. Most alkaloids were stable during the incubations and did not change (Figure 5). A decrease in the concentration was observed only for pilocarpine, monocrotaline, and gramine (Figures 6–8).

Pilocarpine showed the highest degradation rate being totally degraded after 12 hr incubation (Figure 6). The concentration of monocrotaline decreased to 38.9% within the first 12 hr and to 4.7% within the next 12 hr (Figure 7), which represents a degradation of 49.4 and 92.1%, respectively (in relation to the expected concentration). In Figure 7B, the gas chromatograms of the monocrotaline samples collected at time 0, 24, and 48 hr incubation are illustrated. It is possible that an ester hydrolysis of monocrotaline is involved in the degradation of this alkaloid by rumen microbes. Similar reaction mechanisms have been postulated for jacobine (Wachenheim et al., 1992a,b) and for the monoester heliotrine, along with modification of the necine base (Russell and Smith, 1968; Lanigan, 1971, 1976). Enzymatic hydrolysis of monocrotaline has been reported from hepatic microsomal incubation of guinea pigs, leading to the nontoxic necic acid and necine base (Dueker et al., 1992). There are substantial evidence and counterevidence for detoxification pathways in liver vs. rumen in the literature concerning pyrrolizidine alkaloids (Swick et al., 1983; Craig et al., 1986, 1992; Cheeke, 1988, 1998; Wachenheim et al., 1992a). Wachenheim et al. (1992b) found that pyrrolizidine alkaloids (PAs) were degraded more rapidly when incubated *in vitro* with ovine or caprine than

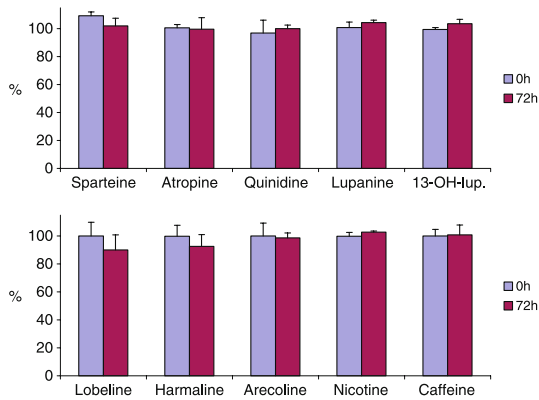

FIG. 5. Recovery of alkaloids after 72 hr incubation in RUSITEC fermenters. Values are given in mean and standard deviation. No significant differences were found between 0 and 72 hr at $P < 0.05$ (*t*-student).

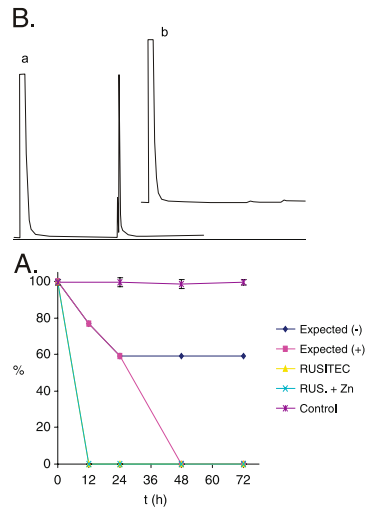

FIG. 6. Fate of pilocarpine in RUSITEC fermenter. (A) Kinetics of pilocarpine contents during fermentation; (B) GLC profiles of the cultures after 0 hr (a) and 12 hr (b); 1 = pilocarpine.

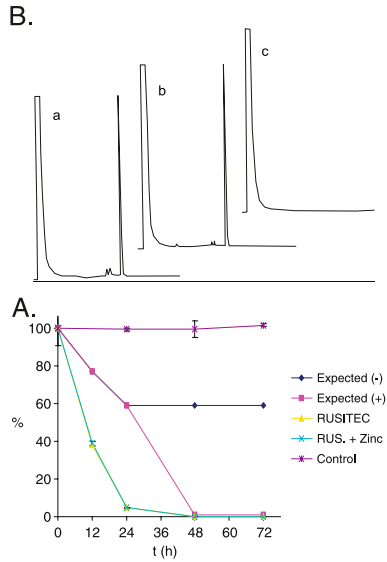

FIG. 7. Fate of monocrotaline in RUSITEC fermenter. (A) Kinetics of monocrotaline contents during fermentation; (B) GLC profiles of the cultures after 0 hr (a), 24 hr (b), and 48 hr (c); 1 = monocrotaline.

with bovine rumen fluid, and by using the "most probable number procedure" for estimating PA-degrading bacteria in rumen, they demonstrated that cattle have these bacteria, but in much reduced numbers than sheep and goats. In contrast, Shull et al. (1976) and Swick et al. (1983) found that *S. jacobaea* is still toxic to rats after incubation with sheep rumen fluid. Nevertheless, the presence of alkaloid-degrading organisms in the rumen is far from ubiquitous in all ruminants. The apparently high frequency of reports on PA-degrading microbes found in the literature, compared with other toxins, seems to be rather a result of the intense investigation of this group of alkaloids and their economical importance in the range management (Swick et al., 1983; Craig et al., 1986, 1992; Cheeke, 1988, 1998; Wachenheim et al., 1992a).

Gramine suffered a lower degradation rate, 35.7 and 38.3%, respectively, of the expected concentration after 12 and 24 hr (Figure 8). The gas chromatograms of the gramine samples collected 0, 24, and 48 hr after incubation in RUSITEC are shown in Figure 8B. Since gramine occurs in young leaves of cereals and these plants are the main component of a roughage-based diet for

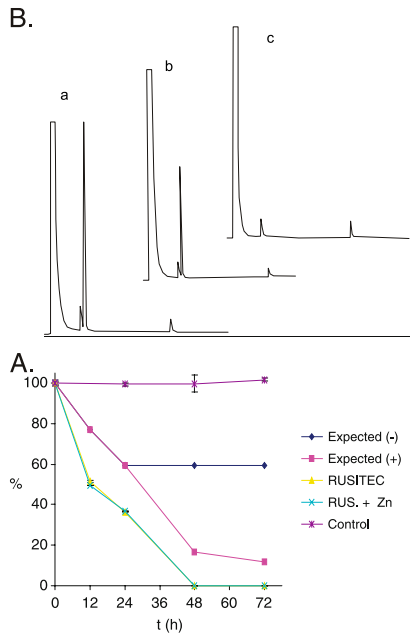

FIG. 8. Fate of gramine in RUSITEC fermenter. (A) Kinetics of gramine contents during fermentation; (B) GLC profiles of the cultures after 0 hr (a), 24 hr (b), and 48 hr (c); 1 = gramine.

ruminants, we would expect to find that rumen microbes from cattle kept in stables and fed on this kind of diet are able to metabolize this alkaloid.

We found no evidence for alkaloid N-oxide formation in any fermenter, which agrees with the hypothesis of Dawson et al. (1997) that oxidative reactions are not to be expected in the rumen because of the relatively low oxygen concentrations present.

In summary, our experiments using the Hungate and HFT approach provide no evidence for alkaloid degradation when rumen microorganisms from naive and unexposed sheep and cattle were assayed. Using the RUSITEC method, which takes a more complex rumen microbial community into account, most alkaloids appear to be stable and were not degraded. Evidence for degradation was obtained for gramine, pilocarpine, and monocrotaline, however, indicating a broader metabolic activity than would have been assumed from the bacterial cultures using Hungate and HFT alone.

Feeding sheep for a period of 4 wk on a diet containing increasing amounts of bitter lupin seeds was not sufficient to induce alkaloid-degrading metabolism in ovine rumen microbes. Nevertheless, rumen microbes could adapt to the presence of lupanine, and the fermentation processes, determined by total gas production, were, therefore, enhanced. Feeding ruminants a diet containing plant toxins can thus modify the composition of the microbial rumen community either by selecting those microorganisms able to tolerate higher concentration of the toxins consumed by the herbivore or by allowing the microorganisms to become adapted to the new conditions.

Most experiments are conducted *in vitro* using rumen fluid from animals (mainly cattle and sheep) kept in stables and fed on controlled grass-based diets. Many authors have reported that previous exposure to a diet containing the plant toxin is, in most cases, a requisite for the rumen microbes to be able to metabolize it or for the ruminant not to be affected by the toxin, suggesting an adaptation of the rumen microorganisms for metabolizing it (Smith, 1992). Allyl cyanide is degraded in the rumen of sheep fed on cabbage, but not in the rumen of grass-fed sheep (Duncan and Milne, 1992). Ethiopian sheep gradually adapted to a diet containing *Acacia angustissima* are protected from its toxins through rumen detoxification, whereas non-adapted animals die within a few days (Odenyo et al., 1997). The rumen flora of nonadapted deer is severely inhibited by the essential oils of Douglas fir needles, whereas no inhibition is found on rumen flora from deer previously adapted to a diet containing up to 50% Douglas fir needles (Oh et al., 1967). The antiprotozoal factor (saponins) of *Sesbania sesban*, an African leguminous tree, affects negatively the rumen protozoa of Scottish sheep but not of feral Ethiopian sheep, and *in vitro* experiments suggest that bacteria are adapted to detoxify this antiprotozoal agent (Newbold et al., 1997; Teferedegne et al., 1999). Culvenor et al. (1984) found that the *in vitro* degradation of *Echium* pyrrolizidine alkaloids incubated

with ovine rumen fluid can be improved up to 22 times when the donor sheep are previously fed with *Echium* for 12 wk. Sheep that have been reared on pyrrolizidine alkaloids can degrade them, but inexperienced sheep cannot. Similarly, the rumen flora of deer that have not previously been fed with Douglas fir needles are severely inhibited by it, but experienced deer can consume a diet of up to 50% needles without showing negative effects (Oh et al., 1967).

The results obtained in this study clearly emphasize that degradation of alkaloids does not occur to a substantial degree in the rumen of naïve ruminants, thus suggesting a prominent role of liver detoxification instead. More work is needed to understand the specific role of the rumen and the liver in the different susceptibility of ruminants to plant toxins and other xenobiotics. More attention should be paid to detoxification activity in hepatic tissues of ruminants and on their previous feeding conditions. In addition, the nature of the ruminant species should be taken into account when comparing interspecific differences. Grazing ruminants, e.g., cattle, have evolved on food plants (e.g., grasses) that are, in general, poorly chemically defended (Cheeke, 1998), depending more on growth habit and physical defenses. In contrast, browsers, e.g., sheep, goat, and deer, have evolved on food plants that are generally well equipped with chemical defenses. Thus, different detoxification mechanisms may have evolved in both groups, and their rumen floras have been subject to different evolutionary pressures.

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COMPARISON OF ANTIFUNGAL AND ANTIOXIDANT ACTIVITIES OF *Acacia mangium* AND *A. auriculiformis* HEARTWOOD EXTRACTS

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Abstract—The effect of heartwood extracts from *Acacia mangium* (heartrot-susceptible) and *A. auriculiformis* (heartrot-resistant) was examined on the growth of wood rotting fungi with *in vitro* assays. *A. auriculiformis* heartwood extracts had higher antifungal activity than *A. mangium*. The compounds 3,4′,7,8-tetrahydroxyflavanone and teracacidin (the most abundant flavonoids in both species) showed antifungal activity. *A. auriculiformis* contained higher levels of these flavonoids (3.5- and 43-fold higher, respectively) than *A. mangium*. This suggests that higher levels of these compounds may contribute to heartrot resistance. Furthermore, both flavonoids had strong 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and laccase inhibition. This suggests that the antifungal mechanism of these compounds may involve inhibition of fungal growth by quenching of free radicals produced by the extracellular fungal enzyme laccase.

Key Words—*Acacia mangium*, *A. auriculiformis*, heartrot, heartwood extracts, flavanone, *Phellinus noxius*, *P. badius*, antifungal activity, antioxidant, laccase inhibition.

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INTRODUCTION

Polyphenolic compounds are common in higher plants (Haslam, 1988) and have properties that afford plant tissues protection from the external environment including antimicrobial (Scalbert, 1991; Field and Lettinga, 1992; Mila et al., 1996), antifeedant (Butler, 1992), and antioxidant properties (Hagerman et al., 1998; Eyles et al., 2004). In woody tree species, polyphenolic compounds accumulate in bark, leaves, and heartwood, while lesser amounts are found in sapwood (Hillis, 1987). Extracts from bark and heartwood of many woody tree species have strong biological activities, such as enzyme inhibition (Mitsunaga et al., 1997; Shimizu et al., 1998; Juntheikki and Julkunen-Titto, 2000), antioxidant activity (Chang et al., 2001; Willför et al., 2003), and antifungal activity (Kishino et al., 1995). Durability of wood (resistance to fungal decay) is often attributed to extractive content (Hart and Hillis, 1974; Scalbert, 1992; Harju et al., 2002).

Acacia mangium is a fast-growing hardwood planted widely in many areas of Asia and is susceptible to heartrot (Lee et al., 1988; Mahmud et al., 1993; Ito and Nanis, 1994; Barry et al., 2004). Heartrot is caused by fungal infections of heartwood via dead or broken branches (Mahmud et al., 1993; Ito and Nanis, 1994). *A. auriculiformis*, a closely related species, and hybrids of *A. auriculiformis* × *A. mangium* are rarely affected by heartrot (Ito and Nanis, 1997). One explanation for the difference in susceptibility is an increased incidence of infections associated with the higher proportion of infection courts provided by large-diameter branches that occur more frequently in *A. mangium* than in *A. auriculiformis* (Ito and Nanis, 1997). Heartwood extractives may also influence susceptibility to fungal infection and require further investigation in these *Acacia* spp. (Barry et al., in press).

Extracts from *A. mangium* heartwood have been investigated (Tachi et al., 1989; Lange and Hashim, 2001; Barry et al., in press). 3,4′,7,8-Tetrahydroxyflavanone (**1**) (Figure 1) and 4′,7,8-trihydroxyfavanone (**2**) are the main flavonoid compounds in healthy *A. mangium* heartwood, but their levels are markedly reduced in decayed heartwood (Barry et al., in press). This decrease is

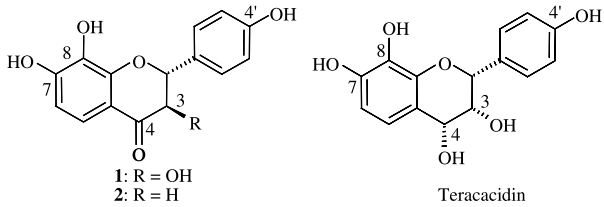


FIG. 1. Structures of compounds **1**, **2**, and teracacidin.

probably caused by enzymatic degradation of phenolic compounds associated with heartrot fungi (Barry et al., in press). While the identities of fungi causing *A. mangium* heartrot are not well known, *Phellinus noxius* has been isolated from early stages of decay in *A. mangium* (Lee and Zakaria, 1993; Lee and Yahya, 1999). *P. badius* has been isolated from the heartrot of *Acacia* spp. (Quraishi and Ahmad, 1973; Stalpers, 1978)

In this study, we compare the antifungal properties of heartwood extracts from *A. mangium* and *A. auriculiformis* against *P. noxius* and *P. badius* using *in vitro* bioassays. Possible antifungal mechanisms (extracts acting as antioxidants or laccase inhibitors) are investigated.

METHODS AND MATERIALS

Wood Materials. Six-year-old trees of *A. mangium* and *A. auriculiformis* were harvested from an experimental plantation in northern Queensland, Australia, in April 2003. Two trees from each species were analyzed. For bioassays, extracts from one tree of each species were used. Each tree was felled and then a billet was cut from a height of 3 m above ground level. The billets were dried at 40°C for 2 wk. The heartwood of each tree was separated from the bark and sapwood, and milled using a Wiley Mill (Thomas Wiley Mill Model 4; Arthur H. Thomas Company, USA). After drying at 40°C for 1 wk, the ground heartwood samples (600 g) were extracted (two times) for 2 d each with 5 l of methanol. Extracts were maintained in the dark at room temperature on a shaker. The methanolic extracts were filtered through Whatman No. 5 filter paper (UK). Solvents were evaporated to dryness on a rotary evaporator at 40°C. The total extracts of *A. mangium* and *A. auriculiformis* wood were abbreviated as M-T (yield = 2.9 and 4.0% based on air dried wood, w/w) and A-T (9.3 and 8.0%), respectively.

Successive Extraction. Extractions were carried out as previously reported (Barry et al., in press). Methanol extracts (13 g) of *A. mangium* and *A. auriculiformis* were dissolved in 10 ml of methanol and extracted with 1 l of diethyl ether, ethyl acetate, and *n*-butanol, successively. Each extraction was conducted at room temperature for 2 hr. Residues were collected by centrifugation (1400 × *g*, 10 min) and supernatants were evaporated at 40°C. Three fractions and the residue were obtained and coded as M-A (yield = 0.94% based on air dried wood, w/w), M-B (0.43%), M-C (0.37%), and M-D (0.35%) for *A. mangium*, and A-A (4.54%), A-B (1.04%), A-C (0.82%), and A-D (0.03%) for *A. auriculiformis*.

Isolation of Flavonoids. 3,4′,7,8-Tetrahydroxyflavanone (**1**), 4′,7,8-Trihydroxyflavanone (**2**), and teracacidin were isolated as previously described (Barry et al., in press). M-A (2 g) was dissolved in *n*-hexane/acetone (1:1, v/v),

applied to silica gel 60 for column chromatography (Merck, Australia), and eluted with *n*-hexane/acetone (7:3 then 1:1) and methanol. The *n*-hexane/acetone (1:1) fraction was further purified by preparative thin layer chromatography (TLC) to isolate compounds **1** and **2**. Preparative TLC was completed using silica gel 60 plates (Merck, Germany) with methanol/dichloromethane (8:92, v/v).

Teracacidin was isolated from the *A. auriculiformis* methanol extract because it occurred in much higher amounts than in *A. mangium*. Fraction A-A (3 g) was dissolved in 300 ml of ethyl acetate and extracted with water (3 × 200 ml) in a separatory funnel. The water-soluble fraction was purified by preparative HPLC using a reverse phase column (Develosil ODS-10, 20 mm i.d. × 250 mm; Nomura Chemical Co. Ltd., Japan) monitored at 280 nm. The solvent system used was as follows: a linear gradient elution for 40 min from 15% to 60% solvent B (methanol) in solvent A (0.01% TFA in H_2O) at a flow rate of 10 ml/min. The elution time of teracacidin was 18 min.

Antifungal Bioassays. The liquid medium bioassay was adapted from Hart and Hillis (1974). *P. noxius* (Melbourne C2558) and *P. badius* (Melbourne C3437) were subcultured on malt agar plates (1% malt, 1.5% agar) for 2 wk at 20°C and 36°C, respectively. A 2-ml aliquot of 13% aqueous ethanol solution containing a fixed amount of *A. mangium* and *A. auriculiformis* methanol extracts was added to 48 ml of 1% malt liquid media to obtain final concentrations of 0.1, 1.0, and 10.0 mg/ml extract. The malt liquid medium was inoculated with a 5-mm-diam plug of fungal culture and incubated at 20°C for 2 wk with shaking. After incubation, the cultures were filtered (Whatman filter paper No. 2, UK), the mycelia washed with methanol, dried at 50°C for 2 d, and the fungal weights measured. Three replicates were prepared for each treatment, and the results were averaged.

The agar dilution bioassay was adapted from Yoshimoto et al. (1984). A 1-ml aliquot of 20% aqueous ethanol solution containing a fixed amount of methanol extract and each fraction of *A. mangium* and *A. auriculiformis* was added to 9 ml of malt agar solution to obtain a final concentration of 0.1%, 0.3%, and 0.5% (w/v) extract. These malt agar solutions (2 ml) were transferred in sterile test tubes to make agar pieces 10 cm in length. The agar was inoculated by placing a 5-mm-diam plug of fungal culture on the edge of the agar (Figure 2) and incubated at 28°C. Mycelia growth was measured sequentially over a period of 30 d. Three replicates were undertaken for each treatment, and the results were averaged. The same method was used for bioassay of the pure compounds that were tested at a concentration of 8 and 15 mM.

Quantitative HPLC Analysis. Quantification of compounds **1**, **2**, and teracacidin was achieved by HPLC (CLASS M10A; Shimadzu Co. Ltd., Japan) analysis of methanol extracts of *A. mangium* and *A. auriculiformis* using a Develosil ODS HG-5 reversed phase column (4.6 mm i.d. × 250 mm; Nomura

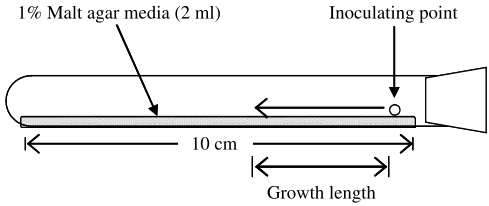


FIG. 2. Agar dilution bioassay method.

Chemical Co. Ltd., Japan) and monitoring at 280 nm. The solvent system used was as follows: a linear gradient for 45 min from 5% to 90% solvent B (methanol) in solvent A (0.01% TFA in H_2O) at a flow rate of 1 ml/min. The amount of each compound was calculated with a calibration curve of each pure compound based on the relationship of the HPLC chromatogram peak area and concentration of the compounds.

Evaluation of 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Radical Scavenging Activity. The DPPH radical scavenging activity was measured as previously reported (Mihara et al., 2004). A test sample (ethanol solution, 2 ml) was added to 0.5 mM DPPH in ethanol (1 ml) mixed with a 50 mM 2-monopholinoethanesulfonic acid buffer (pH 7.4, 2 ml), and the mixture was shaken vigorously. After 15 min incubation at 25°C, the relative decrease in absorbance of the test sample mixtures at 517 nm was measured on a Jasco V-520 UV/Vis spectrophotometer (JASCO Co. Ltd., Japan). The DPPH radical scavenging activity was expressed as the relative activity compared to the 0.5 mM butylhydroxytoluen (BHT). Quercetin and gallic acid were tested as positive controls of natural antioxidants (Amić et al., 2003; Eyles et al., 2004). The experiment was performed in duplicate, and the following formula was used to calculate results:

$$\text{DPPH radical scavenging activity}\,(\%) = \{(\text{ethanol alone}) - (\text{test sample})\}/\{(\text{ethanol alone}) - (\text{BHT})\} \times 100$$

Laccase Inhibitory Activity. This assay was conducted by using syringaldazine as a substrate (Leonowicz and Grzywnowicz, 1981). Laccase (EC 1.10.3.2) was purchased from Sigma-Aldrich Japan Co. Ltd. Each extract was dissolved in 10% aqueous dimethyl sulfoxide (DMSO) to give 0.3 mg/ml of sample solution. A 0.05-ml aliquot of sample solution was mixed with 0.9 ml of 0.1 M phosphate buffer (pH 6.5) and 0.067 ml of 0.5 mM syringaldazine ethanol solution. A 0.1-ml aliquot of laccase solution (288 U/ml) was added to the above mixture. After incubation at 30°C for 30 min, the absorbance at 525 nm was measured. For the control, 10% aqueous DMSO was used instead of the

sample solution. The experiment was performed in duplicate, and the following formula was used to calculate activity:

$$\text{Laccase inhibition (\%)} = \{(\text{control}) - (\text{test sample})\}/\text{control} \times 100$$

Enzymatic Reaction Kinetics. A 0.1-ml aliquot of syringaldazine ethanol solution (0.13–0.5 mM) was mixed with 0.74 ml of 0.1 M phosphate buffer (pH 6.5) and 0.06 ml of 0.1 mM teracacidin or 10% aqueous DMSO. A 0.1-ml aliquot of commercial laccase solution (288 U/ml) was added to the above mixture and incubated at 30°C. Absorbance at 525 nm was measured over 20 min. Reaction rates were calculated from the difference between absorbance at 15 and 20 min (absorbance increased linearly between 10 and 25 min).

Statistical Analysis. All statistical analyses were completed with SAS for Windows Version 8 (SAS Institute, 1999). For the liquid bioassay, a three-way ANOVA was conducted on log-transformed data with fixed factors of extract type (*A. mangium* or *A. auriculiformis*), extract concentration, and fungus type (*P. badius* or *P. noxius*). For the agar dilution bioassay testing different fractions, a two-way ANOVA was conducted on log-transformed data for fixed factors extract type and concentration. The ANOVA was conducted on the data from the final measurement at 30 d.

RESULTS

Comparison of Antifungal Activity. A. auriculiformis methanol extracts had strong antifungal effects on the growth of *P. noxius* and *P. badius*. *A. mangium* extracts showed no effect on *P. noxius* growth and little inhibition of *P. badius* compared to *A. auriculiformis* (Figure 3). ANOVA of the whole bioassay showed high significance ($P < 0.001$), and all main factors were significant, including fungal species and extract type ($P < 0.001$) and extract concentration ($P < 0.001$). Second-order interactions were significant between extract type × concentration ($P < 0.001$) and fungal species × extract concentration ($P = 0.039$).

The largest difference between the two *Acacia* spp. extracts was at the highest concentration (10 mg/ml) on the growth of *P. badius* (Figure 3). *A. auriculiformis* extract inhibited fungal growth, while *A. mangium* extract did not. The inhibitory effect of *A. auriculiformis* extracts on growth of both *P. noxius* and *P. badius* increased in a concentration-dependent manner.

An antifungal bioassay of fractions obtained by successive extraction was conducted with an agar dilution method using *P. badius* only because *P. noxius* growth appeared to be inhibited by ethanol (Figure 3). Fraction A-D was not tested due to low yield (56 mg). Only the results from the highest concentration

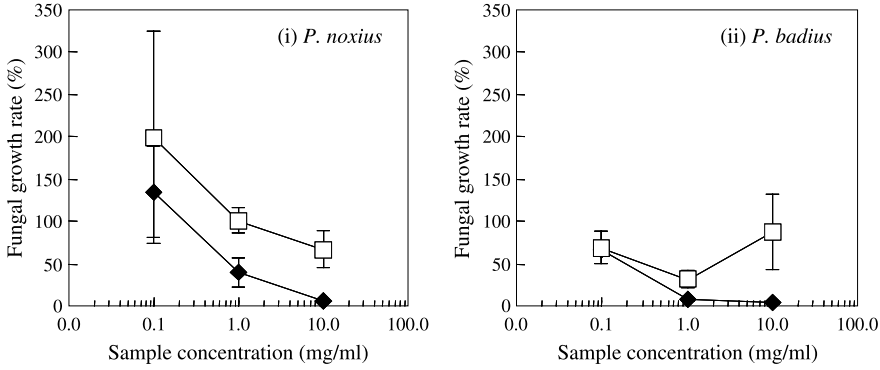


FIG. 3. Antifungal activities of methanol extracts from *A. auriculiformis* (filled diamond) and *A. mangium* (open square) heartwood against (i) *P. noxius* and (ii) *P. badius*. Error bars indicate ±SEM of three replicates. Fungal growth rates represented as percentage of fungal growth of water control. Fungal growth of ethanol control for *P. noxius* and *P. badius* was 59.3% (±9.5) and 94.7% (±18.8), respectively.

(0.5%) are presented (Figure 4). The test was statistically significant based on the two-factor fixed ANOVA ($P < 0.001$), and extract type ($P < 0.001$) contributed to this more than concentration ($P < 0.001$). However, the interaction between the factors was significant ($P = 0.007$). *A. auriculiformis* methanol extract (A-T) and fractions A-A, A-B inhibited growth of *P. badius*. Only the diethyl ether fraction of *A. mangium* (M-A) was inhibitory to *P. badius* (Figure 4). Other fractions of *A. mangium* and A-C accelerated fungal growth. HPLC analyses confirmed the presence of compounds **1**, **2**, and teracacidin in fractions A-A, A-B, and M-A, while A-C, M-B, M-C, and M-D showed amalgamation of broad peaks.

The same assay was conducted using compound **1** and teracacidin at two different concentrations. Compound **2** was not tested due to low yield. Both compound **1** and teracacidin were inhibitory against *P. badius* at a concentration of 15 mM, but not at 8 mM (Figure 5). Compound **1** showed slightly stronger activity than teracacidin at similar concentrations. *A. auriculiformis* heartwood contained 3.5-fold of compound **1**, 1.5-fold of compound **2**, and 43-fold of teracacidin per unit weight of dry wood compared to *A. mangium* (Table 1).

Antioxidant and Laccase Inhibitory Activity. The DPPH radical scavenging activities of *A. mangium* and *A. auriculiformis* extracts were determined (Table 2). DPPH radical scavenging activity was defined as the effective concentration of extract fraction needed to decrease the initial DPPH radical by 50% (EC_{50}). The EC_{50} value of A-T was ca. 3-fold higher than that of M-T. Fractions A-A, A-B, and M-A also had strong antioxidant activity. Furthermore,

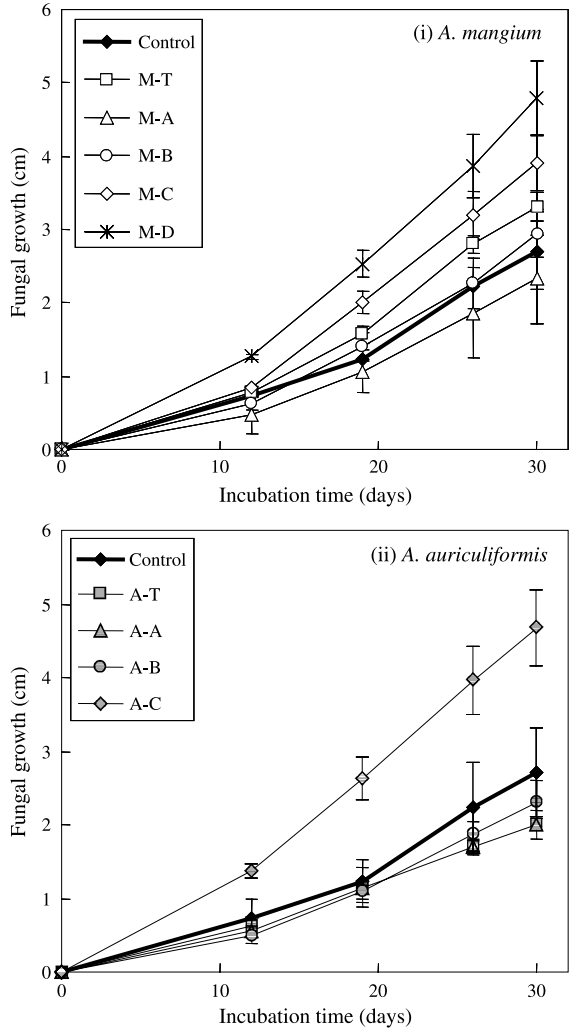
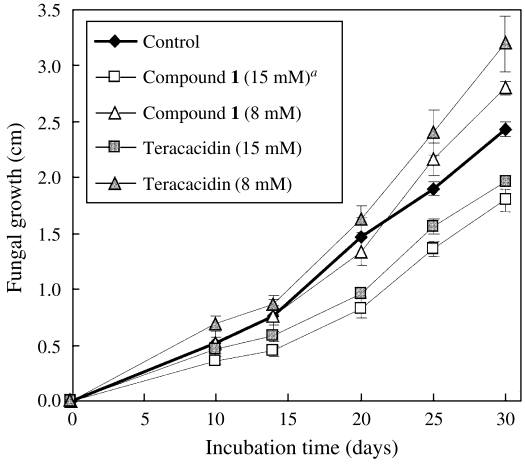


FIG. 4. Antifungal activities of successive extracts from (i) *A. mangium* and (ii) *A. auriculiformis* heartwood methanol extracts against *P. badius*. The concentration of extracts was 0.5% per agar medium. Error bars indicate ±SEM of three replicates. M-T and A-T correspond to methanol extracts of *A. mangium* and *A. auriculiformis* heartwood, respectively. M-A-C and A-A-C correspond to fractions obtained from *A. mangium* and *A. auriculiformis*, respectively, by successive extractions with diethyl ether, ethyl acetate, and *n*-butanol. M-D and A-D correspond to the extract residues.



a. Final concentration of compounds in the agar medium.

FIG. 5. Antifungal activities of compound **1** and teracacidin against *P. badius*. Error bars indicate ±SEM of three replicates.

compounds **1**, **2**, and teracacidin (the main compounds of A-A) had strong antioxidant activity.

Compounds **1**, **2**, and teracacidin all had strongly inhibitory laccase activity (Table 2). The rank order of laccase inhibition by these compounds was the same as the DPPH radical scavenging activity (compound **2** > compound **1** > teracacidin). Fractions that were strongly antioxidant (M-A, M-B, A-T, A-A, and A-B) all inhibited laccase activity. Lineweaver–Burk plots of teracacidin activity toward syringaldazine as a substrate is shown in Figure 6. The results suggest that, for teracacidin, the type of laccase inhibition is uncompetitive.

Relationships between antifungal, antioxidant, and laccase inhibitory activity of each extract and fraction were explored using plots and calculating

TABLE 1. CONTENTS OF COMPOUNDS **1**, **2**, AND TERACACIDIN IN *Acacia* SPECIES HEARTWOOD

	A. mangium		*A. auriculiformis*	
	% Per[a] extract (w/w)	% Per wood	% Per extract	% Per wood
Compound **1**	4.52	0.15	6.25	0.53
Compound **2**	1.23	0.04	0.68	0.06
Teracacidin	4.85	0.17	83.94	7.35

[a]The mean of extracts from two individual trees.

TABLE 2. ANTIOXIDANT AND LACCASE INHIBITORY ACTIVITIES OF HEARTWOOD EXTRACTS FROM *Acacia* SPECIES

Sample	EC_{50} value of DPPH radical scavenging[a] (μg/ml)	(nmol/ml)	Laccase inhibition (%)[b]
M-T[c]	24.7	–	36.5
M-A	9.5	–	57.3
M-B	13.4	–	78.4
M-C	32.6	–	10.1
M-D	61.9	–	n.d.
A-T	6.4	–	92.4
A-A	5.9	–	92.0
A-B	7.5	–	91.0
A-C	22.1	–	14.9
A-D	>80	–	11.9
Compound **1**	5.1	17.7	85.5
Compound **2**	4.1	15.0	87.7
Teracacidin	6.0	20.5	73.3
Gallic acid[d]	1.7	10.0	95.8
Quercetin	2.3	27.6	98.2

[a] Effective concentration of antioxidant necessary to decrease the initial DPPH radical by 50%.
[b] Final concentration of each extract and fraction was 15 μg/ml. For compounds **1**, **2**, teracacidin, gallic acid, and quercetin, that was 33.5 μM.
[c] See Figure 4.
[d] Positive control for DPPH radical scavenging assay.

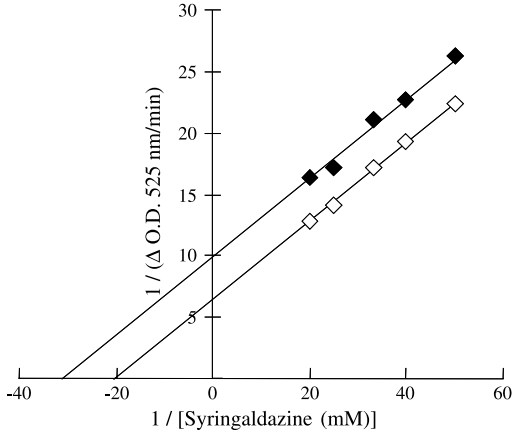
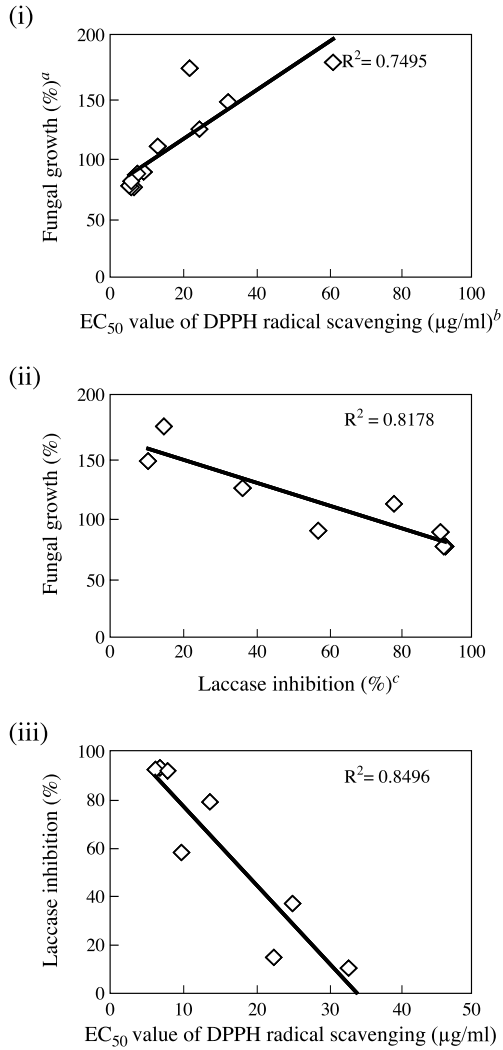


FIG. 6. Lineweaver–Burk plot of laccase and syringaldazine in the presence (filled diamond) or absence (open diamond) of teracacidin.



a. Fungal growth rate of *P. badius* on malt agar plate containing 0.5% of *Acacia* spp. extracts on the basis of control fungal growth after incubated 30 days.

b, *c*. see Table 2.

FIG. 7. Relationship between (i) antifungal and antioxidant activities, (ii) antifungal activity and laccase inhibition, and (iii) laccase inhibition and antioxidant activity of *Acacia* spp. extracts.

correlation coefficients between each factor (Figure 7). Significant correlations (R^2 = 0.75–0.85) were obtained between all three factors.

DISCUSSION

A. auriculiformis heartwood extracts have stronger antifungal properties than *A. mangium* against *P. noxius* and *P. badius*. *A. mangium* heartwood extracts generally did not inhibit the mycelial growth of *P. noxius* and *P. badius* (Figure 3). Previous studies have shown that methanolic extracts of *A. mangium* contained ca. half the amount of total phenols compared to *A. auriculiformis* (Barry et al., in press). This may explain why *A. auriculiformis* extract (A-T) was inhibitory to fungal growth while *A. mangium* (M-T) was not, and also implies that the heartrot susceptibility of *A. mangium* may be related to lower quantities of active compounds in the extracts.

In the antifungal assays of each fraction obtained by successive extraction, the diethyl ether fractions (M-A and A-A) of both *Acacia* spp. showed antifungal activity (Figure 4). Compounds **1**, **2**, and teracacidin were present in highest concentrations in M-A and A-A. Compound **1** and teracacidin inhibited the growth of *P. badius* at 15 mM, but at 8 mM mycelial growth was accelerated compared to the controls (Figure 5). This indicates that a twofold increase in the concentration of these compounds can result in significant biological consequences for fungal growth. Quantitative analysis by HPLC revealed that *A. auriculiformis* contained 0.53% (w/w) of compound **1** and 7.3% of teracacidin in dry wood (Table 1). This is a substantially higher concentration compared to the *in vitro* biologically effective concentration of 15 mM (ca. 0.5% per agar medium, w/w). *A. mangium* contained only 0.15% (w/w) of compound **1** and 0.17% of teracacidin in dry wood (Table 1) and as the concentration of 8 mM (ca. 0.3% per agar medium, w/w) did not show *in vitro* antifungal effects, it can be postulated that *A. mangium* heartwood does not contain a sufficient concentration of antifungal compounds to inhibit *P. badius*. These results suggest that the higher amount of antifungal compounds in *A. auriculiformis* contribute to its heartrot resistance.

The *n*-butanol fractions (M-C and A-C) and M-D stimulated *P. badius* growth (Figure 4). The acid butanol reaction and high BSA adsorption ability of these fractions, as reported previously (Barry et al., in press), suggests that they consist of proanthocyanidins. In cultures containing proanthocyanidins, growth of several wood-rotting basidiomycete fungi also was accelerated (Mitsunaga et al., 1999). Therefore, the acceleration of *P. badius* growth could be induced by proanthocyanidins of *A. mangium*. Decolorization of samples was observed in agar medium containing M-D or A-C. It is possible that the proanthocyanidins in these fractions were decomposed by enzymes produced by *P. badius*

and then the products utilized as a nutrient source. Hashida et al. (2001) have shown that another white rot fungus, *Lentinus tigrinus*, decomposed proanthocyanidin from the bark of *A. mearnsii*. Our previous study reported that *A. mangium* had twofold higher proanthocyanidins per heartwood extracts compared to *A. auriculiformis* (Barry et al., in press). This may be another possible reason why heartwood extracts of *A. mangium* showed lower inhibitory effects against *Phellinus* spp. than that of *A. auriculiformis* (Figures 3 and 4).

The fractions (M-A, A-T, A-A, and A-B) that showed antifungal activity against *P. badius* also had strong antioxidant activity (Table 2). Because compounds **1**, **2**, and teracacidin showed high DPPH radical scavenging ability, it is suggested that the antioxidant properties of A-A and A-T can be attributed to these compounds. A-T contained 1.4-fold more of compound **1** and 17-fold more of teracacidin compared to M-T (Table 1).

The radical scavenging ability of several flavonoids could be due to the hydroxylation pattern of the B ring (Amić et al., 2003). The pyrogallol and catechol pattern of the B ring may impart radical scavenging activity. However, compounds **1**, **2**, and teracacidin have a hydroxyl group at the C-4′ position of the B ring (Figure 1). These compounds have a pyrogallolic hydroxylation pattern in the A ring that may be responsible for their antioxidant properties. Teracacidin showed lower antioxidant activity than compound **1** or **2**, which may be due to the absence of a 4-carbonyl group (Figure 1). This group could also play a role in the antioxidant behavior of flavonoids (Amić et al., 2003).

P. noxius and *P. badius* produce laccase (R. Mihara, unpublished data), which is typical of white rot fungi. Phenol oxidases such as laccase and polyphenol oxidase (PPO) oxidize phenolics by radical reactions (Guillén et al., 2000). The kinetics study of laccase with teracacidin as an inhibitor suggests that teracacidin might not act directly on the active site of laccase, but instead inhibit the radical reaction after laccase forms a complex with syringaldazine (Figure 6). Laccase activity is directly proportional to mycelial growth of a white rot fungus, *Agaricus bisporus* (Wood, 1979). Therefore, the antifungal fractions of *A. auriculiformis* and *A. mangium* may inhibit fungal growth as a result of quenching the radicals produced by the extracellular enzymes of *P. noxius* and *P. badius*. This hypothesis is further supported since the antifungal fractions that inhibited laccase activity also showed high DPPH radical scavenging activity (Table 2; Figure 7). The tannin fractions (M-C, A-C, and A-D) of *Acacia* spp., however, did not inhibit laccase activity. The structures of proanthocyanidins in these *Acacia* spp. extracts are unknown (Barry et al., in press) and further investigation is required.

While many previous studies have reported the antifungal and antimicrobial activities of polyphenols, the precise mechanisms involved with such activities are not clear. Enzyme inhibition by the astringent character of tannin and inhibition of the electron transport system on mitochondria have been

investigated (Scalbert, 1991). In this study, proanthocyanidin fractions (A-C, M-C, and M-D) did not show antifungal and laccase inhibitory activities. This suggests that the antifungal properties of *Acacia* spp. extracts were not due to the astringent character of proanthocyanidins (tannins). However, antifungal activity, DPPH radical scavenging activity, and laccase inhibition were all correlated with each other (Figure 7).

Mechanisms of wood degradation by wood-rotting fungi have been described. Backa et al. (1993) hypothesized that hydroxyl radicals were produced from the fungal degradation of wood. These hydroxyl radicals were produced by an extracellular, low molecular weight substance and as the rate of wood degradation increased so did the amounts of hydroxyl radicals (Tanaka et al., 1999). Therefore, the antifungal property of *A. auriculiformis* heartwood may be attributed to the scavenging of hydroxyl radicals as well as to laccase inhibition.

We focused attention on heartwood extracts of *A. mangium* and *A. auriculiformis* and found significant differences between the antifungal properties of both species that relates to the incidence of heartrot observed in these species during field studies. We tested extracts from two individual trees of both species; however, more trees should be sampled. It would be of interest to further investigate the antifungal property of hybrids of *A. mangium* and *A. auriculiformis*, of which some have been shown to be resistant to heartrot (Ito, 2002).

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CHEMICAL RESPONSE OF *Picea glehnii* SEED-EPIPHYTIC *Penicillium* SPECIES TO *Pythium vexans* UNDER *IN VITRO* COMPETITIVE CONDITIONS FOR MYCELIAL GROWTH

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Abstract—The potential protection of *Picea glehnii* seedlings from damping-off by seed-epiphytic *Penicillium* species was investigated. We studied the chemical response of seed-epiphytic *Penicillium* species (*Pen. cyaneum*, *Pen. damascenum*, and *Pen. implicatum*) to *Pythium vexans*, a damping-off fungus, *in vitro*. *Penicillium* species were cultured singly or cocultured with *Pyt. vexans* for 14 or 18 d, and mycelial growth, pH of culture filtrate, antifungal activity of the culture filtrate against *Pyt. vexans*, and the amount of antifungal compound produced by each *Penicillium* species, were examined. The filtrate of both the single culture of *Penicillium* and the coculture of *Penicillium* and *Pyt. vexans* showed antifungal activity against *Pyt. vexans*. In a coculture with *Pyt. vexans*, *Pen. cyaneum* produced an antifungal compound (patulin) as in the single culture. *Pen. damascenum* cocultured with *Pyt. vexans* produced an antifungal compound (citrinin), as it did in the single culture and in larger amounts on day 10. *Pen. implicatum* produced two antifungal compounds, frequentin and palitantin, and the ratio of frequentin (with higher antifungal activity than palitantin) to palitantin was higher in the coculture with *Pyt. vexans* than in the single culture. Our results indicate that these *Penicillium* species have the ability to produce antifungal compounds and to keep antifungal activity under competitive condition with *Pyt. vexans*. The chemical

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response of these *Penicillium* species to *Pyt. vexans* may contribute to protect *P. glehnii* seedlings from damage by *Pyt. vexans*.

Key Words—Seed-epiphytic fungi, *Penicillium damascenum*, *Penicillium implicatum*, *Penicillium cyaneum*, *Pythium vexans*, citrinin, frequentin, palitantin, patulin, *Picea glehnii*.

INTRODUCTION

Picea glehnii (Fr. Schm.) Masters is one of the dominant conifers in northern Japan (Hokkaido) (Tatewaki, 1958; Matsuda, 1989), where several pathogenic fungi can damage young seedlings in natural forest-regeneration areas (Takahashi, 1980; Endo et al., 1985). We have studied seed-epiphytic microorganisms that can protect *P. glehnii* seedlings from damping-off caused by *Pythium vexans*. We previously screened seed-epiphytic microorganisms that produce antifungal compounds against *Pyt. vexans*, and isolated three *Penicillium* species that produce particular antifungal compounds (Yamaji et al., 1999). *Pen. cyaneum* produces patulin, *Pen. damascenum* produces citrinin, and *Pen. implicatum* produces palitantin and frequentin. The damage to *P. glehnii* seedlings *in vitro* caused by *Pyt. vexans* is inhibited by coinoculation with seed-epiphytic *Pen. damascenum*. *Pen. damascenum* inhibits damping-off of the seedlings by (1) occupying the space around the seedling roots and (2) producing an antifungal compound citrinin (Yamaji et al., 2001). *Pen. cyaneum* and *Pen. implicatum* did not significantly protect seedlings from damage by *Pyt. vexans* because they did not grow vigorously in the coinoculation test (Yamaji et al., 2001).

How does *Penicillium* sp. interact with *Pyt. vexans* under competitive conditions when grown together? The antagonistic phenomena were classified into two categories: primary interaction, occurring mainly by direct contact between the two organisms, and secondary interaction, occurring mainly by changes in the environment between them (Dickinson and Lucas, 1982). In this study, we examined the chemical response of seed-epiphytic *Penicillium* species to *Pyt. vexans* under *in vitro* conditions competitive for their mycelial growth. We set up two culture conditions, the single culture of *Penicillium* species and the coculture (*Penicillium* species and *Pyt. vexans*). We examined (1) mycelial weight, (2) pH of culture filtrate, (3) antifungal activity of culture filtrate against *Pyt. vexans*, and (4) amount of antifungal compound produced by each *Penicillium* fungus.

METHODS AND MATERIALS

Fungi and Medium. P. glehnii seed-epiphytic *Pen. cyaneum* (Bainier et Sartory) Biourge (strain no. PGS-T5), *Pen. damascenum* Baghdadi (strain no.

PGS-O7), and *Pen. implicatum* Biourge (strain no. PGS-S16), which produced antifungal compounds against *Pyt. vexans* de Bary (Yamaji et al., 1999), and a damping-off fungus *Pyt. vexans*, which was isolated from the roots of *P. glehnii* seedlings in a nursery at Hokkaido University (Kasuya, 1995), were used. Potato dextrose (PD) broth (Wako, Tokyo, Japan) and potato-dextrose agar (PDA) containing 2% agar (Wako, w/v) were used for fungal cultivation.

Inoculation of Penicillium Fungus and Pyt. vexans. In the single culture of *Penicillium*, a spore suspension (10^5 spores/500 μl sterile water) was inoculated into a PD medium (30 ml) in an Erlenmeyer flask (50 ml). In the coculture, a mycelial disk (8 mm, i.d.) of *Pyt. vexans* grown on PDA for 2–3 d was inoculated into the medium simultaneously with a spore suspension of *Penicillium*. A single culture of *Pyt. vexans* was also prepared. The cultures were incubated in the dark at 25°C under stationary conditions for 2, 4, 6, 8, 10, 12, or 14 d for *Pen. cyaneum* and *Pen. damascenum*, and 2, 4, 6, 8, 10, 12, 14, 16, or 18 d for *Pen. implicatum.* Three replications were prepared for each incubation period and treatment.

Measurements

Mycelial Dry Weight. Fungal mycelia was recovered from broth culture by vacuum filtration on filter paper (Advantec, Tokyo, Japan) and was rinsed with deionized water three times. The culture filtrate was used to measure antifungal activity and to quantify the antifungal compounds. The filter paper with mycelia was dried in an oven at 110°C for 3 hr. Mycelial dry weight was calculated as the difference between the dry weights of filter paper with and without mycelia. In the coculture, it was impossible to separate the two fungi, therefore the total dry weight of the mycelia of the two fungi was measured. The values in the three cultures per incubation time were averaged.

pH. pH of three culture filtrates per incubation time was measured with an ISFET pH meter (Beckman Coulter, Inc., Fullerton, CA, USA), and the values were averaged.

Antifungal Activity. A mycelial disk (8 mm i.d.) of *Pyt. vexans* grown on PDA for 2–3 d was placed at the center of a PDA plate (90 mm i.d.). The culture filtrate (125 or 250 μl) was refiltered with a 0.22-μm syringe filter (Advantec), and aseptically dispensed into a sterile cylinder (6 mm i.d. × 10 mm, stainless steel) placed 1.5 cm from the margin of the *Pyt. vexans* disk. After a 36-hr incubation in the dark at 25°C, inhibitory zones (mm) were measured from the margin of the cylinder to the mycelia. Each test was conducted three times per culture filtrate, so the values of nine cylinders per incubation time were averaged.

Quantification of Compounds

Chromatographic Equipment. For analyses of patulin and citrinin, high-performance liquid chromatography (HPLC) equipped with a Hitachi L-6000 Intelligent pump (Hitachi, Tokyo, Japan) and a Hitachi L-4200 UV–VIS detector (Hitachi) set at 254 nm was used. For analyses of palitantin and frequentin, HPLC equipped with a Hitachi L-6320 Intelligent pump (Hitachi) and a Hitachi L-4250 UV–VIS detector (Hitachi) set at 254 nm was used.

Chemicals. All chemicals used as internal standards (3, 5-di-*tert*-buthylphenol, 4-*n*-butoxybenzoic acid, and orcinol) were of analytical grade (Kanto Co. Ltd., Tokyo, Japan). Chloroform, acetonitrile, and 2-propanol for elution were of HPLC grade (Wako). Patulin, citrinin, palitantin, and frequentin were isolated as previously reported (Yamaji et al., 1999, 2001) and used to make standard curves.

Chromatographic Conditions, Extraction, and Purification of Patulin. HPLC was performed using a LiChrosorb DIOL column (250 × 6 mm i.d.; Merck, Darmstadt, Germany), with chloroform as the eluent at a flow rate of 0.5 ml/min at ambient temperature. The culture filtrate was adjusted to pH 2 with 2 M HCl, then extracted with ethyl acetate (10 ml) three times. The combined organic layer was dried over Na_2SO_4, followed by vacuum concentration to dryness. The residue was then dissolved in 2 ml of chloroform and subjected to a Diol September-pak cartridge (Sep-pak Plus, short body type; Waters, Milford, MA, USA) preequilibrated with the same solvent. Patulin was eluted with 4 ml of chloroform, and the eluate was dried *in vacuo* and redissolved in 2 ml of chloroform including 3,5-di-*tert*-buthylphenol (1 mg/ml) as an internal standard. An aliquot (5 μl) of the solution thus prepared was analyzed. Each analysis was conducted per culture filtrate, and three values per incubation time were averaged. When concentrations of patulin were high, the sample solution was diluted before analysis.

Citrinin. HPLC was performed by using an Inertsil PREP-ODS column (250 × 5 mm i.d., G. L. Sciences Inc., Tokyo, Japan) with 0.25 M phosphoric acid–acetonitrile–2-propanol (55:35:10) as the eluent (Phillips et al., 1980) at a flow rate of 1.0 ml/min, at ambient temperature. The culture filtrate was extracted with ethyl acetate as described above. The residue was dissolved in 2 ml of water–acetonitrile–2-propanol (55:35:10) and subjected to a C18 September-pak cartridge (Sep-pak Plus, short body type; Waters) preequilibrated with the same solvent. Citrinin was eluted with 8 ml of water–acetonitrile–2-propanol (55:35:10), then the eluate was dried *in vacuo* and redissolved in 2 ml of acetonitrile including 4-*n*-butoxybenzoic acid (100 μg ml^{-1}) as an internal standard. An aliquot (5 μl) of the solution thus prepared was analyzed. Three replicates of every culture filtrate were analyzed and averaged. When the concentration of citrinin was high, the sample solution was diluted prior to analysis.

Palitantin and Frequentin. HPLC was performed by using a YMC-Pack Polymer C_{18} column (250 × 6.0 mm i.d., YMC, Tokyo, Japan) with Na_2HPO_4/NaOH (pH10)–CH_3CN (4:1) as the eluent at a flow rate of 1.0 ml/min at ambient temperature. Na_2HPO_4/NaOH (pH10) buffer was prepared according to Perrin and Dempsey (1974). The culture filtrate was extracted with ethyl acetate as described above. The residue was dissolved in 2 ml of acetonitrile–water (1:4) and applied to a C18 September-pak cartridge (Sep-pak plus, short body type; Waters) preequilibrated with the same solvent. The cartridge was rinsed with 3 ml of acetonitrile–water (1:4), and palitantin and frequentin were eluted with 8 ml of acetonitrile–water (1:1). The eluate was dried *in vacuo* and redissolved in 2 ml of acetonitrile, including orcinol (340 μg/ml) as an internal standard. An aliquot (5 μl) of the solution thus prepared was analyzed. Three replicates of each culture filtrate were analyzed and averaged. The solution of frequentin or palitantin was diluted as necessary before analysis.

Standard Curve. Patulin (31.3, 62.5, 125, 250, 500, and 1000 μg/ml acetone) or citrinin (125, 250, 500, 1000, and 2000 μg/ml acetone), or palitantin and frequentin (125 and 125, 250 and 250, 500 and 500, 1000 and 1000, 2000 and 2000 μg/ml acetone) was added to each PD medium (29 ml). Three replicate flasks were prepared for each concentration of compound. The medium was extracted with ethyl acetate, purified, and dissolved in the solvent including the standard compound by the methods described above. Analysis of three replicate samples was conducted and results were averaged. The standard curve was obtained via the internal standard method.

Statistical Analysis. Two-factor factorial ANOVA (Statview 5.1.0-Mac) with days and treatments as independent factors was used to examine differences in dry weight, pH, antifungal activity, and amount of antifungal compound between the single culture and the coculture. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Single Culture of Pen. cyaneum and Coculture with Pyt. vexans

Mycelial Dry Weight. In the single culture, the dry weight of *Pen. cyaneum* increased until day 10 (115 mg), and decreased slightly thereafter (Figure 1a). In coculture with *Pyt. vexans*, the total dry weight of *Pen. cyaneum* and *Pyt. vexans* increased until day 10 and then became stable (107–108 mg) (Figure 1a). Dry weight in the single culture of *Pyt. vexans* increased from day 4 to 106 mg on day 16 and then decreased on day 18 (Figures 1a and 5a). The *total* dry weight of *Pen. cyaneum* and *Pyt. vexans* in the coculture was not significantly different from that of *Pen. cyaneum* in the single culture (Figure 1a). Since the

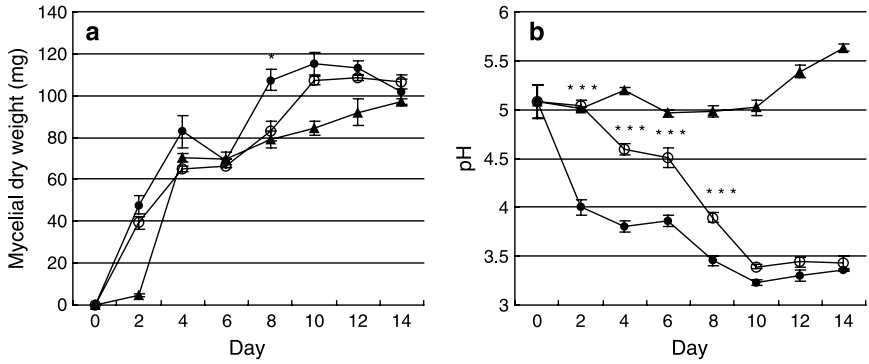

FIG. 1. Single culture of *Pen. cyaneum* and coculture with *Pyt. vexans* (1). (a) Mycelial dry weight. (b) pH. Filled circle: the single culture of *Pen. cyaneum*. Open circle: the coculture of *Pen. cyaneum* with *Pyt. vexans*. Filled triangle: the single culture of *Pyt. vexans*. Statistical analysis was conducted to compare the single culture with the co-culture with *Pyt. vexans*. Bars: ±SE. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

growth of both *Pen. cyaneum* and *Pyt. vexans* was observed in the coculture, the growth of *Pen. cyaneum* must be less in the coculture compared to the single culture.

pH. In the single culture of *Pen. cyaneum*, pH decreased quickly by day 2 and became stable after day 10 (pH 3.23–3.36) (Figure 1b). In the coculture with *Pyt. vexans*, pH gradually decreased until day 10 and became stable thereafter (pH 3.39–3.44) (Figure 1b). pH in the single culture of *Pyt. vexans* was nearly stable until day 10 (pH 4.96–5.19) and increased to 5.63 by day 14 and decreased thereafter to 5.28 (Figures 1b and 5b). pH in the coculture with *Pyt. vexans* was higher than that in the single culture of *Pen. cyaneum* on days 2, 4, 6, and 8 ($P < 0.001$). Thus, the growth of *Pyt. vexans* in the coculture might delay the decrease of pH (Figure 1b).

Antifungal Activity and Amount of Patulin. In the single culture of *Pen. cyaneum*, antifungal activity appeared on day 6, increased until day 10, and became stable thereafter (inhibitory zones; 12.6–13.7 mm) (Figure 2a). Patulin was detected on day 4 and increased rapidly until day 8 (66.8 μg/125 μl), and slightly thereafter (Figure 2b). In the coculture with *Pyt. vexans*, antifungal activity appeared on day 8, increased until day 12 (15.0 mm), and then decreased slightly (13.4 mm on day 14) (Figure 2a). Patulin was detected on day 4, but it increased rapidly after day 6, and the concentration stabilized after day 10 (65.7–70.6 μg/125 μl) (Figure 2b). The antifungal activity and the amount of patulin on days 10, 12, and 14 were not significantly different between the cultures of *Pen. cyaneum* with and without *Pyt. vexans* ($P > 0.05$, Figure 2). On

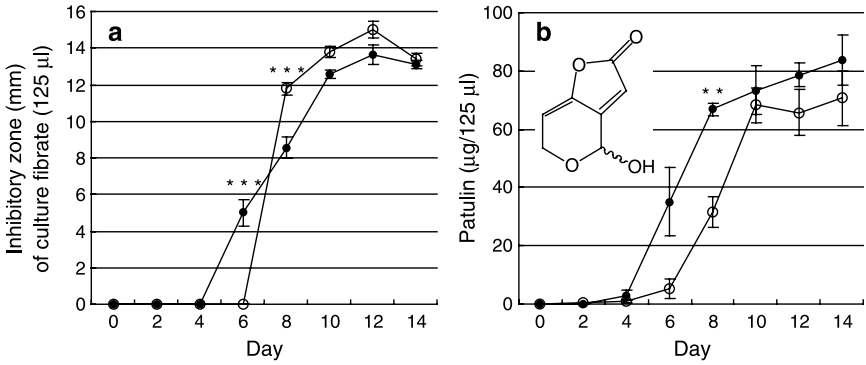

FIG. 2. Single culture of *Pen. cyaneum* and coculture with *Pyt. vexans* (2). (a) Antifungal activity. (b) Amount of patulin. Filled circle: the single culture *Pen. cyaneum*. Open circle: the coculture of *Pen. cyaneum* with *Pyt. vexans*. Bars: ±SE. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. The measurement unit µg/125 µl was used in panel b because the inhibitory zones of culture filtrates (125 µl) were examined in panel A. The culture filtrate in the single culture of *Pyt. vexans* did not show any antifungal activity against *Pyt. vexans*. No antifungal compound was detected in the single culture filtrate of *Pyt. vexans*.

day 8, the antifungal activity in the coculture was higher than in the single culture ($P < 0.001$), although the amount of patulin was smaller than that in the single culture ($0.001 < P < 0.01$) (Figure 2). Twenty-five to 100 µg/disk of patulin causes a 14-mm inhibitory zone against *Pyt. vexans* (Yamaji et al., 1999) (the antifungal results using paper disks were not much different from those using cylinders when antifungal compounds in our study were examined). However, the inhibitory zone caused by the culture filtrate of the single culture on day 8 (66.8 µg/125 µl) was much smaller than 14 mm. *Pen. cyaneum* grew more vigorously in the single culture than that in the coculture on day 8 (Figure 1a), suggesting that some metabolites produced in the single culture may have suppressed the activity of patulin. *Pen. cyaneum*, hence, produces patulin even under competitive conditions with *Pyt. vexans*.

Single Culture of Pen. damascenum and Coculture with Pyt. vexans

Mycelial Dry Weight. In the single culture, the dry weight of *Pen. damascenum* increased until day 12 and stabilized thereafter (144 mg) (Figure 3a). In coculture with *Pyt. vexans*, the total dry weight of *Pen. damascenum* and *Pyt. vexans* increased until day 6, fluctuated slightly, then decreased to 102 mg

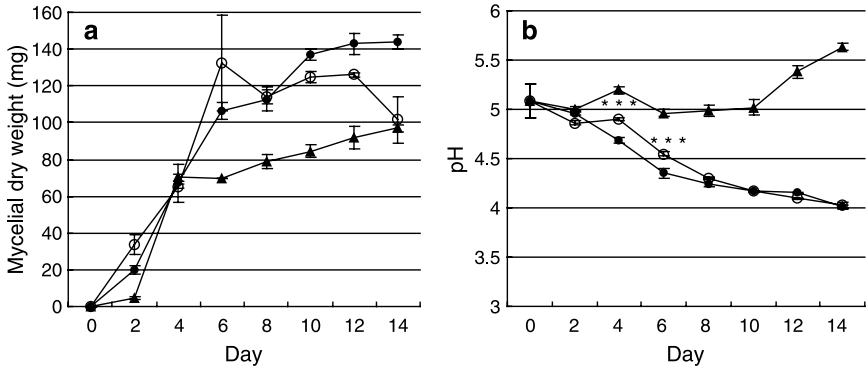

FIG. 3. Single culture of *Pen. damascenum* and coculture with *Pyt. vexans* (1). (a) Mycelial dry weight. (b) pH. Filled circle: the single culture of *Pen. damascenum*. Open circle: the coculture of *Pen. damascenum* with *Pyt. vexans*. Filled triangle: the single culture of *Pyt. vexans*. Statistical analysis was conducted to compare the single culture with the coculture with *Pyt. vexans*. Bars: ±SE. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

by day 14 (Figure 3a). There were no significant differences between *total* mycelial dry weights of the two fungi in the coculture and the dry weight of *Pen. damascenum* in the single culture ($P > 0.05$). Again, as growth of both *Pen. damascenum* and *Pyt. vexans* was observed in the coculture, the growth of *Pen. damascenum* is less in the coculture.

pH. In both the single culture of *Pen. damascenum* and the coculture with *Pyt. vexans*, pH decreased until day 8 and became stable thereafter (Figure 3b). However, pH in the coculture on days 4 and 6 was higher than that in the single culture ($P < 0.001$). The growth of *Pyt. vexans* in the coculture, thus, might delay the pH decrease (Figure 3b).

Antifungal Activity and Amount of Citrinin. In the single culture of *Pen. damascenum*, antifungal activity appeared on day 4 and continued to increase until day 14 (inhibitory zone; 9.4 mm on day 14) (Figure 4a). Citrinin was first detected on day 4, continued to increase until day 8, and became nearly stable thereafter (32.2–39.3 μg/250 μl) (Figure 4b). In the coculture with *Pyt. vexans*, antifungal activity appeared on day 6 and became nearly stable after day 8 (inhibitory zone; 9.4–10.6 mm) (Figure 4a). Citrinin was first detected on day 4 and rapidly increased to its highest value on day 10 (68.9 μg/250 μl). After day 10, citrinin decreased (25.6 μg/250 μl on day 14) (Figure 4b). Antifungal activity in the coculture was higher than that in the single culture on days 6, 8, and 10 but lower on day 4 ($P < 0.001$). However, the amount of citrinin was not different between the two cultures for 14 d except on day 10 (Figure 4b). Although 33 μg/disk of citrinin causes a 9.5-mm inhibitory zone against *Pyt.*

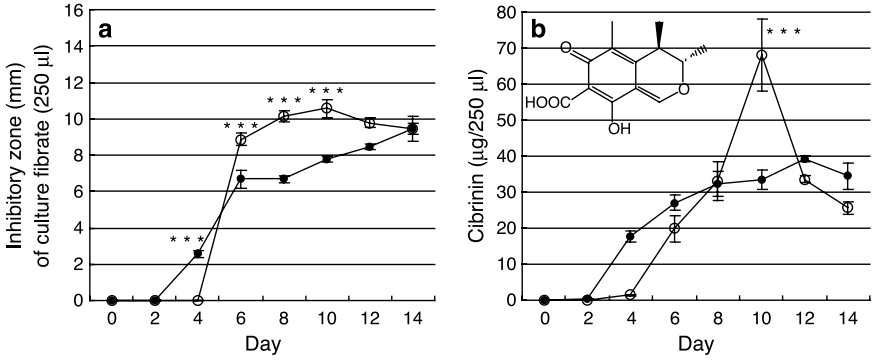

FIG. 4. Single culture of *Pen. damascenum* and coculture with *Pyt. vexans* (2). (a) Antifungal activity. (b) Amount of citrinin. Filled circle: the single culture of *Pen. damascenum*. Open circle: the coculture of *Pen. damascenum* with *Pyt. vexans*. Bars: ±SE. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. The measurement unit µg/250 µl was used in panel b because the inhibitory zones of culture filtrates (250 µl) were examined in panel a. The culture filtrate in the single culture of *Pyt. vexans* did not show any antifungal activity against *Pyt. vexans*. No antifungal compound was detected in the single culture filtrate of *Pyt. vexans*.

vexans (Yamaji et al., 1999), the inhibitory zones caused by the culture filtrate of the single culture on day 8 (32.2 µg/250 µl), day 10 (33.5 µg/250 µl), and day 12 (39.3 µg/250 µl) were smaller than 9.5 mm. These results suggest that some metabolites produced in the single culture may have suppressed the antifungal activity of citrinin. The largest amount of citrinin on day 10 (68.9 µg/250 µl) in the coculture did not appear to affect inhibition (10.6 mm, Figure 4a). Our results indicate that *Pen. damascenum* under competitive conditions with *Pyt. vexans* produces citrinin constantly and more abundantly (day 10) than in the single culture.

Single Culture of Pen. implicatum and Coculture with Pyt. vexans

Mycelial Dry Weight. In the single culture, dry weight of *Pen. implicatum* increased until day 10 (115 mg on day 10) and became nearly stable thereafter (98–112 mg) (Figure 5a). In coculture with *Pyt. vexans*, the total dry weight of *Pen. implicatum* and *Pyt. vexans* increased irregularly until day 18 (120 mg on day 18) (Figure 5a). The *total* dry weight of the two fungi in the coculture was heavier than that of *Pen. implicatum* in the single culture on day 4 ($0.001 < P < 0.01$), but was not significantly different thereafter (Figure 5a). The mycelial growth of both *Pen. damascenum* and *Pyt. vexans* was observed in the coculture; therefore, the growth of *Pen. implicatum* is lower in the coculture with *Pyt. vexans*, at least on days 8, 10, 12, and 14.

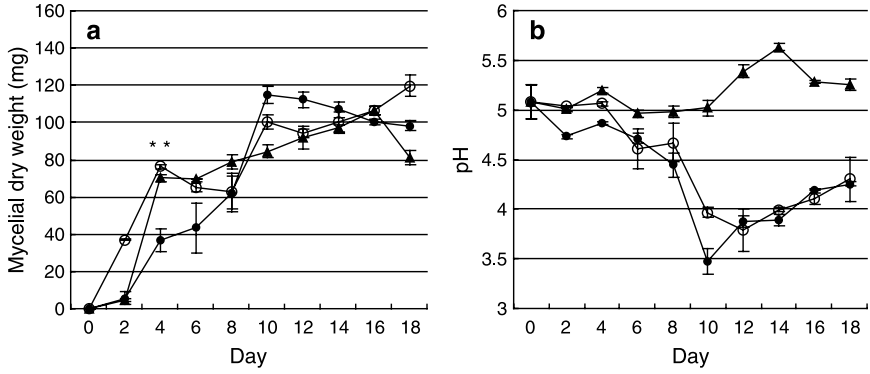

FIG. 5. Single culture of *Pen. implicatum* and coculture with *Pyt. vexans* (1). (a) Mycelial dry weight. (b) pH. Filled circle: the single culture of *Pen. implicatum*. Open circle: the coculture of *Pen. implicatum* with *Pyt. vexans*. Filled triangle: the single culture of *Pyt. vexans*. Statistical analysis was conducted to compare the single culture with the coculture with *Pyt. vexans*. Bars: ±SE. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

pH. Both in the single culture of *Pen. implicatum* and in the coculture with *Pyt. vexans*, pH decreased during the first 10 d and increased slightly thereafter (Figure 5b). On day 10, pH was 3.47 in the single culture and 3.97 in the coculture. There were no significant differences in pH between the single culture and the coculture during the cultural period ($P > 0.05$).

Antifungal Activity and Amount of Palitantin and Frequentin. In both the single culture of *Pen. implicatum* and the coculture with *Pyt. vexans*, antifungal activity appeared on day 10 and increased until day 14 (Figure 6a). Antifungal activity after day 14 was stable in the single culture (inhibitory zone; 11.8–12.0 mm), but decreased in the coculture (inhibitory zone; 8.80–10.1 mm) (Figure 6a). In the single culture, frequentin was first detected on day 8, increased rapidly until day 16 (21.1 μg/125 μl), and decreased thereafter (Figure 6b). Palitantin was detected on day 4, continued to increase until day 14 (10.7 μg/125 μl on day 14), and decreased thereafter (Figure 6c). In the coculture with *Pyt. vexans,* frequentin increased after day 10 until day 16 (15.8 μg/125 μl on day 16), and then decreased thereafter (Figure 6b). The amount of palitantin in the coculture was low during the cultural period (0–3.6 μg/125 μl) (Figure 6c). The antifungal activity in the coculture was not significantly different from or lower (on days 10, 12, and 18, $P < 0.001$) than that in the single culture of *Pen. implicatum* (Figure 6a). In contrast, the pattern of the antibiotic production was different between the single culture and the coculture (Figure 6b and c). The maximum ratio of frequentin to palitantin was 3.67:1 in the single culture, whereas it was 5.87:1 in the coculture during the 18-d culture

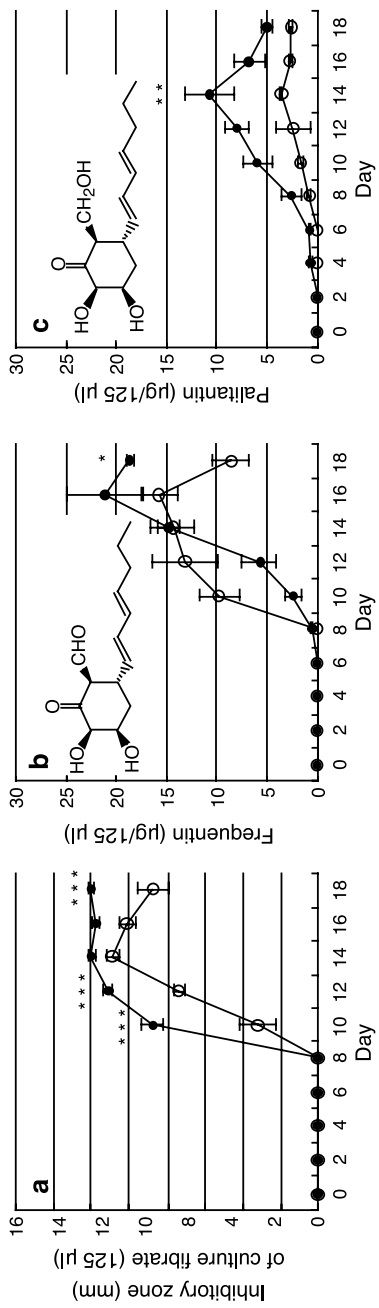

FIG. 6. Single culture of *Pen. implicatum* and coculture with *Pyt. vexans* (2). (a) Antifungal activity. (b) Amount of frequentin. (c) Amount of palitantin. Filled circle: the single culture of *Pen. implicatum*. Open circle: the coculture of *Pen. implicatum* with *Pyt. vexans*. Bars: ±SE. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$. The measurement unit µg/125 µl was used in panels b and c because the inhibitory zones of culture filtrates (125 µl) were examined in panel a. The culture filtrate in the single culture of *Pyt. vexans* did not show any antifungal activity against *Pyt. vexans*. No antifungal compound was detected in the culture filtrate of *Pyt. vexans*.

period. Frequentin shows higher antifungal activity than palitantin against *Pyt. vexans* (Yamaji et al., 2001). *Pen. implicatum* in the coculture produced a larger amount of frequentin than palitantin. *Pen. implicatum*, thus, may maintain high antifungal activity by changing the ratio of antifungal compounds produced.

Each *Penicillium* species in our study showed a particular chemical response to *Pyt. vexans*. Several researchers have reported chemical responses between microorganisms. Coculture of *Lentinus edodes* (Basidiomycete) and *Trichoderma* spp. (a pathogenic fungus against *L. edodes*) showed higher antifungal activity against *Trichoderma* than that of the single culture of *L. edodes* (Tokimoto et al., 1987). *L. edodes* produced two induced antifungal compounds against *Trichoderma* spp. in coculture, and a higher production depended on the kind of medium (Tokimoto et al., 1987). Coculture of *Aspergillus flavus* and *Aspergillus nidulans* produced two induced antibiotics, hydroxyaspergillic acid and an aspergillic acid-like compound, that were not produced when either fungus was grown alone. (Perry et al., 1984). Soil bacteria produced volatile organic compounds (VOCs) that affected the mycelial growth and enzyme activity of several fungi (Mackie and Wheatley, 1999). Fungal response to the bacterial VOCs varied with medium, species, and age of the fungus and the bacteria. These reports and our results suggest that the chemical responses between microorganisms vary with the species, environment, and age.

Pen. cyaneum, *Pen. damascenum*, and *Pen. implicatum* have the ability to produce antifungal compounds and to keep antifungal activity under conditions competitive with *Pyt. vexans*. *Pen. damascenum* can grow vigorously around *P. glehnii* seedling roots and protect seedlings from damping-off when co-inoculated with *Pyt. vexans* (Yamaji et al., 2001). In that report, modified Melin Norkrans Medium (MMN) without glucose was used for seedling growth, thus carbon for fungal growth was supplied from only *P. glehnii* seedlings. *Pen. damascenum* seems to utilize carbon exuded from the seedling roots and to resist *Pyt. vexans* by constant or abundant production of citrinin even under competitive conditions. In contrast, *Pen. cyaneum* and *Pen. implicatum*, which did not protect *P. glehnii* seedlings from damage by *Pyt. vexans* (Yamaji et al., 2001), may not fully utilize the root exudate when grown together with *Pyt. vexans* around *P. glehnii* seedling roots. Composition of root exudates (amino acids and soluble carbon compounds) changes with plant age in trees (Grayston et al., 1996), and this may influence competition between microorganisms in the rhizosphere. When *Pen. cyaneum* and *Pen. implicatum* compete with *Pyt. vexans* for nutrients, *Pen. cyaneum* seems to produce patulin constantly, while *Pen. implicatum* seems to change the proportion of frequentin and palitantin. Both these chemical responses to *Pyt. vexans* may contribute to protection of *P. glehnii* seedlings from damage by *Pyt. vexans*.

Penicillium species are common saprophytes in the soil and have been isolated in significant amounts from the surface of seeds of various plants

(Peterson, 1959; Watanabe, 2002). As well as interactions between symbiotic microorganisms, we should also consider the possible interaction of free-living microorganisms in the rhizosphere. Rhizospheric microorganisms are never independent of other microorganisms and plants. Therefore it is important to clarify the chemical interactions between them.

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INTERSPECIFIC VARIATION IN DEFENSE SECRETIONS OF MALAYSIAN TERMITES FROM THE GENUS *Bulbitermes*

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Abstract—The chemicals of the defense secretions of Malaysian *Bulbitermes*, *B. singaporensis*, *B. germanus*, *B. sarawakensis*, and *Bulbitermes* sp. B, show that *B. singaporensis* is distinct from the other species, which are themselves closely related; the genetic distance between *B. singaporensis* and *B. germanus* is 0.71. *B. singaporensis* contains tetracyclic kempane, and *B. germanus* and *B. sarawakensis* contain tricyclic trinervitene; *Bulbitermes* sp. B contains a mixture of kempane and trinervitene. The mono- and diterpenoid compositions are species-specific.

Key Words—*Bulbitermes*, defense secretions, diterpenes, interspecific variations, Nasutitermitinae.

INTRODUCTION

The genus *Bulbitermes* belongs to the subfamily Nasutitermitinae, whose soldiers possess conical, nozzle-shaped projections on their heads from which defensive secretions are ejected. Behaviorally, *Bulbitermes* species forage under covered trails that run from their arboreal nests up tree trunks; they feed on dead plant material. Only soldiers and workers move in the covered trails; the soldiers protect the trails by positioning themselves at the sides. If the cover is disturbed, the soldiers defend their foraging trail by ejecting a gluey secretion at the predator (Prestwich, 1979a). The Malaysian *Bulbitermes* termites are less studied than the free-ranging *Lecessititermes* (Goh et al., 1990), *Hospitalitermes* (Chuah et al., 1983, 1986), and *Longipeditermes* (Goh et al., 1984), and, in fact,

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even the biology is relatively unknown. This paper presents the compositional and structural variations of the defense secretions of the soldier termites of *Bulbitermes singaporensis* (Prestwich et al., 1981a), *B. germanus*, *B. sarawakensis*, and an unidentified species which will be referred to as *Bulbitermes* sp. B (Tho, 1992).

METHODS AND MATERIALS

Termite Material. Soldier termites used for the present studies were collected from within closed canopy dipterocarp forests from the following localities in Peninsular Malaysia (Figure 1): (1) Gunong Jerai—an upper hill dipterocarp forest in the state Kedah; (2) Muka Head Field Station—a coastal hill dipterocarp forest reserve in Penang; (3) Maxwell Hill—an upper hill dipterocarp forest bordering on the oak montane forest type; (4A) Gombak;

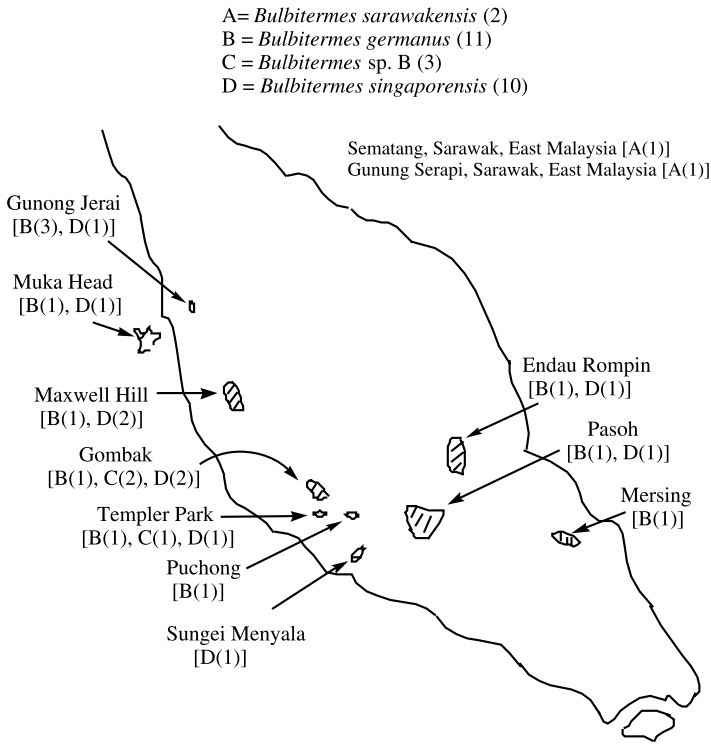


FIG. 1. Distribution of *Bulbitermes* in Peninsular Malaysia.

(4B) Templer Park; (4C) Puchong; 4A–C are hill dipterocarp forests in the state of Selangor; (5) Sungei Menyala—a lowland dipterocarp forest in the state of Negeri Sembilan; (6) Pasoh—a lowland dipterocarp forest in the state of Negeri Sembilan that has been the site for intensive ecological studies under the auspices of the International Biological Program; (7) Endau Rompin—a forest reserve area in the states of Pahang and Johor; (8) Mersing—a lowland dipterocarp forest in the state of Johor; and (9) Sematang and Gunung Serapi—an upper hill dipterocarp forest in the state Sarawak, East Malaysia. The species are labeled as A, B, C, and D, and the number of colonies collected were indicated (Figure 1).

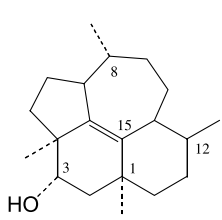
With minimum disturbance, the soldiers were collected in plastic bags and chilled to −10°C, after which they were decapitated and the heads (ca. 20) were crushed and extracted by hexane. The crude extracts were then filtered, and the solvent was partially removed *in vacuo* before analysis (Chuah et al., 1983, 1990).

Analytical Methods. Gas chromatography (GC) analysis of monoterpenes and diterpenes was performed on an HP 5790A instrument fitted with a flame ionization detector. The oven temperature was programmed at 6°C/min from 80 to 150°C and then at 8°C/min to 300°C. Identification of the monoterpenes and diterpenes was performed on an HP 5972A MSD mass spectrometer interfaced with an HP-6890 series GC fitted with an HP-5 5% phenyl methyl siloxane capillary column (30 m × 0.25 mm i.d., 0.3-μm film thickness). The mass spectra were recorded at an ionization voltage of 70 eV with an ion source temperature of 180°C. The monoterpenes (I–V) were characterized by GC-MS spectroscopy and coinjection with commercially available (Tokyo Kasei) standards (Table 1). Similarly, diterpenes (VI–XX, Figure 2) were characterized by MS and NMR spectroscopy (Vrkoc et al., 1978a,b; Prestwich, 1979a,b; Dupont et al., 1981) and by coinjection with compounds previously isolated (Chuah et al., 1983, 1986, 1989; Goh et al., 1982, 1984, 1990). Isolation of

TABLE 1. INTERSPECIFIC VARIATION OF MONOTERPENE COMPOSITION (%) IN *Bulbitermes*

Compounds	*B. singaporensis*[a]	*B. germanus*[b]	*B. sarawakensis*[c]	*Bulbitermes* sp. B[d]
α-Pinene (I)	25–38	55–69	53–60	29–33
β-Pinene (II)	53–64	23–32	25–30	54–61
Myrcene (III)	0–1	0–1	0–1	0–1
Limonene (IV)	7–10	4–12	5–11	3–8
Terpinolene (V)	1–4	1–3	1–3	0–2

[a]Range for 10 colonies.
[b]Range for 11 colonies.
[c]Range for two colonies.
[d]Range for three colonies.



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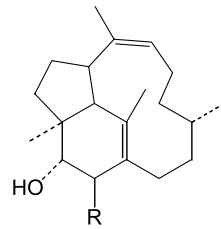
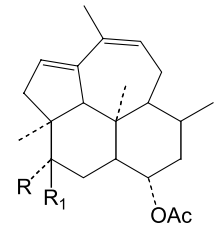
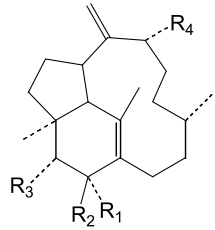


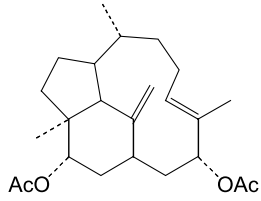
VIII R=H
XII R=OH



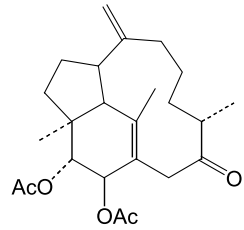
XIV R , R_1=O
XV R=H, R_1=OAc
XVI R=OAc, R_1=H



VII R_1=R_2=R_4=H, R_3=OH
IX R_1 , R_2=O, R_3=OH, R_4=H
X R_1=R_4=H, R_2=OAc, R_3=OH
XI R_1=R_4=H, R_2=R_3=OH
XIII R_1=H, R_2=R_3=OH, R_4=OAc
XVIII R_1=H, R_2=R_3=OAc, R_4=OH
XX R_1=H, R_2=R_3=R_4=OAc



XVII



XIX

FIG. 2. Diterpenes from defense secretions of *Bulbitermes*.

individual compounds from the crude secretion (ca. 30 mg extracted from approximately 1500 soldier termites) was aided by a Waters 440 HPLC instrument fitted with a 25 × 0.64-cm. Ultrasphere silica gel column. Elution of the diterpenes was by a gradient of 2–10% ethyl acetate in petroleum ether (bp 60–80°C). The diterpenes isolated by HPLC were checked for purity by GC and TLC on Merck 5 × 10-cm, 0.25-mm silica gel 60 plates. Proton and carbon NMR spectra were recorded on a Jeol JNM-GSX 270 spectrometer. The isolated known diterpenes (VI–XX) were identified by their ^{1}H NMR, ^{13}C NMR, and MS spectroscopy (Chuah et al., 1983, 1986, 1989; Goh et al., 1982, 1984, 1990).

TABLE 2. INTERSPECIFIC VARIATION OF DITERPENE COMPOSITION (%) IN *Bulbitermes*

Compounds	*B. singaporensis*[a]	*B. germanus*[b]	*B. sarawakensis*[c]	*Bulbitermes* sp. B[d]
15-Ripperten-3α-ol (VI)		8–12	9–11	3–6
Trinervita-1(15), 8(19)-dien-3α-ol (VII)		0–1	0–1	
Trinervita-1(15), 8(9)-dien-3α-ol (VIII)		5–8	4–7	7–10
2-Oxotrinervita-1(15), 8(19)-dien-3α-ol (IX)		4–9	5–8	4–6
Trinervita-1(15), 8(19)-dien-2β, 3α-diol-2-*O*-acetate (X)		3–7	4–6	1–3
Trinervita-1(15),8(19)-dien-2β, 3α-diol (XI)		44–55	43–54	35–42
Trinervita-1(15),8(9)-dien-2β, 3α-diol (XII)		6–12	7–10	12–15
Trinervita-1(15), 8(19)-dien-2β,3α, 9α-triol-9-*O*-acetate (XIII)		8–13	9–11	1–2
3-Oxokempa-6, 8-dien-14α-ol- 14-*O*-acetate (XIV)	40–46			
Kempa-6,8-dien-3β, 14α-diol-3, 14-*O*-diacetate (XV)	21–23			4–6
Kempa-6,8-dien-3α, 14α-diol-3, 14-*O*-diacetate (XVI)	17–21			5–8
Trinervita-11(12), 15(17)-dien-3α, 13α-diol-3, 13-*O*-diacetate (XVII)	14–18			
Trinervita-1(15), 8(19)-dien-2β,3α, 9α-triol-2,3-*O*-diacetate (XVIII)		1–4	1–3	3–5
13-Oxotrinervita-1(15), 8(19)-dien-2β, 3α-diol-2,3-*O*- diacetate (XIX)		1–2	1–2	1–2
Trinervita-1(15), 8(19)-dien-2β,3α,9α -triol-2,3,9-*O*-triacetate (XX)		1–2	1–2	

[a]Range for 10 colonies.
[b]Range for 11 colonies.
[c]Range for two colonies.
[d]Range for three colonies.

TABLE 3. CHROMATOGRAPHIC PROPERTIES OF DITERPENES FROM *Bulbitermes*

Compounds	Mol. wt.	R_f (color)[a]	OV-1[b]	OV-17[c]
15-Ripperten-3α-ol (VI)	288	0.48 (sky blue)	21.8	24.1
Trinervita-1(15),8(19)-dien-3α-ol (VII)	288	0.58 (wisteria violet)	22.0	24.3
Trinervita-1(15),8(9)-dien-3α-ol (VIII)	288	0.54 (violet)	22.3	24.7
2-Oxotrinervita-1(15),8(19)-dien-3α-ol (IX)	302	0.61 (lilac)	22.8	25.5
Trinervita-1(15),8(19)-dien-2β, 3α-diol-2-*O*-acetate (X)	346	036 (pink)	23.5	26.2
Trinervita-1(15),8(19)-dien-2β,3α-diol (XI)	304	0.23 (pink)	23.5	26.2
Trinervita-1(15),8(9)2β,3α-diol (XII)	304	0.16 (pink)	24.0	26.7
Trinervita-1(15),8(19)-dien-2β, 3α,9α-triol-9-*O*-acetate (XIII)	362	0.14 (deep purple)	24.5	27.2
3-Oxokempa-6,8-dien-14α-ol-14-*O*-acetate (XIV)	342	0.54 (green)	24.9	27.2
Kempa-6,8-dien-3β,14α-diol-3, 14-*O*-diacetate (XV)	386	0.58 (green)	25.5	28.0
Kempa-6,8-dien-3α,14α-diol-3, 14-*O*-diacetate (XVI)	386	0.57 (deep purple)	25.9	28.0
Trinervita-11(12),15(17)-dien-3α, 13α-diol-3,13-*O*-diacetate (XVII)	388	0.59 (blue)	26.3	29.5
Trinervita-1(15),8(19)-dien-2β,3α, 9α-triol-2,3-*O*-diacetate (XVIII)	404	0.42 (greenish blue)	27.1	28.8
13-Oxotrinervita-1(15),8(19)-dien-2β, 3α-diol-2,3-*O*-diacetate (XIX)	402	0.56 (violet)	27.7	29.0
Trinervita-1(15),8(19)-dien-2β, 3α,9α-triol-2,3,9-*O*-triacetate (XX)	446	0.52 (orange)	27.9	31.0

[a]TLC on silica gel G, solvent ethyl acetate/dichloromethane (5:95, v/v). Stained by vanillin.
[b]1.6 m × 3 mm 3% OV-101 on 100–120 Gas Chrom. Q; retention indices relative to *n*-alkanes.
[c]1.6 m × 3 mm 3% OV-17 on 100–120 Gas Chrom Q.

The entire gas chromatographic profile of each defense secretion was typically obtained by on-column injection at 90°C and then programmed at 8°C/min to 300°C on 1.6 m × 3-mm 3% OV-101 and 1.6 m × 3-mm 3% OV-17 glass columns. Retention indices of the diterpenes were relative to *n*-alkane

TABLE 4. GENETIC DISTANCES AMONG *Bulbitermes*

Species	*B. germanus*	*B. sarawakensis*	*Bulbitermes* sp. B
B. singaporensis	0.71	0.72	0.40
B. germanus		0.01	0.10
B. sarawakensis			0.09

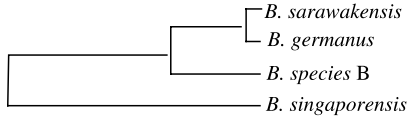


FIG. 3. Genetic relationship among *Bulbitermes*.

standards, which were spiked into the chromatograms by coinjection with the samples. The quantification of monoterpenes and diterpenes was made relative to C_{14} and C_{20} *n*-alkanes, respectively. The data are summarized in Tables 1 and 2. The R_f values of diterpenes on silica gel TLC plates, solvent ethyl acetate/dichloromethane (5:95, v/v), are listed in Table 3.

The genetic distance between two species was calculated (Table 4) by using the mean relative proportions of all the mono- and diterpene compounds following the formula of Nei (1972) on the basis of the assumption that the defense chemicals are genetic:

$$D_{XY} = -\mathrm{In}\left[\frac{\sum X_i Y_i}{\sqrt{\left[\sum X_i^2 \sum Y_i^2\right]}}\right]$$

A dendrogram using the unweighted pair-group arithmetic average clustering method was constructed (Figure 3).

RESULTS AND DISCUSSION

The evolution and development of the frontal gland in the subfamily Nasutitermitinae, of which over 500 species have been reported (Tho, 1992), are known to parallel the development of chemicals for the defense of the termite colony against predators (Prestwich and Collins, 1981). The biosynthesis of such chemicals provides the termites with a vast range of chemicals against hostile organisms. Conversely, the distribution of chemicals provides an understanding of the genetic variations among termite species. The chemical variations in the *Bulbitermes* species show differences in the distribution of monoterpenes (Table 1) and diterpenes (Table 2) except for *B. germanus* and *B. sarawakensis*. The species can be differentiated by such variations, which also encompass the presence or absence of certain diterpenes that generally have complex structural types and compositional patterns. Intraspecifically, there are only small, but detectable, quantitative variations in monoterpene and diterpene composition.

The genetic distances between *B. germanus* of Peninsular Malaysia and *B. sarawakensis* of East Malaysia indicate that the two taxa are closely related. In a previous study, Tho (1992) described the separation only in terms of color differences of the head and femur; the shade is somewhat darker for *B. germanus*. In the present study, the genetic distance between *B. germanus* and *Bulbitermes* sp. B is a marginally larger value (0.10), which indicates genetic proximity, as *B. germanus* differentiates morphologically, having a larger and thicker nasus. The dendrogram (Figure 3) provides evidence that *B. germanus* and *B. singaporensis* are distinct (genetic distance = 0.71). Morphologically, the soldier of *B. singaporensis* is generally smaller than that of *B. germanus*. The nasus of *B. germanus* is relatively less broad-based in profile, whereas *B. singaporensis* has a broad-based profile.

Previous studies on the terpenoid distribution among termite species in different parts of the world (Prestwich et al., 1979b, 1981a,b; Braekman et al., 1980, 1983, 1984; Dupont et al., 1981; Baker and Walmsley, 1982; Gush et al., 1985; Valterova et al., 1986) have documented considerable variation; the variation can be used as a chemotaxonomic characteristic (Prestwich, 1983) in establishing interrelationships within the genus. The present investigation of the chemical defense secretions from *Bulbitermes* will eventually contribute toward a better understanding of the taxonomic position of the genus in relation to the other genera in the subfamily Nasutitermitinae. The generic distinction between *Bulbitermes* and *Nasutitermes* is based largely on the constricted head capsule in *Bulbitermes* soldiers. As demonstrated in this study, the major chemical components of the defense secretion of *B. singaporensis* (Prestwich et al., 1981a) is close to that of *Nasutitermes havilandi* (Chuah et al., 1989). On the other hand, *Nasutitermes matangensis* (Prestwich et al., 1981b; Chuah et al., 1989) appears to be distinct from these two species.

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DIET-RELATED MODIFICATION OF CUTICULAR HYDROCARBON PROFILES OF THE ARGENTINE ANT, *Linepithema humile*, DIMINISHES INTERCOLONY AGGRESSION

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Abstract—Territorial boundaries between conspecific social insect colonies are maintained through a highly developed nestmate recognition system modulated by heritable and, in some instances, nonheritable cues. Argentine ants, *Linepithema humile*, use both genetic and environmentally derived cues to discriminate nestmates from nonnestmates. We explored the possibility that intraspecific aggression in the Argentine ant might diminish when colonies shared a common diet. After segregating recently field-collected colony pairs into high or moderate aggression categories, we examined the effect of one of three diets: two hydrocarbon-rich insect prey, *Blattella germanica* and *Supella longipalpa*, and an artificial (insect-free) diet, on the magnitude of aggression loss. Aggression diminished between colony pairs that were initially moderately aggressive. However, initially highly aggressive colony pairs maintained high levels of injurious aggression throughout the study, independent of diet type. Each diet altered the cuticular hydrocarbon profile by contributing unique, diet-specific cues. We suggest that acquisition of common exogenous nestmate recognition cues from shared food sources may diminish aggression and promote fusion in neighboring colonies of the Argentine ant.

Key Words—Argentine ant, cuticular hydrocarbons, diet, invasive ants, nestmate recognition, unicoloniality.

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INTRODUCTION

Social insects have evolved a highly developed recognition system that forms the basis of social structure and communication. The signals used in nestmate recognition are primarily under genetic control; however, exogenous cues derived from nest materials (Gamboa et al., 1986; Stuart, 1987) or diet (Jutsum et al., 1979; Obin and Vander Meer, 1988; Le Moli et al., 1992; Liang and Silverman, 2000) may also play a role. Cuticular hydrocarbons have long been considered important mediators of nestmate recognition in ants (Vander Meer and Morel, 1998), with recent evidence supporting a direct role (Lahav et al., 1999; Liang and Silverman, 2000). However, the relative contribution of heritable and environmentally derived cues, including hydrocarbons, to the recognition profile is not known. Irrespective of the source, workers must learn colony-specific cues and must be able to properly evaluate cues present on newly encountered workers. Recognition cues are generally dynamic and may change throughout the life of the colony (Vander Meer et al., 1989) and exhibit seasonal variation (Ichinose, 1991). Therefore a worker must continually update its perception of colony odor in response to endogenous and external changes.

The Argentine ant, *Linepithema humile*, is one of several invasive ants in which the relative loss of territorial behavior is thought to contribute to invasion success. Introduced *L. humile* populations are unicolonial and frequently very large, and they often dominate native ant species (Suarez et al., 1999). Nestmate recognition in the Argentine ant is influenced by genetic (Tsutsui et al., 2000, 2003; Suarez et al., 2002) and environmental (Chen and Nonacs, 2000; Liang and Silverman, 2000) inputs. Holway et al. (1998) and Suarez et al. (2002) reported that aggression persisted between *L. humile* colonies despite maintenance under uniform rearing conditions, whereas Chen and Nonacs (2000) observed a decrease in *L. humile* intercolony aggression following 2 mo of laboratory rearing. Whereas Tsutsui et al. (2000) demonstrated a significant inverse relationship between *L. humile* genetic similarity and intercolony aggression, the colony pairs used by Holway et al. (1998), Chen and Nonacs (2000), and Suarez et al. (2002) were not subjected to genetic analysis. Therefore the observed changes (or lack thereof) in aggression may have resulted from different degrees of genetic similarity, with aggression between the most dissimilar pairs unlikely to change despite similar rearing conditions.

Whereas it has been suggested that loss of genetic diversity is primarily responsible for the unicolonial population structure observed in introduced Argentine ant populations (Tsutsui et al., 2000), the role of shared environmental cues such as diet in promoting unicoloniality is unknown. Shared dietary components, specifically hydrocarbons, for colonies displaying low intercolony genetic differentiation, may mask subtle inherent between-colony distinctions, thereby promoting fusion of adjacent colonies. Liang and Silverman (2000) and

Silverman and Liang (2001) demonstrated the potential of prey hydrocarbons to alter nestmate recognition in the Argentine ant, with worker exposure to specific prey eliciting aggression from colony mates. Of the many different prey exposed to *L. humile* workers, contact with the brown-banded cockroach, *Supella longipalpa*, induced the highest level of intracolony aggression (Liang et al., 2001). *S. longipalpa* has several hydrocarbons that are identical or similar to those of *L. humile*, which may be important in *L. humile* nestmate recognition (Liang et al., 2001). We propose that the opposite process can occur, whereby key *S. longipalpa*-acquired recognition chemicals attenuate differences between *L. humile* colonies, thus diminishing intercolony aggression. We compare intercolony aggression levels before and after continuous exposure to diets including *S. longipalpa*, *Blattella germanica*, or artificial diet and also measure changes in key prey-specific hydrocarbons on *L. humile* cuticle. We hypothesize that diets with *S. longipalpa* will diminish aggression the most. By documenting changes in intercolony aggression following exposure to sources of exogenous recognition cues, we hope to develop a deeper understanding of the dynamic nature of Argentine ant nestmate discrimination and its potential role in structuring populations in this invasive insect.

METHODS AND MATERIALS

Collection and Rearing of Laboratory Colonies. We used 11 colonies of Argentine ants (*L. humile*) from 11 sites in the southeastern USA—North Carolina (six): Chapel Hill (chh), Emerald Isle (emi), Greenville (gnc), Jacksonville (jac), Shallotte (sch), and Winston-Salem (for); South Carolina (two): Greenville (hto) and Greer (gwm); and Georgia (three): Barnesville (bch), Fayetteville (fay), and Griffin (grf). Ants were collected from a variety of habitats, including landscaped residential lots, natural wooded areas, or sand dunes. For each location, we established three large colonies consisting of 5000–10,000 workers, a few hundred queens, and numerous brood. Colonies were maintained in soil-free, Fluon-coated trays. Nests were plastic dishes filled with moist grooved plaster. Colonies were reared on one of three diets, each of which included a 25% sucrose solution *ad libitum* and hard-boiled eggs once a week: artificial noninsect diet (Bhatkar and Whitcomb, 1970), *S. longipalpa* male and female adults, or *B. germanica* male and female adults. All colonies were maintained at 24 ± 1°C, 50 ± 10% RH, and a 12:12 hr light/dark cycle.

Aggression Tests (Nestmate Recognition Bioassay). We assessed the initial level of aggression between 18 colony pairs (listed below) with an assay that measured the level of aggression in single worker introductions into a foreign colony. This behavioral assay has low variance among replicates within the same colony pairing (Roulston et al., 2003). Individual intruder workers were

collected on a toothpick and introduced into rearing trays (52 × 38 cm) containing a resident colony (~10,000 workers). The responses of resident workers toward the intruder were recorded, and aggression was scored using the 0–4 scale of Suarez et al. (1999). The intruder was discarded after each trial, and subsequent trials were conducted when the residents were no longer visibly agitated (5–10 min). Ten replicates per colony pair were performed: five replicates with colony 1 as the resident and five replicates with colony 1 as the intruder. The observer who recorded the aggression level did not know the identity of the interacting colonies and was unfamiliar with the hypothesis being tested. All assays to estimate the initial aggression levels were performed within a week of collection and extraction of ants from the original nesting substrate. Data were analyzed as the maximum score per trial (Roulston et al., 2003).

Our preliminary observations indicated a possible relationship between the initial level of aggression displayed by a colony pair and that colony pair losing aggression over time, with pairs having high initial aggression maintaining it over time and colonies with moderate levels of initial aggression becoming nonaggressive. We define moderate aggression as an average score of 3.0 or lower and high aggression as a score of 3.0 or higher on a 0–4 scoring scale (Suarez et al., 1999). This assignment is based on aggression above level 3 being injurious (biting, stinging), whereas aggression below level 3 is noninjurious (mutual antennation, avoidance). Eight colony pairs were moderately aggressive: gnc–fay, for–emi, chh–bch, for–gnc, chh–grf, chh–hto, gwm–sch, and gwm–fay, and 10 colony pairs were highly aggressive: jac–fay, jac–sch, jac–chh, emi–bch, jac–hto, emi–grf, emi–chh, emi–hto, emi–sch, and jac–bch. Aggression assays and hydrocarbon analyses were repeated 140 d later for all three dietary regimes to assess changes in nestmate recognition patterns and to determine whether behavioral changes were consistent with hydrocarbon patterns. Aggression assays were performed again at day 224 to determine whether aggression had further declined with prolonged laboratory rearing.

Extraction, Isolation, and Chemical Analysis of Cuticular Hydrocarbons. Ants were killed by freezing (−20°C) prior to hydrocarbon extraction. External lipids were extracted from the cuticle by immersing 10 whole thawed ants in 1-ml hexane for 10 min, followed by a brief second rinse. The samples were gently shaken for the first and last 20 sec of the soak period. Hexane extracts were concentrated under nitrogen to ~100 μl and applied to prewetted (hexane) Pasteur pipette minicolumns filled with 500 mg of silica gel (63–200 mesh size, Selecto Scientific, GA, USA). The hydrocarbon fraction was eluted with 6-ml hexane and blown to dryness under nitrogen. The extract was redissolved in 5-μl hexane, and 1 μl was analyzed (two ant equivalents). Gas chromatography (GC) was carried out using an HP 5890 gas chromatograph equipped with a DB-1 column (30 m × 0.25 mm × 0.25 μm film thickness) and interfaced with a G1045A Chemstation (version A05.01). Oven temperature was held at 40°C

for 2 min, then increased to 200°C at 20°C/min, and then to 310°C at 40°C/min. The injector and flame-ionization detector were at 270 and 320°C, respectively. Helium was the carrier gas, and the make-up gas was nitrogen. Quantitative data were obtained by integrating the peaks and calculating the percent area under each peak. Specific peak identity was determined with hydrocarbon standards and by matching diagnostic peaks with those from prior studies (Jurenka et al., 1989; Liang et al., 2001).

Statistical Analyses. The significance of main effects (diet and initial aggression category) and their interaction was tested by using a mixed model ANOVA (PROC MIXED) in SAS 8.1 (SAS Institute, 2002). Upon finding that the effect of diet was not the same in the two aggression categories, we tested for the effect of diet on aggression loss within each of the two aggression categories with colony pairing and diet treated as random and fixed variables, respectively (ANOVA, PROC MIXED). Differences between the three dietary treatments within and across aggression categories were determined with least-squares means. To analyze the magnitude of aggression loss, we used absolute, rather than relative, aggression loss values.

We used linear discriminant analysis (LDA) (Statgraphics Plus, v. 5.1) to examine hydrocarbon divergence patterns between field-collected colonies (Initial) and the same colonies raised on each of the three diets (*Blattella*, *Supella*, and Artificial). The analysis was performed using standardized variables, and an LDA matrix was constructed with 11 colonies, belonging to each of four treatments (Initial, *Blattella*, *Supella*, and Artificial), using 27 peak percentages of the most abundant cuticular hydrocarbons. Significance tests comparing diets used the MANOVA procedure (PROC GLM). The degree of dispersion around the centroids (i.e., the degree of differentiation between colonies within a treatment) was calculated by averaging standard deviations for each of the 11 colonies across all 27 hydrocarbons within each treatment. To test whether Argentine ants acquired key prey-specific hydrocarbons, we first identified key diagnostic hydrocarbons provided by each prey. For *B. germanica*, we selected peaks corresponding to 11-, 13-, and 15-methylnonacosane and 3-methylnonacosane. Both hydrocarbons are relatively abundant in adult *B. germanica*, comprising approximately 14.5 and 10.3% of the total hydrocarbons, respectively (Jurenka et al., 1989). Furthermore, our preliminary analysis indicated that both hydrocarbons were readily acquired by Argentine ants. For *S. longipalpa*, we selected 15,19-dimethylheptatriacontane present in *S. longipalpa* at 19.0% and acquired by Argentine ants from *S. longipalpa* prey (Liang and Silverman, 2000; Liang et al., 2001). To compare changes in individual hydrocarbon levels (average level on day 140 vs. average level on day 0), we used one of two types of *t*-tests, depending on the equality of variances. A parametric *t*-test was used when the variances were homogenous. In cases where the variances were unequal, we used the Welch *t*-test with a Satterthwaite correction (Zar, 1999).

RESULTS

Analysis of the behavioral data revealed that the interaction between diet and aggression was significant (ANOVA, $F_{2,15.1} = 13.38$, $P < 0.001$). Because the diet effects were not the same in the two aggression categories, a separate analysis of diet effects for each aggression category was performed. Colony pairs experienced a significant reduction in initial aggression, irrespective of the diet (Table 1 and Figure 1). The aggression scores in colonies that were initially moderately aggressive and reared on either of the two cockroach diets decreased by ~40% ($P = 0.91$, Table 2). Ants raised on the artificial diet, however, experienced an ~70% loss in initial aggression scores, which was significantly higher than that experienced by ants raised on either *B. germanica* ($P < 0.001$) or *S. longipalpa* ($P < 0.001$). Argentine ants displaying high initial aggression experienced relatively little change in aggression, approximately 8% loss for each of the three dietary regimes. This decrease, although relatively low, was statistically significant for each of the three diets (Table 1), and the magnitude of aggression loss did not differ between dietary categories (Table 2; ANOVA, $F_{2,18} = 0.72$, $P = 0.50$). A comparison of the magnitude of aggression loss between the aggression categories revealed that moderately aggressive colony pairs lost a significantly higher proportion of their initial aggression across all dietary treatments, relative to colony pairs showing high initial aggression (Table 2). Results of aggression tests performed 84 d after the first testing revealed no further aggression loss in any of the aggression/diet categories ($P > 0.05$).

To provide another measure of the magnitude of aggression loss in both aggression categories, we recorded changes in the proportion of injurious/

TABLE 1. INITIAL AGGRESSION LEVELS AND AGGRESSION LOSS IN MODERATELY AND HIGHLY AGGRESSIVE COLONY PAIRINGS REARED UNDER THREE DIETARY REGIMES

Aggression category	Initial aggression level[a]	Aggression loss				
			Change			
		Diet	End	Absolute	Relative	P^b
Moderate	2.79 ± 0.08 ($n = 8$)	*Supella*	1.7 ± 0.2	1.1 ± 0.2	39.5 ± 7.1%	<0.001
		Blattella	1.8 ± 0.2	1.0 ± 0.2	36.7 ± 5.7%	<0.001
		Artificial	0.8 ± 0.2	2.0 ± 0.2	73.0 ± 7.0%	<0.001
High	4.00 ± 0.00 ($n = 10$)	*Supella*	3.7 ± 0.1	0.3 ± 0.1	8.3 ± 2.2%	0.002
		Blattella	3.8 ± 0.1	0.2 ± 0.1	5.5 ± 2.1%	0.009
		Artificial	3.8 ± 0.1	0.2 ± 0.1	5.8 ± 2.4%	0.018

[a] Initial aggression levels are between pairs of field-collected colonies. Values reported are mean ± SE.
[b] ANOVA on absolute change (SAS Institute, 2002).

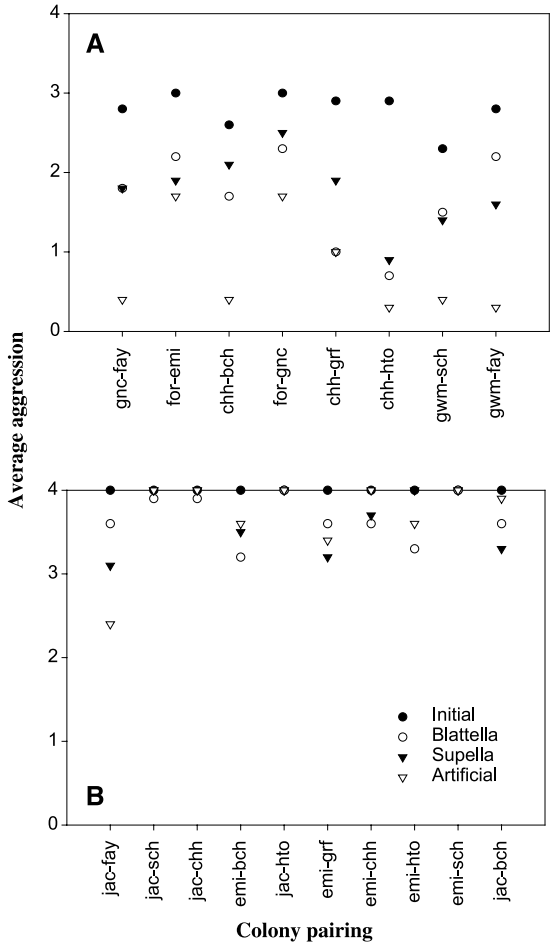

FIG. 1. Changes in intraspecific aggression in moderately (A) and highly (B) aggressive colony pairings. Field-collected colonies (Initial, closed circles) raised on one of three diets: *Blattella* (open circles), *Supella* (closed triangles), and artificial (open triangles). Mean values are reported ($n = 10$) with error bars omitted for clarity. See Methods and Materials for explanation of colony codes.

noninjurious encounters between colony pairs. Our initial aggression classification (moderate vs. high) was based on a distinction between injurious and noninjurious aggression. We classified aggression above level 3 as injurious (biting, stinging) and aggression below level 3 as noninjurious (mutual antennation, avoidance). Although the magnitude of aggression loss was statistically

TABLE 2. COMPARISON OF AGGRESSION LOSS WITHIN AND AMONG AGGRESSION CATEGORIES

Diet	Aggression	Aggression loss	Diet	Aggression	Aggression loss	P^a
Supella	Moderate	1.1 ± 0.2	*Blattella*	Moderate	1.0 ± 0.2	0.91
Supella	Moderate	1.1 ± 0.2	Artificial	Moderate	2.0 ± 0.2	<0.001
Blattella	Moderate	1.0 ± 0.2	Artificial	Moderate	2.0 ± 0.2	<0.001
Supella	High	0.3 ± 0.1	*Blattella*	High	0.2 ± 0.1	0.42
Supella	High	0.3 ± 0.1	Artificial	High	0.2 ± 0.1	0.25
Blattella	High	0.2 ± 0.1	Artificial	High	0.2 ± 0.1	0.73
Supella	High	0.3 ± 0.1	*Supella*	Moderate	1.1 ± 0.2	0.001
Blattella	High	0.2 ± 0.1	*Blattella*	Moderate	1.0 ± 0.2	<0.001
Artificial	High	0.2 ± 0.1	Artificial	Moderate	2.0 ± 0.2	<0.001

[a]Least squares analysis (SAS Institute, 2002).

significant in each of the two aggression categories, our results indicated that in contrast to pairs displaying moderate aggression, the incidence of injurious fights between highly aggressive pairs remained high. Therefore we question the biological importance of the statistically significant aggression loss between highly aggressive colony pairs. Among moderately aggressive pairs, initially 69 of 80 (86%) encounters resulted in an aggression score of 3 or 4 (three out of 80 had an aggression score of 4). At the end of the study, only 36% of *Supella*-fed ants (corresponding to a 58% reduction in the number of injurious encounters), 36% of *Blattella*-fed ants (58% reduction), and 19% of ants on the artificial diet (78% reduction) displayed level 3 aggression. In contrast, among highly aggressive colonies, initially all encounters (100/100) scored level 4. At the end of the study, 96% of *Supella*-fed ants (4% reduction), 97% of *Blattella*-fed ants (3% reduction), and 98% of ants on the artificial diet (2% reduction) still engaged in injurious aggression. Level 4 aggression remained in 71% of *Supella*-fed ants, 78% of *Blattella*-fed ants, and 79% of ants fed with artificial diet.

Argentine ants acquired significant levels of prey hydrocarbons (Figure 2)—*Blattella*: 11-, 13-, and 15-methylnonacosane ($P < 0.001$), 3-methylnonacosane ($P < 0.001$) and *Supella*: 15,19-dimethylheptatriacontane ($P < 0.001$). Because *S. longipalpa* hydrocarbons overlap the long-chain hydrocarbon profile of *L. humile* (Liang et al., 2001), field-collected colonies of Argentine ants possess relatively high initial levels of hydrocarbons specific to *S. longipalpa* (Figure 2C). However, there are certain qualitative and quantitative differences between *L. humile* hydrocarbons and those provided by *S. longipalpa*. Consequently, providing Argentine ants with *S. longipalpa* prey would likely alter the relative ratio of hydrocarbons already present as well as introduce new hydrocarbons. Furthermore, the results of discriminant analysis revealed marked

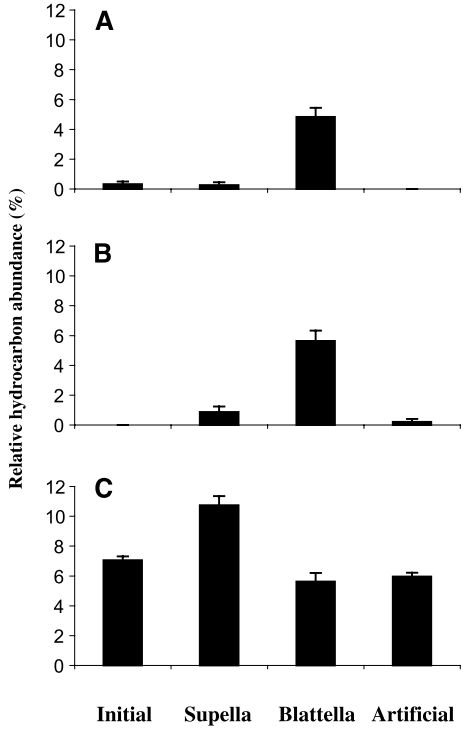

FIG. 2. Relative abundance (% total area) of key prey-derived hydrocarbons in field-collected colonies of *L. humile* (Initial) and colonies provisioned with one of prey diets: *S. longipalpa* (*Supella*) and *B. germanica* (*Blattella*). Hydrocarbons (A) and (B) are *Blattella*-derived and (C) is *Supella*-derived. (A) 11-, 13-, and 15-methylnonacosane; (B) 3-methylnonacosane; (C) 15,19-dimethylheptatriacontane. Means ± SE for 11 colonies are presented.

divergence in cuticular hydrocarbon composition between field-collected colonies (Initial) and colonies raised on either *B. germanica*, *S. longipalpa*, or the artificial diet (Figure 3): Initial vs. *Blattella* (MANOVA, Wilk's lambda = 0.0028, $F_{26,5} = 67.04$, $P < 0.001$), Initial vs. *Supella* (Wilk's lambda = 0.010, $F_{26,5} = 18.16$, $P = 0.002$), and Initial vs. Artificial (Wilk's lambda = 0.010, $F_{26,5} = 18.94$, $P = 0.002$). The divergence between the *Blattella* and *Supella* diets was also significant (Wilk's lambda = 0.0066, $F_{26,5} = 28.88$, $P < 0.001$). Estimates of intracolony variability within treatments revealed that field-collected colonies (Initial) had the lowest variance (1.029), followed by colonies on the artificial diet (1.035), *Supella* (1.147), and *Blattella* (1.209). Changes in the proportions of the 27 most abundant cuticular hydrocarbons are summarized

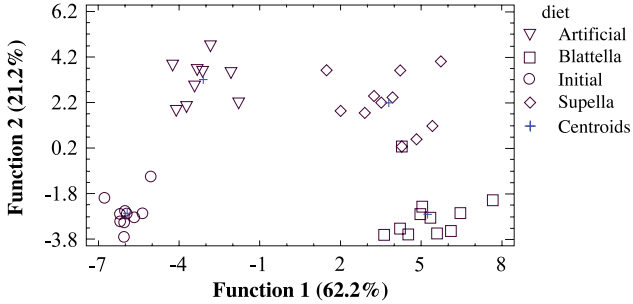

FIG. 3. Linear discriminant analysis of the 27 predictor variables (relative proportions of hydrocarbons) for 11 colonies of *L. humile* each provided a unique diet (*B. germanica*, *S. longipalpa*, and artificial).

in Table 3. Overall, colonies raised on all three diets experienced changes in the proportions of all hydrocarbons. In colonies raised on the artificial diet, six hydrocarbons significantly increased and eight hydrocarbons significantly decreased (52% of hydrocarbons changed significantly). In colonies raised on *Blattella*, five hydrocarbons significantly increased and 12 hydrocarbons significantly decreased (63% change). Colonies raised on *Supella* experienced the greatest change (67%), with seven hydrocarbons increasing and 11 hydrocarbons decreasing over the course of the study.

DISCUSSION

Our results provide further support for Argentine ant nestmate recognition being modulated, in part, by environmental factors (Chen and Nonacs, 2000; Liang and Silverman, 2000), in that rearing under controlled conditions reduces aggression between colonies. Prior studies (Liang and Silverman, 2000; Liang et al., 2001) demonstrated that exogenous hydrocarbons disrupted *L. humile* colony integrity by inducing aggression between fragments of the same colony. Herein, we determined that shared diet reduced intercolony aggression to an extent that might promote fusion of dissimilar colonies.

Changes in aggression were minimal between colony pairs that were initially highly aggressive, despite worker ants acquiring prey hydrocarbons. Therefore limited change in the pattern of intraspecific aggression within the highly aggressive pairings suggests that exogenous recognition cues did not override intrinsic cues, which may help to explain the results of other ant studies in which aggression persisted over time (Le Moli et al., 1992; Heinze et al., 1996; Holway et al., 1998; Stuart and Herbers, 2000; Suarez et al., 2002). In

TABLE 3. TWENTY-SEVEN MOST ABUNDANT CUTICULAR HYDROCARBONS AND THEIR CHANGES FOLLOWING REARING ON LABORATORY DIETS

Hydrocarbon	Diet		
	Artificial	*Blattella germanica*	*Supella longipalpa*
n-Heptadecane	0	0	+
Nonadecene	0	+	+*
n-Tricosane	−	−	−
n-Pentacosane	−	−	−
n-Heptacosane	+*	+*	+*
n-Octacosane	+*	+	+*
n-Nonacosane	+*	+*	+*
11-, 13-, and 15-Methylnonacosane	−	+*	−
3-Methylnonacosane	+	+*	+*
n-Triacontane	+	+*	+
n-Hentriacontane	+*	−	+
11-, 13-, and 15-Methylhentriacontane	−	−	−*
Dimethylhentriacontane	+*	−	+
Trimethylhentriacontane	−*	−	+
n-Tritriacontane	−	−*	−*
13- and 15-Methyltritriacontane	−*	−*	−*
5,15- and 5,17-Dimethyltritriacontane	−	−*	−*
5,15,19-Trimethyltritriacontane	−	−*	+*
13-, 15-, and 17-Methylpentatriacontane	−*	−*	−*
15,19-Dimethylpentatriacontane	+*	−*	−*
5,15- and 5,17-Dimethylpentatriacontane	−*	−*	−*
5,13,17- and 5,15,19-Trimethylpentatriacontane	−	−*	−*
3,13,17- and 3,15,17-Trimethylpentatriacontane	−*	−*	−
13-, 15-, 17-, and 19-Methylheptatriacontane	−*	−*	−*
15,19-Dimethylheptatriacontane	−*	−	+*
5,15- and 5,17-Dimethylheptatriacontane	−*	−*	−*
5,15,19- and 5,13,17-Trimethylheptatriacontane	−	−*	−*
Significant changes	+(6), −(8)	+(5), −(12)	+(7), −(11)

*Significant change in hydrocarbon proportion ($P < 0.05$; Student's *t*-test).

contrast, colonies in pairings that were initially moderately aggressive were strongly affected by the imposition of diet-based cues. We predicted that exposure to cockroach prey, especially *S. longipalpa*, would provide abundant hydrocarbons that could minimize between-colony recognition disparities, thereby producing the greatest decline in aggression. However, intraspecific aggression in ants fed with artificial diet diminished further than aggression in ants fed with cockroaches, indicating that the greatest change in behavior may be a consequence of the field-to-lab transition. Hydrocarbon profiles of *L. humile* raised on the artificial diet changed significantly over time; however, the hydrocarbons

(and/or chemical cues) affecting this behavioral change are unclear. Moreover, the difference in the degree of aggression loss between the two prey diets was not significant, an unexpected result given that long-chain hydrocarbons from *S. longipalpa* (33 carbons or more) added to the native *L. humile* cuticular profile induced intracolony aggression, whereas other prey items from diverse insect taxa (including *B. germanica*), having fewer or none of these long-chain hydrocarbons, induced little or no within-colony aggression (Liang et al., 2001). The action of prey hydrocarbons in promoting intercolony unity appears to differ from that inducing intracolony aggression. Perhaps the difference in the way prey hydrocarbons alter nestmate recognition in *L. humile* is related to quantitative differences in hydrocarbon levels needed to affect a behavioral change. Thus, where two antagonistic fragments were created from a single colony (Silverman and Liang, 2001), the acquisition of one or a few key hydrocarbons produced a recognizable shift in an otherwise uniform profile. In the current study, the acquisition of foreign prey hydrocarbons appeared insufficient to completely override intrinsic distinctions between colonies.

Our findings may provide insights into the evolution of unicoloniality in introduced populations of the Argentine ant. Proposed mechanisms for the transition from multi- to unicoloniality include reduced heterozygosity due to genetic drift (Tsutsui et al., 2000) and introduction to new habitats with relaxed ecological constraints (Giraud et al., 2002), with unicoloniality maintained via selection against rare recognition alleles (Tsutsui et al., 2003). Whereas Tsutsui et al. (2000, 2003) and Giraud et al. (2002) demonstrated the importance of genetic, ecological, and behavioral factors in the transition from multicoloniality to unicoloniality, we suggest that shared environmentally derived recognition cues may promote unicoloniality by masking inherent between-colony distinctions, thereby allowing adjacent colonies to fuse. Competing Argentine ant colonies will most likely interact with each other at advancing invasion fronts where they are likely to compete for nesting sites and/or food resources that provide similar nestmate recognition cues. In areas where mutually aggressive colonies exploit a common locally abundant food source, two mechanisms may work independently or together to promote fusion of neighboring colonies: diet sharing and intraspecific "dear enemy" phenomenon, whereby competing animals respond less aggressively to threats by neighbors than strangers (Temeles, 1994; Heinze et al., 1996; Langen et al., 2000). Diet sharing through cooperative use of locally abundant food sources may provide sufficient levels of critical hydrocarbons to alter nestmate recognition and promote fusion of colonies. Argentine ants, like other invasive ants, are generalist feeders (Newell and Barber, 1913; Markin, 1970), tending homoptera and scavenging living and dead arthropods. In addition, shared cues originating from nesting material may diminish intercolony aggression. The "dear enemy" phenomenon may also play a role in the Argentine ant's transition from multicoloniality to

unicoloniality. An increase in the frequency of encounters between aggressive colonies may decrease the frequency of aggression between them, especially in areas where food is abundant and intraspecific competition is limited (Foitzik and Heinze, 1998). Furthermore, repeated interactions in the field were essential for maintaining recognition among spatially isolated nests in the polydomous *Leptothorax longispinosus* with nonaggressive field colonies becoming hostile after 3 mo of laboratory rearing, thereby indicating a possible role for common environment-derived cues and/or contact between colonies in reducing aggression (Stuart and Herbers, 2000). Where Argentine ants interact and compete for resources that provide common recognition cues, cooperation among nests through a reduction in intercolony aggression may be promoted. However, an exception may occur within the native range, where *L. humile* is multicolonial despite neighboring colonies presumably having access to shared environment-derived cues. Native *L. humile* exhibit relatively high genetic diversity and high intercolony aggression (Tsutsui et al., 2000); consequently, environmentally derived recognition cues may be limited in diminishing intercolony aggression. However, colonies from invasive populations having weaker genetic structure (Tsutsui and Case, 2001; Buczkowski et al., 2004) may be more likely to fuse when exogenous cues mask colony distinctions, thereby fostering the development of unicoloniality.

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EVIDENCE OF NATURAL HYBRIDIZATION BETWEEN TWO SYMPATRIC SIBLING SPECIES OF *Bactrocera dorsalis* COMPLEX BASED ON PHEROMONE ANALYSIS

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Abstract—*Bactrocera carambolae* and *B. papayae* are major fruit fly pests and sympatric sibling species of the *B. dorsalis* complex. They possess distinct differences in male pheromonal components. In the 1990's, wild *Bactrocera* fruit flies with morphological traits intermediate between those of *B. carambolae* and *B. papayae* were often captured in traps baited with methyl eugenol (ME). Chemical analyses of rectal glands of ME-fed males revealed that the laboratory F_1, F_2, and backcross hybrids possessed ME-derived sex pheromonal components ranging from that typical of *B. papayae* to that of *B. carambolae* without any specific trend, which included a combination of pheromonal components from both parental species within an individual hybrid. ME-fed hybrids without any ME-derived pheromonal components were also detected. Further chemical analysis of rectal glands from wild *Bactrocera* males, after ME feeding in the laboratory, showed a combination of pheromonal components similar to that found in the ME-fed, laboratory-bred hybrids. These findings present circumstantial evidence for the occurrence of a natural hybrid of the two *Bactrocera* species.

Key Words—*Bactrocera carambolae*, *B. papayae* (=*B. dorsalis*), hybrid, methyl eugenol, rectal gland, male pheromone, 2-allyl-4,5-dimethoxyphenol, (*E*)-coniferyl alcohol, 6-oxo-1-nonanol.

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INTRODUCTION

Two sibling species of the *Bactrocera dorsalis* complex (Diptera: Tephritidae), *B. carambolae* Drew and Hancock and *B. papayae* Drew and Hancock, are major fruit pests in Southeast Asia, particularly Malaysia, Thailand, and Indonesia. These sympatric species are found readily throughout the peninsular Malaysia, with *B. carambolae* and *B. papayae* being more abundant in the southern and central regions, respectively (Tan and Nishida, 1996).

Following a taxonomic revision by Drew and Hancock (1994), *B. carambolae* can be differentiated morphologically from *B. papayae* by three key traits: (1) wing costal band slightly enlarged at apex of R_{4+5}, (2) the presence of a dark spot on foreleg femora, and (3) the appearance of a bar-shaped pattern of the lateral abdominal band on terga III–V. Subsequently, Iwaizumi et al. (1997) proposed the use of genital characters to differentiate sibling species within the *B. dorsalis* complex. However, identification of wild fruit flies based on these morphological and genitalia traits has been difficult and unreliable because they often show considerable variation (Iwaizumi et al., 1997; Iwahashi, 1999). Nevertheless, distinctive differences were found in volatile components of the male rectal gland, genetic polymorphism in intron sequences, and ribosomal DNA analyses (Perkins et al., 1990; He and Haymer, 1997; Armstrong and Cameron, 2000).

Both species are strongly attracted to and compulsively feed on methyl eugenol (ME), a potent male attractant frequently used in area-wide fruit fly control programs. Males of *B. papayae*, after consuming ME, produced 2-allyl-4,5-dimethoxyphenol (DMP) and (*E*)-coniferyl alcohol (CF) (Nishida et al., 1988; Tan and Nishida, 1996), whereas males of *B. carambolae* produced only CF (Tan and Nishida, 1996; Wee, 2000) in addition to their major endogenous rectal gland component, 6-oxo-1-nonanol (OXO), and other minor components (Perkins et al., 1990). These pheromonal compounds are sequestered and stored in the rectal gland prior to release during courtship at dusk.

Since the early 1990's, wild *Bactrocera* fruit flies with intermediate morphological traits between *B. carambolae* and *B. papayae* (hereafter referred to as intermediates) often have been recovered from traps baited with ME in Malaysian ecosystems where these species are known to coexist (Wee, 2000). This has raised speculation that natural hybridization might have occurred. Nevertheless, the intermediates have been regarded as morphological variants of their respective species (Iwahashi, 1999). Attempts to mate the two sibling species in the laboratory yielded viable hybrids with similar morphological traits to those of the field intermediates (Wee and Tan, 2000a; Wee, 2000). However, to date, there is no conclusive evidence as to whether natural hybridization between the sibling species occurs in the wild.

Here, we report evidence of natural hybrids between *B. carambolae* and *B. papayae* using gas chromatographic analyses of pheromonal components

accumulated in the male rectal glands after ME consumption, involving laboratory-bred hybrids and wild flies, respectively.

METHODS AND MATERIALS

Chemicals. 1,2-Dimethoxy-4-[2-propenyl]benzene (methyl eugenol; >99.8% purity) was obtained from Agrisense-BCS Ltd. (UK). Authentic samples (>96% purity) of CF (96% *trans*), DMP, and OXO were synthesized and supplied by R. Nishida (Kyoto University, Japan).

Insects. Laboratory strains of *B. carambolae* and *B. papayae* were pure-bred lines for more than 30 generations. These fruit fly species possessed the phenotypic traits typical of *B. carambolae* or *B. papayae*, respectively, according to Drew and Hancock (1994). Fruit fly colonies were cultured as described in Wee and Tan (2000b). Male and female flies were segregated by the fourth day after adult eclosion. Virgin males and females of each species were kept in separate cages with food and water in an insectary that had 12 D:12 L regimes with room temperature of 25–29°C and 83–90% RH.

Laboratory Crosses. All crosses were conducted in a screen cage ($40 \times 40 \times 40$ cm^3) placed indoors. Equal numbers (50–100) of sexually mature males of one species and females of the other species were transferred into the experimental cage in the morning and were acclimatized for at least 8 hr before courtship began. At 21:00 hr, under red light, each mating pair was carefully collected in a specimen vial, labeled, and allowed to continue mating until dawn.

The pure-bred lines of *B. carambolae* (BC) and *B. papayae* (BP) were crossed reciprocally to produce F_1 hybrids (BC ♀ × BP ♂ and BP ♀ × BC ♂). Six additional sources of flies, comprising a variety of the three key morphological traits in combination (that is, pattern of wing costal band at R_{4+5} and abdominal band at terga III–V, and absence/presence of a femoral spot on prothoracic legs) between BC and BP, were obtained from the following crosses of flies: (1) reciprocal crosses of F_1 hybrids were self-crossed to produce F_2 generations; (2) F_1 hybrids (from BP ♀ × BC ♂) were backcrossed with the parental species (BP ♀ × hybrid ♂ and hybrid ♀ × BC ♂); and (3) F_1 hybrids (from BC ♀ × BP ♂) were backcrossed with the parental species.

The emerged flies were maintained on artificial diet until sexual maturity (Wee and Tan, 2000b; Wee, 2000) and then allowed to feed on ME. This was performed on 30–74 male flies for each of the above crosses.

Wild Fruit Fly Collections. Wild fruit flies were obtained either by field trapping using ME-baited trap or collecting field-infested fruits from various locations in peninsular Malaysia (northern region: Penang; central region: Perak; and southern region: Johor), which were subsequently reared in the laboratory.

For field trapping, improved and modified clear traps from Tan (1985) (Figure 1) baited with ME were set up at sites located near hillside forests. The trap was designed in such a way as to prevent the trapped flies from feeding on the ME source, thus avoiding regurgitation and anal feeding behavior (Tan, 2000). These traps were used only once (trapping conducted between 08:00 and 14:00 hr) and discarded after use. Trapping was conducted on different dates in July 2000. Occasionally, flies were caught using specimen vials before they could enter the trap. In contrast, male flies raised from rotten fruits were maintained until sexual maturity (20–28 d), and allowed to feed on ME for

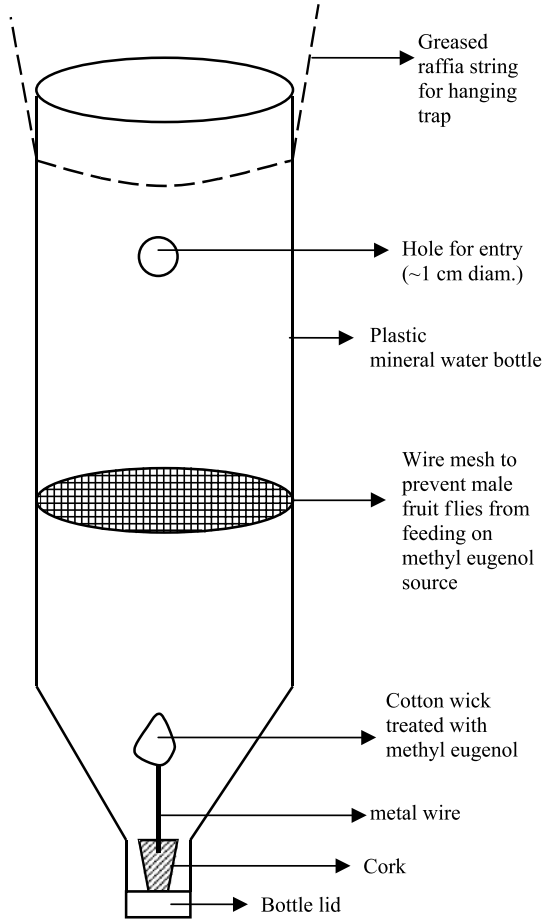

FIG. 1. Methyl eugenol-baited trap used to trap wild *Bactrocera* fruit fly males.

subsequent pheromonal analysis. A total of 167 wild males (Penang, 106; Perak, 11; and Johor, 50) were analyzed for pheromonal components.

Consumption of ME. In the laboratory, each male fruit fly was allowed to feed on 0.1 μl ME dispensed on a small piece of filter paper (1×1 cm^2; Whatman® No. 1) for 10 min during their peak response to ME (10:00–11:00 hr) (Tan, 1985). After 24 hr, male rectal glands were excised and soaked individually in 20–50 μl of redistilled ethanol spiked with an internal standard (1-dodecanol to monitor evaporation of sample solvent and not for quantification) placed in a screw-cap glass vial, and stored at −20°C for further analysis.

Rectal gland samples were obtained from (1) parental males of *B. carambolae* and *B. papayae* ($N = 30$ each), (2) males of laboratory hybrids from F_1 and F_2 generations plus backcrosses, and (3) wild males from both field trapping and male flies raised from field-infested fruits.

Pheromone Analyses. Rectal glands in ethanol (see above) were carefully homogenized with a fine glass rod followed by 5 min sonication. After centrifugation at ~1,000 *g* for 2–3 min, 1 μl aliquots of the supernatant were subjected to gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses, respectively.

Analysis of rectal gland volatiles was conducted using splitless injections on a Shimadzu GC-14A gas chromatograph, with an HP Ultra-1 column (Agilent, 25 m × 0.25 mm × 0.33 μm) programmed from 80°C/1 min to 240°C at 10°C/min. The peak areas were calibrated with those of the authentic standard samples using a C-R6A integrator (Shimadzu). Chemical identification was performed by comparison with the retention time, use of authentic internal standards, and mass spectral fragmentation patterns of authentic standards.

GC-MS analyses were performed with an HP 5989B mass spectrometer (electron impact at 70 eV) connected to an HP-5MS column (30 m × 0.25 mm, 0.33 μm film thickness) using the same running conditions as above.

RESULTS

The rectal gland of laboratory-reared *B. papayae* males following ME consumption contained CF and DMP, whereas CF was detected in the rectal gland of *B. carambolae* male along with its endogenously produced rectal gland component, OXO (Figure 2A and B). Identification of the rectal gland components was confirmed by comparison with the mass spectra of authentic standards (Figure 3A, B, and C).

Rectal gland analysis of ME-fed laboratory hybrids revealed the presence of various combinations of components between the two parental species (Table 1). A majority of the ME-fed F_1 hybrid males (68–84%) contained only CF in their

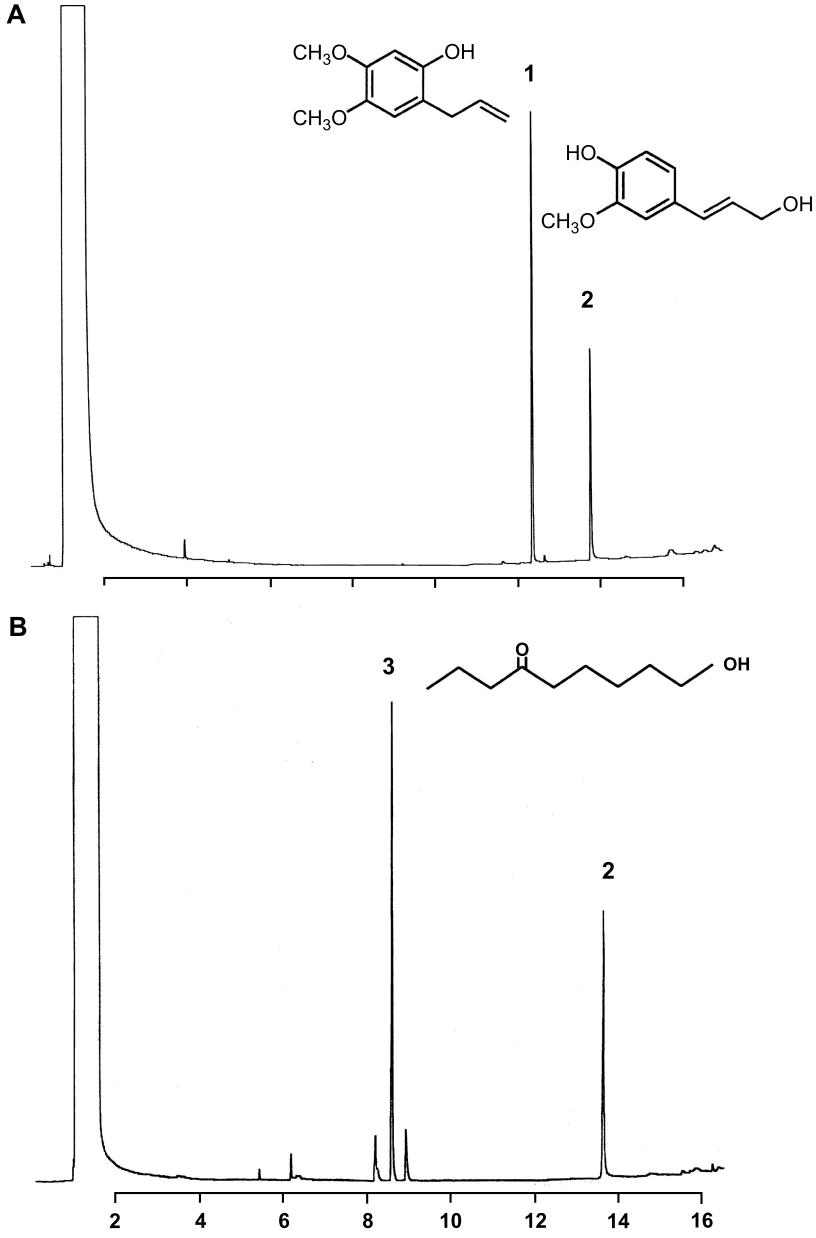

FIG. 2. Gas chromatograms of laboratory male rectal gland of (**A**) *Bactrocera papayae* and (**B**) *B. carambolae*, 24 hr after methyl eugenol consumption. (**1**) 2-Allyl-4,5-dimethoxyphenol, (**2**) (*E*)-coniferyl alcohol, and (**3**) 6-oxo-1-nonanol.

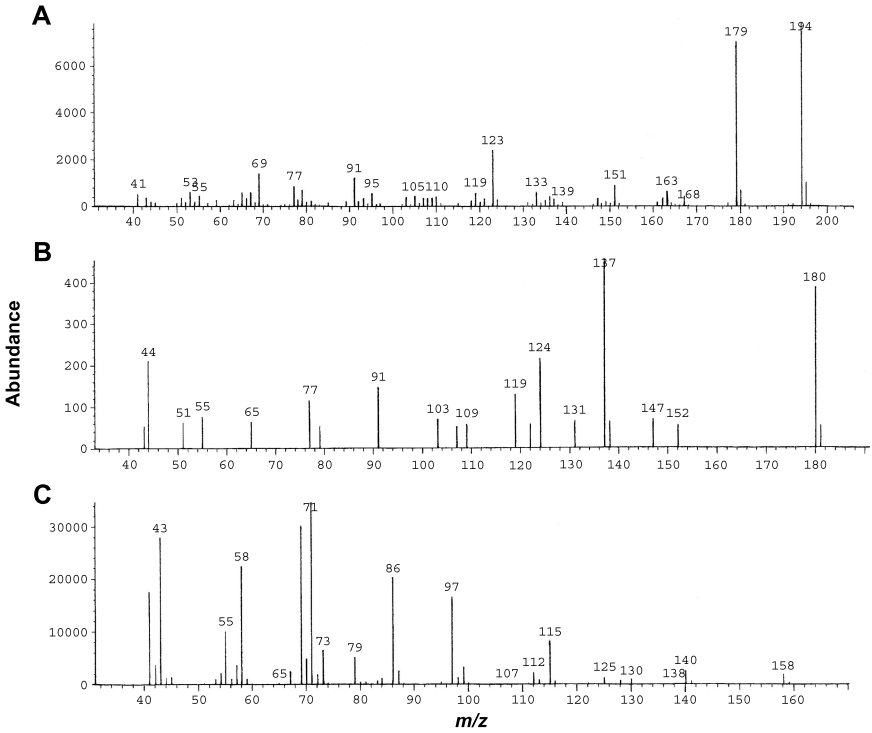

FIG. 3. Mass spectra of (**A**) 2-allyl-4,5-dimethoxyphenol and (**B**) (*E*)-coniferyl alcohol, the methyl eugenol-derived sex pheromonal components in male rectal gland of *B. papayae* and *B. carambolae* (with absence of **A**); and (**C**) 6-oxo-1-nonanol, the major endogenous component of sexually mature male *B. carambolae*.

rectal glands, whereas these percentages showed gradual decrease to below 30% when the respective F_1's were self-crossed (i.e., F_2) or backcrossed to the parental species (Table 1).

Between 14 and 18% of F_1 males possessed both CF and DMP in their rectal gland, which is typical for *B. papayae* males which have consumed ME. Percentages of hybrids that possessed this combination increased when they were subjected to self-crossing (Table 1). Backcrossing F_1 hybrids with parental *B. papayae* further increased the percentages of individuals bearing the *B. papayae* rectal gland blend (Table 1).

Similarly, the occurrence of *B. carambolae* blend (OXO + CF) was low among the F_1 hybrids but showed an increase in the F_2 generation. Backcrossing to the parental *B. carambolae* increased the percentage of hybrids having the *B. carambolae* rectal gland blend (Table 1).

TABLE 1. RECTAL GLAND CONTENTS PROFILE (%) OF HYBRIDS F_1, F_2 AND BACKCROSSED PROGENIES RESULTING FROM INTERBREEDING BETWEEN SIBLING SPECIES OF THE *dorsalis* COMPLEX, *B. carambolae* (BC) AND *B. papayae* (BP), AFTER METHYL EUGENOL CONSUMPTION

Progeny	F_1		F_2		Backcrosses			
Crosses: Female × Male (progeny designation)	BC × BP (R)	BP × BC (S)	R × R	S × S	BC × R	R × BP	BP × S	S × BC
Chemical combination	(N = 74)	(N = 50)	(N = 30)	(N = 50)	(N = 53)	(N = 50)	(N = 50)	(N = 49)
CF	68.9	84.0	33.3	36.9	17.0	28.0	26.0	10.2
DMP + CF[a]	17.6	14.0	43.4	43.5	5.7	66.0	64.0	–
OXO + CF[b]	8.0	2.0	23.3	15.2	75.4	–	–	73.4
OXO + DMP + CF	4.1	–	–	4.4	1.9	2.0	–	–
DMP	1.4	–	–	–	–	4.0	–	–
OXO	–	–	–	–	–	–	–	8.2
None	–	–	–	–	–	–	10.0	8.2

Notes: CF = (*E*)-coniferyl alcohol; DMP = 2-allyl-4,5-dimethoxyphenol; OXO = 6-oxo-1-nonanol; R = hybrid of cross between female BC and male BP; S = hybrid of cross between female BP and male BC.

[a]Combination of rectal gland components typical of a *B. papayae* male after methyl eugenol consumption.

[b]Combination of rectal gland components typical of a *B. carambolae* male after methyl eugenol consumption.

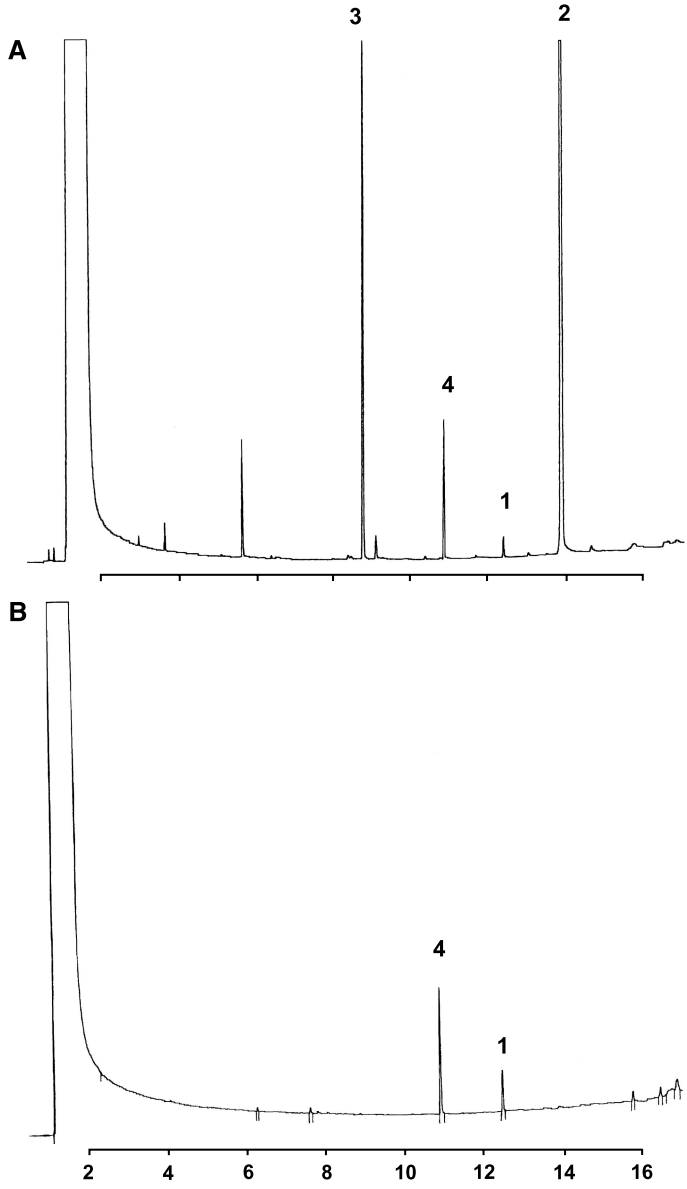

FIG. 4. Gas chromatograms of wild *Bactrocera* male rectal gland, 24 hr after methyl eugenol feeding in the laboratory: (**A**) pheromone profile featuring a combination of both *B. carambolae* and *B. papayae* pheromonal components and (**B**) pheromone profile containing DMP only. (**1**) 2-Allyl-4,5-dimethoxyphenol, (**2**) (*E*)-coniferyl alcohol, (**3**) 6-oxo-1-nonanol, and (**4**) 1-dodecanol as internal standard.

A unique rectal gland blend that featured the combination of both parental species, i.e., OXO + CF + DMP, was detected among the F_1 and F_2 generations as well as when hybrids of *B. carambolae* female and *B. papayae* male were backcrossed with their parental species (Table 1). A low percentage (1–4%) of hybrids possessed DMP only in their rectal glands when *B. carambolae* females interbred with *B. papayae* males, and when their F_1 hybrids were backcrossed to the male parental species (Table 1). Hybrids with OXO component only or without any rectal gland production were not found in F_1 or F_2 generations, but appeared in the backcrossed hybrids of *B. papayae* females and *B. carambolae* males (Table 1).

Rectal gland analyses revealed that several wild males with intermediate morphological traits possessed the unique rectal gland blend from the combination of both *B. carambolae* and *B. papayae* parents, i.e., OXO + CF + DMP (one male each from Penang and Johor; Figure 4A). Apart from this, there were also males detected with DMP only (one male from Perak and two males from Johor; Figure 4B) and without any pheromonal components (three males from Johor).

Interestingly, two wild males collected from Johor of the typical *B. papayae* phenotype were found to possess OXO + CF, which are the typical rectal gland blend of a *B. carambolae* male. In addition, there was a male with the *B. carambolae*-like morphology that contained CF and DMP (typical rectal gland blend of *B. papayae*) in its rectal gland.

DISCUSSION

Among the 52 sibling species in the *B. dorsalis* complex, four (*B. carambolae*, *B. dorsalis*, *B. papayae*, and *B. philippinensis*) have been observed to interbreed in the laboratory. *B. carambolae* interbreeds with *B. dorsalis* (McInnis et al., 1999) and with *B. papayae* (Wee and Tan, 2000a), and *B. dorsalis* interbreeds with *B. papayae* (Tan, 2000). Sexual compatibility was also observed between wild *B. dorsalis* and wild *B. philippinensis* (Medina et al., 1998). However, to date, there is no documented evidence of interspecific mating of these species in the field.

Whereas pheromonal analyses were suggested to be of use in chemotaxonomy of *Bactrocera* flies (Krohn et al., 1992), this paper describes for the first time the use of pheromonal analyses to detect evidence of natural hybrids of the ME-attracted *Bactrocera* species. The distinctive pheromonal components, together with the common phenylpropanoid CF, detected in *B. carambolae* and *B. papayae* were consistent with previous reports (Nishida et al., 1988; Perkins et al., 1990; Tan and Nishida, 1996; Wee, 2000). Therefore, the detection of pheromonal combinations between that of *B. papayae* and *B. carambolae* in a

wild *Bactrocera* fruit fly similar to that of the laboratory hybrids suggests that natural hybridization between *B. carambolae* and *B. papayae* had occurred in the wild.

Wild *Bactrocera* species with intermediate morphological characteristics have been observed regularly since the 1990's (Wee, 2000). Therefore, if natural hybridization happened in the past, some degree of self-crossing among the F_1 or backcrossing to the parental species must have occurred throughout many generations. Laboratory studies have shown that intermediate pheromone combination, such as OXO + CF + DMP, even though at low percentages, persist in F_1, F_2, and backcross generations. Thus, the detection of two wild males with OXO + CF + DMP rectal gland combination in the northern (Penang) and southern region (Johor) of peninsular Malaysia is consistent with the results of our laboratory studies.

The fact that low percentages of captured wild males possessed combinations of pheromonal components may reflect the rarity of the interbreeding that occurs in the wild. However, based on the sample size of trapped wild males, a low capture of those that do interbreed in the field cannot be discounted. Fewer hybrids would suggest higher chances of the hybrid individuals backcrossing with the parental species than among the hybrid themselves. In this study, a higher number of wild males was detected containing DMP only or without any pheromonal components compared with intermediates detected that possess OXO, DMP, and CF. This is also consistent with the laboratory interbreeding results that showed a higher percentage for such occurrence when the F_1 hybrids were backcrossed with the parental species.

The results showed that more hybrids were detected in Johor. Both *B. carambolae* and *B. papayae* are dusk-mating species and exhibit similar mating behavior (Wee, 2000). Although *B. papayae* preceded *B. carambolae* in mating at a slightly higher light intensity (1,000 *versus* 600 lx), their courtship and mating period overlapped (Wee, 2000). A wind tunnel study has shown that females of both species respond by upwind anemotactic flight to conspecific and heterospecific males during courtship period (Wee and Tan, 2000a). In field cages that simulated heterospecific sexual encounters of females, *B. papayae* females showed a much higher prereproductive isolation than *B. carambolae* females (Wee and Tan, 2000a; unpublished data). This suggests that females are able to distinguish between conspecific and heterospecific males at close range/contact during courtship encounters. However, *B. carambolae* females were less discriminating than *B. papayae* females in interspecific encounters (Wee and Tan, 2000a). The fact that *B. carambolae* is more abundant in Johor, and that *B. carambolae* females are nonselective in mate preference may account for natural interbreeding in the field. Moreover, *B. papayae* males are more aggressive in mating behavior than *B. carambolae* males, which could lead to

higher chances of securing a female, be it a conspecific or a heterospecific female (Wee, 2000; Wee and Tan, 2000a). This may explain the higher incidence of wild hybrids captured in Johor.

In contrast, in areas where *B. carambolae* population density is low, high prereproductive isolation in the case of *B. papayae* females may also contribute to the low recovery of hybrids in the wild. However, in the absence of conspecific males, females of both *B. papayae* and *B. carambolae* mate with heterospecific males (Wee, 2000; Wee and Tan, 2000a). In addition, the production of a common ME-derived sex pheromone, in both *B. papayae* and *B. carambolae* males, would further promote and facilitate interspecific encounters and mating in the wild because natural sources of ME are plentiful, especially in the tropics where there are many plant species that contain ME (Tan and Nishida, 2000; Tan et al., 2002; Nishida et al., 2004). ME, as a pheromone precursor (Tan and Nishida, 1996, 1998), enhances male mating competitiveness in *B. dorsalis*, *B. papayae*, and *B. philippinenses* after ME consumption (Shelly and Dewire, 1994; Shelly et al., 1996; Tan and Nishida, 1996, 1998; Hee and Tan, 1998; Shelly, 2000). This may account for the occurrence of the two hybrid flies in the Perak and Penang regions.

Pheromonal analysis also showed that even a wild *Bactrocera* species with the typical phenotype of *B. carambolae* can possess a typical rectal gland blend of a *B. papayae* male and vice versa. Thus, species identification based solely on morphological traits may not be totally reliable, at least in the *B. dorsalis* complex, and especially for the two sympatric species. The discovery of this phenomenon certainly further complicates the existing problem in the taxonomic status of *B. carambolae* and *B. papayae*, the latter of which was recently shown to be neither a distinct biological nor genetic species from *B. dorsalis* (Hendel) (Naeole and Haymer, 2003; Tan, 2003).

Interbreeding between a *B. carambolae* female and *B. papayae* male produced F_1 males with rectal gland blends closer to a *B. papayae* male. However, backcrossing this F_1 male to *B. carambolae* female parent yielded a high percentage (75%) of hybrids having *B. carambolae* rectal gland blend. Therefore, pheromone production in the *Bactrocera* species appears to be a sex-limited trait whereby genes responsible for pheromone production show a 100% penetrance in males only, and although females inherited the same gene, the gene was not expressed in the female progeny.

In contrast, based on these laboratory interbreeding results, backcrossing of the hybrids to the parental species over many generations may produce not only higher percentages of hybrids that resemble the parental species in terms of rectal gland blend, but also, in terms of morphological traits. Therefore, at the time of this investigation, a true hybrid may look like the parental species in all aspects, and, thus, escape any morphological/pheromonal means of screening. Only those minority categories with unique intermediate rectal gland blends

may be detected by pheromone analysis. A more sensitive molecular technique may help in the determination of the hybrids' polymorphism/genetic make-up.

In *B. papayae*, the *in vitro* conversion of ME into its metabolites involves the cytochrome P-450 mixed-function monooxygenases (Lim et al., 1998). Hybridization has resulted in a lower penetrance observed in more than 80% of the F_1 hybrids of the reciprocal crosses, and in some cases, a low percentage of the backcross offspring has been found not to produce either CF or DMP after ME consumption. This lowered and "zero" penetrance may have resulted from the complex interaction between segregated maternal and paternal genes during hybridization of sibling species, causing alteration/deletion or inhibition of the gene responsible for ME conversion, thus disabling the respective gene(s). However, this does not discount other possibilities that may contribute to this lowered penetrance for the sex-limited trait.

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SEX PHEROMONES OF FIVE OLETHREUTINE SPECIES (LEPIDOPTERA: TORTRICIDAE) ASSOCIATED WITH THE SEEDLINGS AND FRUITS OF MANGROVE PLANTS IN THE RYUKYU ISLANDS, JAPAN: IDENTIFICATION AND FIELD EVALUATION

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Abstract—The sex pheromones of three *Cryptophlebia*, one *Centroxena*, and one *Eucosma* species (Lepidoptera: Olethreutinae) inhabiting mangroves in the Ryukyu Islands, Japan, were studied with coupled gas chromatography-electroantennographic detection, and GC-mass spectrometry (GC-MS). The larvae of each *Cryptophlebia* species are specifically associated with viviparous seedlings from one of three mangrove Rhizophoraceae plants. Whereas three EAG-active alcohol components, (*Z*)-8-dodecen-1-ol, (*E*)-8-dodecen-1-ol, and dodecan-1-ol, in a ratio of 100:12:4, were identified from the pheromone gland extract of female of *C. horii* (host: *Bruguiera gymnorrhiza*), two other sibling species produced the corresponding acetates, i.e., (*Z*)-8-dodecenyl acetate, (*E*)-8-dodecenyl acetate, and dodecyl acetate, in a 100:2:3 ratio from *Cryptophlebia palustris* (host: *Rhizophora stylosa* in Iriomote-jima Island) and in a 100:7:13 ratio from *C. amamiana* (host: *Kandelia candel* in Amami-oshima Island). The double bond positions of the monounsaturated components were confirmed by GC-MS analyses of their adducts with dimethyl disulfide. On the other hand, the larvae of *Centroxena* sp. feed on fruits of *Sonneratia alba*, another mangrove plant in the Sonneratiaceae, and the extract of the female pheromone glands contained (8*E*,10*E*)-dodecadienyl acetate and dodecyl acetate in a ratio of 100:5. The double bond position of

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the diunsaturated compound was confirmed by GC-MS analysis of its adduct with 4-methyl-1,2,4-triazoline-3,5-dione. (*E*)-9-Dodecenyl acetate was exclusively identified in the pheromone gland extract of *Eucosma coniogramma* females reared from seedlings of *B. gymnorrhiza*. Although the roles of minor components have not been revealed by field tests, synthetic lures baited with the main pheromone component of each species successfully attracted the target males, confirming that the sex pheromone is one of the most important factors for their reproductive isolation.

Key Words—Sex pheromones, Tortricidae, Olethreutinae, mangrove, Rhizophoraceae, Sonneratiaceae, reproductive isolation, DMDS, MTAD.

INTRODUCTION

The range of the Ryukyu Islands is approximately 800 km in the East China Sea starting from Tane-ga-shima Island (latitude 30°43′N) near the south end of Kyushu to the Yaeyama Islands (~24°N) near Taiwan. This archipelago includes Amami-oshima Island (28°15′N) and Okinawa-jima Island (26°35′N), and mangroves spread throughout the mouths of the rivers on several islands. In Iriomote-jima Island (24°19′N), one of the main islands among the Yaeyama Islands, mangroves mainly consist of seven plant species in five families such as *Bruguiera gymnorrhiza*, *Rhizophora stylosa*, and *K. candel* in the Rhizophoraceae and *Sonneratia alba* in the Sonneratiaceae. The variety of these plants decreases northward, and mangroves in Tane-ga-shima Island consist solely of *K. candel* (Table 1). To date, 37 lepidopteran species have been recorded from the mangroves in the Ryukyu Islands, including three *Cryptophlebia* spp. in Olethreutinae (Lepidoptera: Tortricidae) (Komai, unpublished). While *Cryptophlebia horii* Kawabe, which is almost exclusively associated with viviparous seedlings from *B. gymnorrhiza*, was newly described in 1987, two other sibling species, *C. palustris* Komai and Nasu and *C. amamiana* Komai and Nasu, the larvae of which feed on *R. stylosa* and *K. candel*, respectively, were recently discovered (Komai and Nasu, 2003). Although *C. amamiana* has not been found in the mangroves with *K. candel* in Iriomote-jima Island, the other two sibling species have been observed there. On this island, viviparous seedlings of *B. gymnorrhiza* were also attacked by another olethreutine species, *Eucosma coniogramma* Clarke (=*E. okubiensis* Kawabe), in addition to *C. horii* (Komai and Nasu, 2003). Furthermore, the fruit of *S. alba* is a host of *Centroxena* sp. in Olethrutinae, known only from Iriomote-jima Island (Komai, unpublished) (Table 1).

Lepidopteran sex pheromones have been chemically identified from adult females of more than 500 species, but information about the species distributed in the tropical and subtropical zones is still limited (Ando, 2005; Ando et al.,

TABLE 1. MANGROVE PLANTS REPRESENTATIVE OF THE RYUKYU ISLANDS, OLETHREUTINE SPECIES ASSOCIATED WITH THEIR VIVIPAROUS SEEDLINGS OR FRUITS, AND MAIN SEX PHEROMONE COMPONENTS IDENTIFIED FROM THE FEMALE MOTHS

Mangrove: host plant		Olethreutine moth: herbivorous insect		Main pheromone component
Family: Species	Distribution	Species (Tribe)	Distribution[a]	
Rhizophoraceae				
Kandelia candel	Southern from Tane-ga-shima (30°43′N)	*Cryptophlebia amamiana* (Grapholitini)	Tane-ga-shima, Amami-oshima	Z8-12:OAc
Bruguiera gymnorrhiza	Southern from Amami-oshima (28°95′N)	*Cryptophlebia horii* (Grapholitini)	Amami-oshima, Okinawa-jima, Miyako-jima, Iriomote-jima, etc.	Z8-12:OH
		Eucosma coniogramma (Eucosmini)	Okinawa-jima, Iriomote-jima	E9-12:OAc
Rhizophora stylosa	Southern from Okinawa-jima (26°35′N)	*Cryptophlebia palustris* (Grapholitini)	Ishigaki-jima, Iriomote-jima	Z8-12:OAc
Sonneratiaceae				
Sonneratia alba	Iriomote-jima (24°19′N)	*Centroxena* sp. (Grapholitini)	Iriomote-jima	E8,E10-12:OAc

[a] Data from Komai and Nasu (2003).

2004; Arn et al., 1997; El-Sayed, 2005; Witzgall et al., 2004). Screening tests with synthetic lures baited with known sex pheromones in the Mekong Delta resulted in attraction of males of several lepidopteran species (Hai et al., 2002), but trials targeting olethreutine species have been unsuccessful (Hai et al., unpublished data). Ecological details of the above olethreutine species and damage of mangroves by their larvae are still unknown, and synthetic pheromones are expected to be a useful tool for these investigations. Therefore, we collected seedlings and fruits of mangrove plants infested with olethreutine larvae in the Ryukyu Islands and studied the sexual communication systems of the adults emerging. This paper deals with the identification of the pheromones of the three *Cryptophlebia* and one *Centroxena* species in the tribe Grapholitini and one *Eucosma* species in the tribe Eucosmini in addition to field evaluation of their synthetic pheromones.

METHODS AND MATERIALS

Insects and Pheromone Extracts. In Iriomote-jima Island, young viviparous seedlings of *B. gymnorrhiza* infected by *C. horii* and *E. coniogramma* were collected from January to February, and matured stems of *R. stylosa*, seedlings infected by *C. palustris* were collected in July. Other viviparous seedlings of *K. candel* infected by *C. amamiana* were found in Amami-oshima Island in January. Fruits of *Sonneratia alba* with *Centroxena* sp. were picked in Iriomote-jima Island from July to August. These host plant materials were incubated at 25°C under a 16L:8D cycle. After eclosion, each moth was sexed and kept separately in a glass tube. From 2- to 3-d-old female moths of each species (about 100 females of *C. horii*, *C. palustrism*, and *Centroxena* sp., 10 females of *E. coniogramma*, and 20 females of *C. amamiana*), abdominal tips containing the pheromone glands were removed 2–3 hr after lights off and soaked in *n*-hexane for 15 min to extract pheromone components. Male moths were used for the tests with gas chromatography (GC) equipped with an electroantennographic (EAG) detector (GC-EAD).

Chemicals. The chemical structures of pheromone components and related compounds are abbreviated as follows: Z = (*Z*)-double bond, E = (*E*)-double bond, number before the hyphen = position of the double bond, number after the hyphen = carbon number of the straight chain, OAc = acetate, and OH = alcohol. All dodecenyl compounds and (8*E*,10*E*)-dococadienyl derivatives used as analytical standards and to prepare lures were supplied by Shin-etsu Chemical Co., Ltd. (Tokyo, Japan) with purity levels greater than 98%. Three geometrical isomers of the 8,10-diene (>95% purity) were synthesized according to an established method (Ando et al., 1985).

Derivatization of Pheromone Components. To confirm the double bond position of monounsaturated compounds, crude pheromone extracts of three *Cryptophlebia* spp. were derivatized with dimethyl disulfide (DMDS). Thus, the extract of ca. 10 female equivalents (FE) of each species was dissolved in a mixture of DMDS (50 μl) and a diethyl ether solution of iodine (60 mg/ml, 5 μl) and warmed at 40°C overnight (Buser et al., 1983; Inomata et al., 2000). After the addition of a 5% sodium thiosulfate solution (0.5 ml), crude products were extracted with *n*-hexane and analyzed by GC-mass spectrometry (GC-MS). For the determination of the diunsaturated compound produced by *Centroxena* sp., a crude extract (81 FE) was treated with a CH_2Cl_2 solution of 4-methyl-1,2,4-triazoline-3,5-dione (MTAD, 10 mg/ml, 10 μl) for 10 min at room temperature after removing *n*-hexane (Young et al., 1990). After changing the solvent to *n*-hexane again, the MTAD adduct was analyzed by GC-MS.

GC-EAD Analyses. The EAG activity of the natural pheromone components of each species was measured by GC-EAD (Struble and Arn, 1984), using at least four antennae cut from different 2- to 3-d-old males. The GC was equipped with a DB-23 capillary column (0.25 mm × 30 m, 0.25 μm, J&W Scientific, Folsom, CA), with a temperature program of 80°C for 1 min, 8°C/min to 210°C, 210°C for 10 min. The effluent from the column was split between the flame ionization detector (FID) and EAD at a ratio of 1:1 (Inomata et al., 2005). An antenna was excised at the base from the corresponding male, and a few distal segments were cut off. *Bombyx mori* saline was used for the electrodes, and the airflow carrying eluted compounds to the antenna was at rate of 7.8 cm/min. EAG activities of synthetic pheromone components and their analogs were measured by the GC-EAD using the same conditions as the gland extracts.

GC-MS Analyses. Electron impact GC-MS was carried out on an HP5973 mass spectrometer system (Hewlett-Packard) equipped with a capillary column operating with a mass range of *m*/*z* 50–500. The ionization voltage was 70 eV, and the ion source temperature was 230°C. For analysis of natural pheromone components and DMDS adducts, a DB-23 column (0.25 mm × 30 m) was used under the same temperature program as the GC-EAD. For analysis of the MTAD adduct, an HP-5 column (0.25 mm × 30 m, 0.25 μm; Hewlett-Packard, Wilmington, DE) was used with temperature program of 100°C for 2 min, 15°C/min to 280°C. The analyses of the untreated extracts were repeated in order to confirm the titers of major components and the mixing ratios of minor components, except for *E. coniogramma*.

Field Tests. Lures were white rubber septa (8 mm OD, Aldrich) impregnated with a *n*-hexane solution of the pheromone components. Each septum was placed at the center of a sticky trap (30 × 27 cm bottom plate with a roof; Takeda Chemical Ind. Ltd., Osaka, Japan), which was set at a height of 1.5 m from the ground. The field evaluation for the lures of each olethreutine species

was carried out in the mangrove swamps where the infested seedlings or fruits were collected.

RESULTS

Identification of the C. horii Female Pheromone. A GC-EAD analysis of the *C. horii* pheromone extract (one FE) showed the occurrence of three EAG-active components, as follows: 170 μV at retention time (Rt) 11.2 min [Kováts relative index (RI) 18.9], 900 μV at 11.5 min (RI 19.2), and 830 μV at 11.8 min (RI 19.4) (Figure 1A). This analytical profile was representatively recorded on antennae of *C. horii* males. The chromatographic behaviors of the three components on a DB-23 capillary column coincided with those of authentic samples of 12:OH, E8-12:OH, and Z8-12:OH, which have EAG activity as shown in Figure 1B. The GC-MS data of these natural components also coincided with those of the authentic samples. The mass spectrum at 10.43 min (RI 18.87), which shows the highest ion at *m*/*z* 168 indicating $[M-18]^+$ of 12:OH, was almost the same as that of commercial 12:OH. The mass spectra at 10.75 min (RI 19.15) and 10.97 min (RI 19.35) including the highest ion at *m*/*z* 166, $[M-18]^+$ of dodecen-1-ol, were identical with those of synthetic E8-12:OH and Z8-12:OH. Z8-12:OH occurred as a major component in the pheromone gland extract, and a total ion chromatogram (TIC) of this GC-MS analysis showed a 100:12:4 ratio of Z8-12:OH, E8-12:OH, and 12:OH (Figure 1C). The titer of the main component was ca. 4 ng/female, and its double bond position was confirmed by GC-MS analysis of the crude pheromone extract (nine FE) treated with DMDS. Figure 1D shows the mass spectrum of a DMDS adduct (Rt 23.82 min) derived from the main component. In addition to M^+ at *m*/*z* 278, diagnostic fragment ions at *m*/*z* 175 and 103 confirmed the original double bond at the 8-position of the monounsaturated alcohol.

EAG Responses and Field Attraction of C. horii Males. In GC-EAD studies, antennae of male *C. horii* responded to synthetic pheromone components and their related compounds, as shown in Figure 2. The main pheromone component, Z8-12:OH, showed the largest average value of the EAG activity among the three identified components at a dose of 0.1 ng, whereas the EAG response did not increase dose-dependently and was less than those of E8-12:OH at doses of 1 and 10 ng. Functional derivatives of the pheromonal alcohols also stimulated the antennae, and interestingly monounsaturated aldehydes were more active than the corresponding acetates. Table 2 shows results of the field evaluation of synthetic lures. For the attraction of *C. horii* males, Z8-12:OH was indispensable, but the roles of the other two minor components were at first unclear (Test A-1). However, when the percentage of

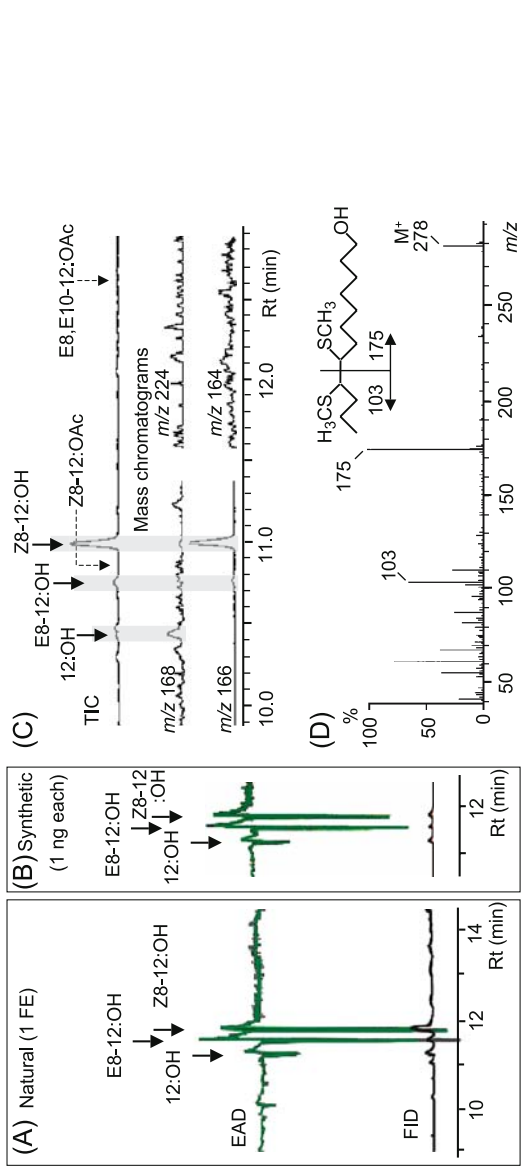

FIG. 1. Analyses of the sex pheromone components of *Cryptophlebia horii* by GC-EAD and GC-MS. (A) GC-EAD of the pheromone extract (one FE) conducted with a flame ionization detector (FID) and an electroantennographic detector (EAD). (B) GC-EAD of synthetic standards (12:OH, E8-12:OH, and Z8-12:OH, 1 ng each). (C) GC-MS analysis of the extract (nine FE); total ion chromatogram (TIC) and mass chromatograms monitoring the diagnostic ions at *m/z* 168 for 12:OH, at *m/z* 166 for E8-12:OH, Z8-12:OH and the corresponding acetates (not detected), and *m/z* 164 and 224 (M^+) for E8,E10-12:OAc (not detected). (D) Mass spectra of a DMDS adduct of Z8-12:OH in the pheromone extract (nine FE).

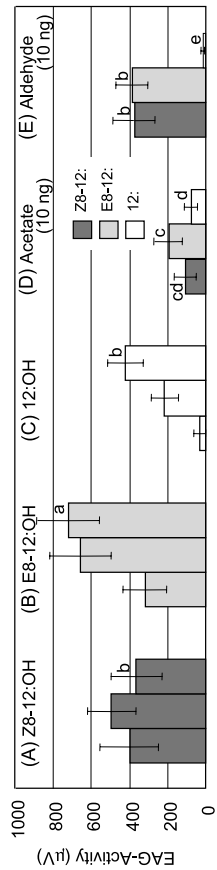

FIG. 2. Electroantennographic (EAG) responses (mean μV ± SE, $N = 10$) of *Cryptophlebia horii* males to synthetic pheromone components (A–C, injection of 0.1, 1, and 10 ng) and some related compounds (D and E, injection of 10 ng). The responses were measured by GC-EAD, which delivered about half of the injected compound to the male antenna. Bars at the dose of 10 ng superscripted by a different letter are significantly different at $P < 0.05$ by Tukey–Kramer test.

TABLE 2. FIELD ATTRACTION OF *Cryptophlebia horii* MALES BY LURES BAITED WITH THE SYNTHETIC C_{12}-CHAIN ALCOHOLS (Z8-12:OH, E8-12:OH, AND 12:OH) IDENTIFIED FROM THE FEMALES AND A RELATED ACETATE (Z8-12:OAC) IN IRIOMOTE-JIMA[a]

	Lure components (mg/rubber septum)			Captured males/trap[b]
Test A-1	Z8-12:OH	E8-12:OH	12:OH	
	1.00	0	0	175 ± 126 a
	1.00	0.12	0	73 ± 26 a
	1.00	0.12	0.04	117 ± 77 a
	1.00	1.12	0.5	93 ± 58 a
	1.00	0.5	0.04	110 ± 42 a
	0	0	0	0
Test A-2	Z8-12:OH	E8-12:OH		
	1.00	0		380 ± 129 a
	0.95	0.05		138 ± 19 a
	0.80	0.20		51 ± 20 ab
	0.50	0.50		21 ± 14 b
	0.10	0.90		6 ± 3 b
	0	0		0
Test A-3	Z8-12:OH	Z8-12:OAc		
	1.00	0		140 ± 61 a
	0.95	0.05		8 ± 3 b
	0.80	0.20		2 ± 2 b
	0.50	0.50		3 ± 1 b
	0.10	0.90		0
	0	0		0

[a] Tested in a mangrove with *Bruguiera gymnorrhiza* using three traps for each lure from March 4 to April 6 (Test A-1), from April 6 to May 18 (Test A-2), and from May 18 to June 30 (Test A-3) in 2004.

[b] Mean ± SE. Values within each test followed by a different letter are significantly different at $P < 0.05$ by Tukey–Kramer test.

the (*E*)-isomer was increased, the number of captured males decreased (Test A-2). Furthermore, the activity of Z8-12:OH was greatly inhibited by the corresponding acetate, Z8-12:OAc (Test A-3), which has not been identified from *C. horii* females but has been found from the other two *Cryptophlebia* spp., as described in the following paragraph.

Identification of C. palustris and C. amamiana Female Pheromone Components. A GC-EAD analysis of the *C. palustris* pheromone extract (one FE) showed at least three EAG-active components: 150 μV at 11.1 min (RI 18.8), 190 μV at 11.35 min (RI 19.05), and 830 μV at 11.6 min (RI 19.3) (Figure 3A). Their chromatographic behaviors and mass spectra coincided with those of authentic samples of 12:OAc, E8-12:OAc, and Z8-12:OAc. The mass spectrum at 10.36 min (RI 18.81) showed the highest ion at *m*/*z* 168 indicating $[M\text{-}60]^+$ of 12:OAc. The mass spectra at 10.64 min (RI 19.05) and 10.86 min (RI 19.25)

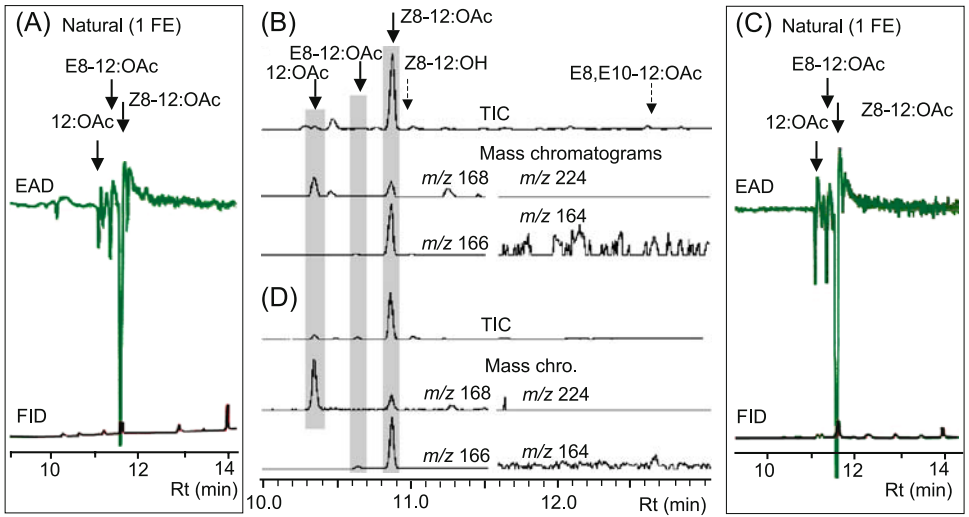

FIG. 3. Analyses of the sex pheromone components of *Cryptophlebia palustris* and *C. amamiana* by GC-EAD and GC-MS. (A) GC-EAD of the *C. palustris* pheromone (one FE). (B) GC-MS analysis of the *C. palustris* pheromone (one FE); TIC and mass chromatograms monitored the diagnostic ions at *m/z* 168 for 12:OAc, at *m/z* 166 for E8-12:OAc and Z8-12:OAc, and the corresponding alcohols (not detected), and *m/z* 164 and 224 (M^+) for E8,E10-12:OAc (not detected). (C) GC-EAD of the *C. amamiana* pheromone (one FE). (D) GC-MS analysis of the *C. amamiana* pheromone (eight FE); TIC and mass chromatograms monitoring the ions at *m/z* 168, 166, 164, and 224.

with the highest ion at *m*/*z* 166, $[M-60]^+$ of dodecenyl acetates, coincided with the spectra of synthetic E8-12:OAc and Z8-12:OAc. Z8-12:OAc occurred as a main component (ca. 4 ng/female), and the chromatogram showed a 100:2:5 ratio of Z8-12:OAc, E8-12:OAc, and 12:OAc in the pheromone gland extract (Figure 3B). The mass spectrum of a DMDS adduct (Rt 22.73 min) with M^+ at *m*/*z* 320, derived from the main component in the crude pheromone extract (three FE), showed two diagnostic fragment ions at *m*/*z* 217 and 103, indicating the original double bond at the 8-position of the monounsaturated acetate.

Similar results were obtained from the analyses of the *C. amamiana* pheromone extract, except for the amounts of the three components (Figures 3C and D). The *C. amamiana* females produced Z8-12:OAc, E8-12:OAc, and 12:OAc in a ratio of 100:7:13, and the titer of Z8-12:OAc was ca. 6 ng/female.

EAG Responses and Field Attraction of C. palustris and C. amamiana Males. Figure 4 shows the EAG dose responses of synthetic pheromone components on male antennae of *C. palustris* and *C. amamiana*, which were measured by a GC-EAD technique. The antennae of both species were most strongly stimulated by the common main pheromone component, Z8-12:OAc, among the three acetates identified. The EAG activity of E8-12:OAc was similar to that of 12:OAc. In *C. palustris* males, the EAG activity of the corresponding aldehyde and alcohol derivatives of the pheromone components was measured, and weak responses of their antennae were recorded.

In a mangrove swamp with *R. stylosa* on Iriomote-jima Island, traps baited with Z8-12:OAc (1 mg/rubber septum) successfully attracted *C. palustris* males; 9 ± 3 males were captured per trap from July 26 to August 22, 2004. However, whereas unbaited traps caught no males, this number was smaller than that expected from a high level of infestation of the viviparous seedlings by the larvae. Although many lures including minor components (E8-12:OAc and 12:OAc) in different ratios have been tested since 2003, no synergistic effects have been observed. In a mangrove swamp with *K. candel* on Amami-oshima Island, a field test for *C. amamiana* was carried out from March 24 to April 6, 2003. Two traps baited with synthetic Z8-12:OAc (1 mg/rubber septum) captured a total of five males, and the minor components showed no synergistic effect.

Identification of the Centroxena sp. Female Pheromone. A GC-EAD analysis of the *Centroxena* sp. pheromone extract (six FE) showed two antennal stimulatory components, as follows: 40 μV at 11.1 min and 380 μV at 13.3 min (RI 20.9) (Figure 5A). Their chromatographic behaviors coincided with those of authentic samples of 12:OAc and E8,E10-12:OAc (Figure 5B). The GC-MS data of these components also coincided with those of authentic samples. The mass spectra at 12.62 min (RI 20.90) with M^+ (*m*/*z* 224) and $[M-60]^+$ (*m*/*z* 164) of dodecadienyl acetate coincided with the spectrum of synthetic E8,E10-12:OAc (Figure 5C). Synthetic standards of its geometrical isomers separately eluted from the DB-23 column [E8,E10-12:OAc 12.57 min (RI 20.85), Z8,Z10-

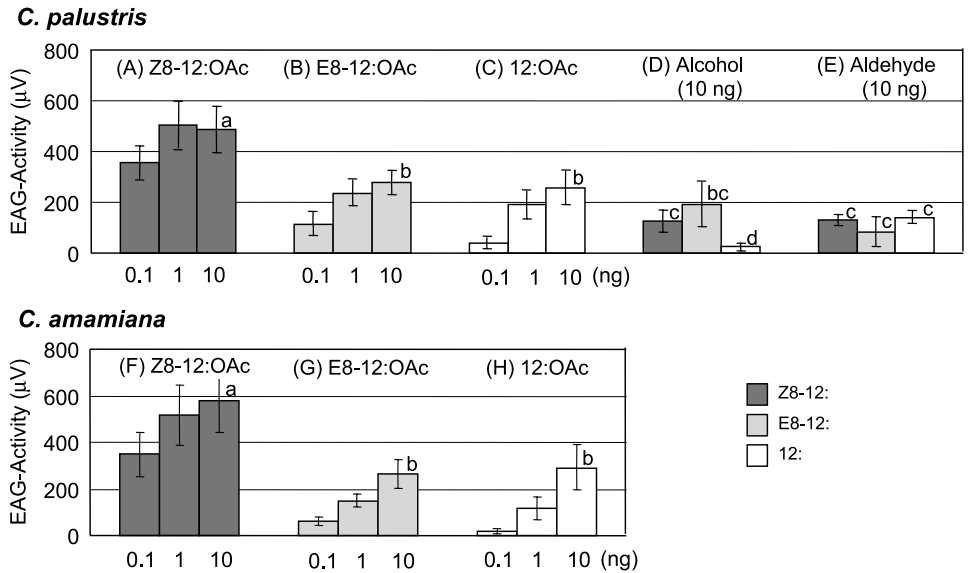

FIG. 4. Electroantennographic (EAG) responses (mean μV ± SE, $N = 10$) of *Cryptophlebia palustris* males to synthetic pheromone components (A–C, injection of 0.1, 1, and 10 ng) and some related compounds (D and E, injection of 10 ng) and those of *C. amamiana* males to synthetic pheromone components (F–H, 0.1, 1, and 10 ng injection). The responses were measured by GC-EAD, which delivered about half of the injected compound to the male antenna. Bars at the dose of 10 ng in each species superscripted by a different letter are significantly different at $P < 0.05$ by Tukey–Kramer test.

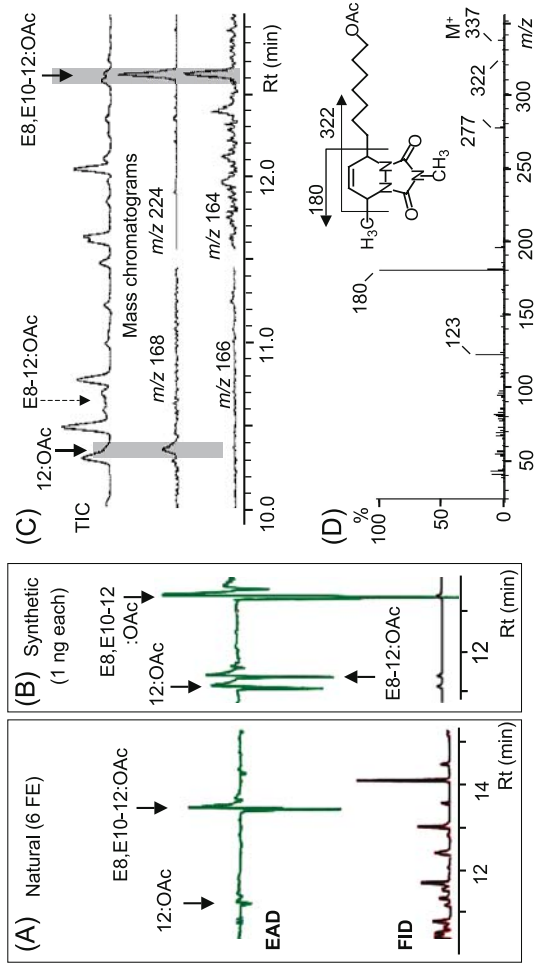

FIG. 5. Analyses of the sex pheromone components of *Centroxena* sp. by GC-EAD and GC-MS. (A) GC-EAD of the pheromone extract (six FE). (B) GC-EAD of synthetic standards (12:OAc, E8-12:OAc, and E8,E10-12:OAc, 1 ng each). (C) GC-MS analysis of the extract (20 FE); TIC and mass chromatograms monitoring the diagnostic ions at *m/z* 168 for 12:OAc, at *m/z* 166 for E8-12:OAc (not detected), and at *m/z* 164 and 224 (M^+) for E8,E10-12:OAc. (D) Mass spectrum of an MTAD adduct of E8,E10-12:OAc in the pheromone extract (81 FE).

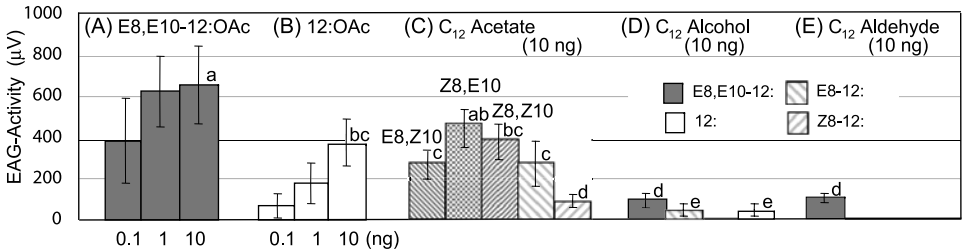

FIG. 6. Electroantennographic (EAG) responses (mean μV ± SE, $N = 10$) of *Centroxena* sp. males to synthetic pheromone components (A and B, injection of 0.1, 1, and 10 ng) and some related compounds (C–E, injection of 10 ng). The responses were measured by GC-EAD, which delivered about half of the injected compound to the male antenna. Bars at the dose of 10 ng superscripted by a different letter are significantly different at $P < 0.05$ by Tukey–Kramer test.

12:OAc 12.80 min (RI 21.07), and E8,Z10-12:OAc 12.82 min (RI 21.09)] and confirmed the 8E,10E configuration of the natural component. The ratio of E8,E10-12:OAc and 12:OAc was 100:5, and the titer of the diunsaturated main component in the pheromone gland extract was ca. 0.6 ng/female. The double bond positions were confirmed by GC-MS analysis of the crude pheromone extract (81 FE) treated with MTAD. The mass spectrum of the MTAD adduct (Rt 14.42 min) derived from the diunsaturated component showed a characteristic base peak at *m*/*z* 180 in addition to $[M\text{-}CH_3]^+$ at *m*/*z* 322 (2%) and M^+ at *m*/*z* 337 (6%) (Figure 5D), proving the original conjugated 8,10-diene structure.

EAG Responses and Field Attraction of Centroxena sp. Males. Figure 6 shows the EAG activity of synthetic standards measured with GC-EAD. Among

TABLE 3. FIELD ATTRACTION OF *Centroxena* sp. AND *Rhadinoscolops koenigianus* MALES TO LURES BAITED WITH THE SYNTHETIC C_{12}-CHAIN COMPOUNDS (E8, E10-12:OAC AND 12:OAC) IDENTIFIED FROM *Centroxena* sp. FEMALES AND RELATED COMPOUNDS IN IRIOMOTE ISLAND[a]

		Captured males/trap[b]	
	Lure components (mg/rubber septum)	*Centroxena* sp.	*R. koenigianus*
Test B-1	E8,E10-12:OAc (0.50 mg)		
	+ none	10 ± 3 a	140 ± 63 a
	+ 12:OAc (0.50 mg)	11 ± 3 a	42 ± 20 b
	+ 12:OAc (1.50 mg)	16 ± 10 a	45 ± 6 b
	none	1 ± 1 b	0
Test B-2	E8,E10-12:OAc (1.00 mg)		
	+ none	7 ± 5 a	19 ± 7 a
	+ 12:OAc (0.10 mg)	6 ± 4 a	35 ± 21 a
	+ E8-12:OAc (0.10 mg)	6 ± 3 a	9 ± 5 a
	+ E8,E10-12:OH (0.10 mg)	12 ± 7 a	25 ± 15 a
	none	0	0
Test B-3	E8,E10-12:OAc (1.00 mg)	10 ± 7 a	16 ± 7 a
	Z8,E10-12:OAc (1.00 mg)	21 ± 15 a	6 ± 2 ab
	E8,Z10-12:OAc (1.00 mg)	8 ± 7 a	1 ± 1 b
	Z8,Z10-12:OAc (1.00 mg)	25 ± 9 a	12 ± 11 a
	none	0	0
Test B-4	E8,E10-12:OAc (1.00 mg)	15 ± 8 ab	49 ± 33 a
	Z8,E10-12:OAc (1.00 mg)	30 ± 9 a	41 ± 15 a
	E8,Z10-12:OAc (1.00 mg)	6 ± 2 b	24 ± 13 a
	Z8,Z10-12:OAc (1.00 mg)	9 ± 2 b	27 ± 8 a
	none	0	0

[a] Tested in a mangrove with *Sonneratia alba* using two traps for each lure from May 1 to July 15 in 2003 (Test B-1), and using three traps for each lure from October 18 to November 24 (Test B-2), from January 25 to April 6 (Test B-3), May 18 to June 30 (Test B-4) in 2004.

[b] Mean ± SE. Values within each test followed by a different letter are significantly different at $P < 0.05$ by Tukey–Kramer test.

the tested compounds, antennae of male *Centroxena* sp. responded most strongly to E8,E10-12:OAc. The other geometrical isomers also elicited considerable EAG activity. In addition to 12:OAc, the monounsaturated acetate (E8-12:OAc) also showed medium activity. Antennal responses to alcohol and aldehyde analogs were weak. Results of field tests with synthetic lures are shown in Table 3. Traps baited with E8,E10-12:OAc attracted males, but the role of 12:OAc was not clear (Tests B-1 and B-2). Interestingly, three other geometrical isomers of E8,E10-12:OAc also effectively attracted the males (Tests B-3 and B-4). In addition, the diunsaturated acetate attracted a large number of males of another olethreutine species, *Rhadinoscolops koenigianus* Fabricius, the attractant of which had not been reported (Table 3). The host plant of this leafroller species is *Melia azedarach*, which is a deciduous arbor tree and is not found in mangroves.

Sex Pheromone of E. coniogramma. A GC-EAD analysis of the *E. coniogramma* pheromone extract (one FE) showed an EAG-active component: 490 μV at 11.4 min (RI 19.1). This chromatographic behavior on a DB-23 column coincided with that of E9-12:OAc, and the geometrical isomer (Z9-12:OAc) eluted separately at 11.7 min (RI 19.35). On a GC-MS analysis with the extract (five FE), the mass spectrum at 10.71 min (RI 19.12) with the highest ion at *m*/*z* 166 was similar to the spectrum of synthetic E9-12:OAc. The titer of

TABLE 4. FIELD ATTRACTION OF *Eucosma coniogramma* MALES BY LURES BAITED WITH SYNTHETIC E9-12:OAC IDENTIFIED FROM THE FEMALES AND RELATED COMPOUNDS, IN IRIOMOTE ISLAND[a]

	Lure components (mg/rubber septum)			Captured males/trap[b]
Test C-1	E9-12:OAc	Z9-12:OAc		
	1.00	0		91 ± 21 a
	0	1.00		1 ± 1 b
	0	0		0
Test C-2	E9-12:OAc	Z9-12:OAc	E9-12:OH	
	1.00	0	0	21 ± 12 b
	0.95	0.05	0	99 ± 45 a
	0.80	0.20	0	1 ± 1 c
	0.50	0.50	0	1 ± 1 c
	0.95	0	0.05	19 ± 13 b
	0.80	0	0.20	22 ± 15 b
	0.50	0	0.50	10 ± 8 b
	0	0	0	0

[a] Tested in a mangrove with *Bruguiera gymnorrhiza* using two traps for each lure from June 9 to July 9 (Test C-1), and using three traps for each lure from July 11 to August 22 (Test C-2) in 2004.
[b] Mean ± SE. Values within each test followed by a different letter are significantly different at $P < 0.05$ by Tukey–Kramer test.

E9-12:OAc was ca. 0.1 ng/female. Results of field tests with synthetic lures are shown in Table 4. Whereas many males of *E. coniogramma* were specifically attracted by E9-12:OAc in the preliminary experiment with single component lures (Test C-1), the evaluation of binary lures revealed that mixing with Z9-12:OAc at 5% effectively increased the activity (Test C-2). E9-12:OH showed no apparent synergistic effect (Test C-2). These results suggest that females secrete the geometrical isomer of E9-12:OAc, but not the alcohol analog, as a minor pheromone component at levels that were undetectable by GC-EAD and GC-MS analyses.

DISCUSSION

Using GC-EAD and GC-MS techniques, the following EAG-active components were identified from the pheromone gland extracts of five olethreutine species collected at mangroves in the Ryukyu Islands: Z8-12:OH, E8-12:OH, and 12:OH (100:12:4) from *C. horii*; Z8-12:OAc, E8-12:OAc, and 12:OAc (100:2:3) from *C. palustris*; the same three acetates (100:7:13) from *C. amamiana*; E8,E10-12:OAc and 12:OAc (100:5) from *Centroxena* sp.; and E9-12:OAc from *E. coniogramma* (Table 1). These compounds are well-known common pheromone components of other olethreutine species. (*Z*)-8-Dodecenyl compounds, the main pheromone components of three *Cryptophlebia* species living in mangroves, have already been identified from several olethreutine species classified into *Grapholita* (subgenus *Aspila*) in the tribe Grapholitini, *Eucosma* and *Proteoteras* in the tribe Eucosmini, and *Hedya* in the tribe Olethreutini. In the genus *Cryptophlebia* (Grapholitini), Z8-12:OAc has been characterized from two species, *C. batrachopa* and *C. leucotreta* (Hall et al., 1984), but both species were recently transferred to the genus *Thaumatotibia* (Komai, 1999). E8,E10-12:OAc has been identified from nine olethreutine species in the genera *Cydia*, *Matsumuraeses*, and *Melissopus* (currently synonymized with *Cydia*) in the Grapholitini and *Epiblema* and *Rhyacionia* in Eucosmini but not in *Centroxena* (Grapholitini). This is the first study of the sex pheromone of any *Centroxena* species.

The tribe Grapholitini is further divided into three groups: the *Grapholita* genus group, the *Cydia* genus group, and the *Dichrorampha* genus group (Komai, 1999). In addition to the sex pheromone, field tests with synthetic compounds have revealed male attraction of more than 90 Grapholitini species (Ando, 2005; Komai, 1999). Males in the *Grapholita* genus group usually were attracted by 8-dodecenyl compounds, suggesting the chemical structures of their real sex pheromones. Conversely, males in the *Cydia* genus group usually were attracted by 8,10-dodecadienyl compounds. The genus *Cryptophlebia* belongs

to the *Grapholita* genus group, and the chemical structures of the pheromones identified from the three *Cryptophlebia* spp. coincide in this respect. The genus *Centroxena* was proposed by Diakonoff in 1971 for a single species *C. ulophora* Diakonoff, and has not been studied much since the original description. This genus also belongs to the *Grapholita* genus group and is closely related to *Cryptophlebia* (Komai, unpublished data). E8,E10-12:OAc identified from *Centroxena* sp. is unusual among this genus group.

E9-12:OAc has been determined as a pheromone component from 10 olethreutine species in *Enarmonia* and *Ancylis* in Enarmoniini, *Gravitarmata*, *Rhyacionia*, and *Zeiraphera* in Eucosmini. This study is the first identification of E9-12:OAc from *Eucosma* species in Eucosmini. In this genus, sex pheromones of two species have been reported, i.e., a mixture of Z8-12:OAc and Z8-12:OH from *E. notanthes* (Hung et al., 2001) and Z7-12:OAc and 7,9-diene derivatives from *E. womonana* (Underhill et al., 1987).

Field tests were carried out in the Ryukyu Islands to evaluate the pheromone components identified from the five species living in mangroves. Although the roles of minor components have not been revealed by this evaluation, each lure baited with the main pheromone component successfully attracted the target males. Four species inhabit Iriomote-jima Island, but their main pheromone components are different, suggesting that diversity of sex pheromones is an important factor in their reproductive isolation. Particularly, male attraction of *C. horii* by Z8-12:OH was strongly inhibited by Z8-12:OAc, which attracted the males of *C. palustris* in the same mangrove swamp mixing with the host plants for both species. Larvae of *C. horii* and *E. coniogramma* bore into seedlings of the same host plant, but male moths of the latter species were attracted by a quite different pheromone component, E9-12:OAc. On the other hand, *C. amamiana* produces the same components as *C. palustris*. *C. amamiana* has not been found in Iriomote-jima Island, and the sexual communication of *C. palustris* is not disturbed by *C. amamiana* on this island (Table 1). The mixing ratios of minor components in their pheromones are interestingly different, but details have not been clarified concerning the sexual communication systems of these two sibling species.

Male moths of *Centroxena* sp. were attracted by not only E8,E10-12:OAc but also by its geometrical isomers, whereas the antennae of male moths showed the highest response to the natural diene among its four geometrical isomers. This result was unexpected, because male moths can generally recognize the geometrical isomer specifically produced by their partners, and are selectively attracted to a synthetic pheromone with the same configuration as the natural component. Isomerization of the diunsaturated compound to the geometrical isomers could possibly occur in the rubber septum used for the formulation and/or after vaporization from the septum (Ideses and Shani, 1988). The septa baited with E8,E10-12:OAc, however, specifically attracted the males of the soybean

pod borer, *Leguminivora glycinivorella* (Matsumura) (Olethreutinae: Grapholitini), in another field test using the same synthetic diunsaturated compounds. *L. glycinivorella* females also produce E8,E10-12:OAc, but its geometrical isomers did not attract male moths (Vang et al., unpublished data). Unspecific attraction of the males of *Centroxena* sp. does not seem to be a result of isomerization. Furthermore, in Iriomote-jima Island, the four isomers attracted many males of *R. koeniganus* (Olethreutine: Enarmoniini). Reproductive isolation between *Centroxena* sp. and *R. koeniganus* would be an interesting subject for future studies.

Whereas *B. gymnorrhiza* (host of *C. horii* and *E. coniogramma*) produces viviparous seedlings all year, seasonal differences in the parasitism rate have been recorded (Kinjo et al., 2001). *R. stylosa* (host of *C. palustris*) bears seedlings from April to August, and *K. candel* (host of *C. amamiana*) bears seedlings from October to May. *S. alba* (host of *Centroxena* sp.) has two main fruit seasons a year beginning in May and October, but the fruits have not been observed in March and April. The host preferences of these olethreutinae species are high, and their seasonal prevalence of occurrence might strongly depend on the foods. However, their life cycles, particularly in a season without food, are not understood. Monitoring by pheromone traps will confirm their habitats and population fluctuations and provide more detail for ecological studies.

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(7*Z*,9*E*)-2-METHYL-7,9-OCTADECADIENE: A SEX PHEROMONE COMPONENT OF *Lymantria bantaizana*[$]

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Abstract—Our objective was to identify the sex pheromone of *Lymantria bantaizana* (Lepidoptera: Lymantriidae) whose larvae feed exclusively on walnut, *Juglans* spp., in China, and Japan. Coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses of pheromone gland extracts revealed a single EAD-active component. Retention index calculations of this compound on four GC columns suggested that it was a methyl-branched octadecadiene with conjugated double bonds. In GC-EAD analyses of 2-methyloctadecenes, (*Z*)-2-methyl-7-octadecene and (*E*)-2-methyl-7-octadecene elicited the strongest antennal responses, suggesting that the double bond positions were at C7 and C9. In comparative GC-EAD analyses of pheromone gland extract and stereoselectively synthesized isomers (*E*,*E*; *E*,*Z*; *Z*,*E*; *Z*,*Z*) of 2-methyl-7,9-octadecadiene, the (*E*,*Z*)- and (*Z*,*E*)-isomer had retention times identical to that of the candidate pheromone, but only the latter isomer elicited strong EAD activity. Results of field experiments in Japan substantiated that (7*Z*,9*E*)-2-methyl-7,9-octadecadiene is the *L. bantaizana* sex pheromone, a compound previously unknown in the

[$] While this manuscript was in press, a recent taxonomic revision of the world's *Lymantria* spp. proposed that *Lymantria bantaizana* should be more properly referred to as *Lymantria* (*Spinotria*) *grisescens bantaizana* Matsumura, 1933 (Schintlmeister, A. 2004. Taxonomy of the Genus *Lymantria* Hübner (1819) (Lepidoptera: Lymantriidae). *Quadrifina* 7:1–248).

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Lepidoptera. Detection surveys in North America for exotic Eurasian forest defoliators could include traps baited with the *L. bantaizana* pheromone.

Key Words—*Lymantria bantaizana*, *Lymantria dispar*, *Lymantria monacha*, *Lymantria fumida*, *Lymantria mathura*, *Lymantria xylina*, (7*Z*,9*E*)-2-methyl-7,9-octadecadiene, sex pheromone.

INTRODUCTION

Lymantria bantaizana Matsumura (Lepidoptera: Lymantriidae) is a little-known species found in Japan, Korea, and northern China (Inoue, 1957; Nam and Kim, 1981; Zhao, 1982). Larvae feed on walnut, *Juglans* spp., in China (Zhao, 1982) and Japan (Gotoh et al., 2004). Unlike all congeners that overwinter in the egg stage, *L. bantaizana* overwinter as caterpillars (Yasunori Kishida, Setagaya-ku, Tokyo, personal communication, August 28, 1997; Gotoh et al., 2004). The flight season of adults is from late June to early August in Japan (Inoue, 1957; Gotoh et al., 2004). Males had not been captured in traps baited with any synthetic pheromone known to date for *Lymantria* spp. When a gravid female was light-trapped in Japan and neonate larvae that hatched from her eggs could be reared to adults, the opportunity arose to identify the pheromone of this unusual *Lymantria* species. We report here the main component of this sex pheromone.

METHODS AND MATERIALS

Laboratory Analyses of Pheromone Extract and General Instrumentation. Abdominal tips with the pheromone gland of calling (pheromone-emitting) 1-day-old virgin female *L. bantaizana* were removed and extracted for 5–20 min in high-performance liquid chromatography (HPLC)-grade hexane. Aliquots of pheromone gland extract and synthetic standards were analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD; Arn et al., 1975) and coupled GC-mass spectrometry (MS), employing equipment and procedures previously described in detail (Gries et al., 2002a). Relative EAD activities of 2-methyloctadecenes (produced by Wittig reactions from corresponding aldehydes and ylids) with (*E*)- or (*Z*)-double-bond geometry were determined in replicated ($N = 3$) GC-EAD analyses, using for each antennal preparation four isomers [(*E*3,*E*4,*E*5,*Z*7); (*Z*3,*Z*4,*Z*5,*Z*7); (*E*6,*E*7,*E*8,*Z*7); (*Z*6,*Z*8,*Z*9,*Z*7); or (*E*9,*E*10,*Z*10,*Z*7)] injected individually in split mode at 30-sec intervals and chromatographed isothermally (200°C; DB-5 column J&W Scientific). Nuclear magnetic resonance (NMR) spectroscopy of synthetic compounds was conducted on Bruker 300 (at 300 MHz for ^{1}H and 75 MHz for ^{13}C) and Varian AS500

(at 499.77 MHz for ^{1}H and 125.68 MHz for ^{13}C) spectrometers, with chemical shifts reported in ppm relative to TMS (^{1}H, δ 0.00) and $CDCl_3$ (^{13}C, δ 77.00). High-performance liquid chromatography (HPLC) of synthetic compounds employed a Waters LC 626 chromatograph equipped with a Waters 486 variable wavelength UV/visible detector set to 210 nm, HP Chemstation software (Rev.A.07.01), and a reverse phase Nova-Pak C18 column (60 Å, 4 μm; 3.9 × 300 mm, Waters) eluted with acetonitrile (1 ml/min).

Syntheses

(7Z,9E)-2-Methyl-7,9-octadecadiene (4) (Figure 1, Scheme 1). 'A solution (4.80 ml) of butyllithium in hexane (2.5 M, 12 mmol) was added dropwise to a stirred solution of 5-chloro-1-pentyne (**1**) (Aldrich Chem. Co.; 1.06 ml, 10 mmol) in 50 ml of THF under argon at −78°C. After 30 min, the mixture was warmed to −65°C, and 2.70 g (12 mmol) of anhydrous $ZnBr_2$ was added. After 45 min at −65°C, the mixture was warmed to −45°C, and a freshly prepared

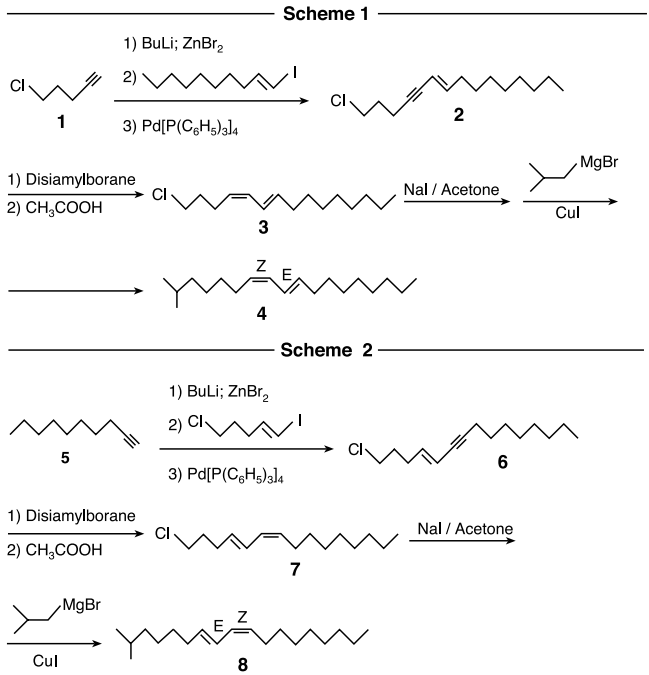

FIG. 1. Syntheses of the (*Z*,*E*)-isomer (Scheme 1) and (*E*,*Z*)-isomer (Scheme 2) of 2-methyl-7,9-octadecadiene.

solution of 6.00 mmol of 1-iodo-(*E*)-1-decene [from 1-decyne, DIBAL and iodine (Gardette et al., 1984)] in 20 ml THF was added, followed immediately by addition in one portion of tetrakis(triphenylphospine)palladium (0.60 g, 0.50 mmol) in 20 ml of THF. After 30 min at −40 to −45°C, the reaction mixture was warmed to and kept at 20°C for 1 hr. The reaction was then quenched with 100 ml of saturated aq. NH_4Cl, extracted with ether (3 × 50 ml), dried overnight with anhydrous Na_2SO_4, and concentrated *in vacuo*. Flash column chromatography (50 g SiO_2, hexane as eluent) afforded 1.42 g (5.28 mmol, 88% yield) of (*E*6)-1-chloropentadeca-6-en-4-yne (**2**). ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, 3H, J = 6.8 Hz); 1.22–1.40 (m, 12H); 1.97 (quint. 2H, H-2, J = 6.7 Hz); 2.07 (q, 2H, H-8, J = 7.3 Hz); 2.48 (dt, 2H, H-3, J = 2.0, 6.7 Hz); 3.65 (t, 2H, H-1, J = 6.7 Hz); 5.43 (td, 1H, H-6, J = 2.0, 15.6 Hz); 6.06 (td, 1H, H-7, J = 7.3, 15.6 Hz). ^{13}C NMR ($CDCl_3$) δ: 14.08, 16.78, 22.65, 28.77, 29.09, 29.22, 29.39, 31.51, 31.85, 32.96, 43.73, 80.18, 86.24, 109.39, 144.09. Anal. calcd. for $C_{15}H_{25}Cl$ (%): C 74.81, H 10.46, found C 74.51, H 10.42.

A solution of **2** (1.32 g, 4.91 mmol) in 50 ml THF was added slowly to freshly prepared (Zweifel and Poiston, 1970) and cooled (−10°C) disiamylborane (6.50 mmol). After stirring for 2.5 hr at 0°C, the mixture was warmed to and kept at room temperature for 5 hr. It was then treated with 6 ml of glacial acetic acid and stirring for 36 hr. Then, 100 ml of water were added, and the reaction mixture was extracted with hexane. Extracts were washed with saturated aq. $NaHCO_3$ and brine, dried with anhydrous $MgSO_4$, and concentrated *in vacuo*. Flash chromatography afforded (*Z*4,*E*6)-1-chloropentadeca-4,6-diene (**3**) (1.22 g, 4.28 mmol, 87% yield, 95% pure by GC). A small sample of **3** was further purified by flash chromatography for spectroscopic analysis. ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, 3H, J = 6.9 Hz); 1.25–1.45 (m, 12H); 1.86 (tt, 2H, H-2, J = 6.6, 6.9 Hz); 2.10 (tdd, 2H, H-8, J = 6.9, 7.5, 1.3 Hz); 2.33 (tdd, 2H, H-3, J = 6.9, 7.5, 1.3 Hz); 3.55 (t, 2H, H-1, J = 6.6 Hz); 5.24 (dt, 1H, H-4, J = 10.8, 7.5); 5.69 (dt, 1H, H-7, J = 15.0, 7.5 Hz); 6.01 (dd, 1H, H-5, J = 10.8, 11.1); 6.30 (dddt, 1H, H-6, J = 11.1, 15.0, 1.3, 1.3 Hz). ^{13}C NMR ($CDCl_3$) δ: 14.09, 22.65, 24.80, 29.22, 29.25, 29.30, 29.44, 31.86, 32.43, 32.85, 44.42, 125.17, 127.24, 130.17, 135.71. Anal. calcd. for $C_{15}H_{27}Cl$ (%): C 74.19, H 11.21, found C 73.85, H 11.02. Without further purification, **3** was dissolved in 50 ml of acetone, and 4.50 g of NaI (30 mmol) were added. After refluxing the mixture for 24 hr, 100 ml of water were added, and the resulting iodide was extracted with hexanes (3 × 50 ml). The combined extracts were washed with 2% aq. $Na_2S_2O_3$ and brine, dried, concentrated, and, at −23°C, added to 50 ml of a THF solution of isobutylmagnesium bromide (13.2 mmol) and CuI (0.25 g, 1.32 mmol). After 20 min, the reaction mixture was allowed to warm to room temperature, quenched with aq. NH_4Cl, extracted with hexane, washed with brine, dried, concentrated *in vacuo*, and filtered through 10 g of silica in hexane. Concentration yielded (7*Z*,9*E*)-2-methyl-7,9-octadecadiene (**4**) (0.81 g, 3.00 mmol, 70% yield based

on **2**, 97% pure based on GC). The (*Z*,*Z*)-isomer contaminant was removed by HPLC, affording >99% geometrically pure **4**. ^{1}H NMR (C_6D_6) δ: 0.93 (d, 6H, J = 6.4 Hz); 0.94 (t, 3H, J = 6.8 Hz); 1.21–1.45 (m, 18H); 1.51 (m, 1H, H-2); 2.14 (td, 2H, H-11, J = 7.1, 6.9 Hz); 2.25 (tdd, 2H, H-6, J = 7.5, 7.5, 1.3 Hz); 5.44 (dt, 1H, H-7, J = 10.8, 7.5 Hz); 5.73 (dt, 1H, H-10, J = 15.0, 7.1 Hz); 6.21 (dd, 1H, H-8, J = 10.8, 10.8 Hz); 6.57 (dddt, 1H, H-9, J = 15.0, 10.8, 1.3, 1.3 Hz). ^{13}C NMR ($CDCl_3$) δ: 14.11, 22.65 (2), 22.68, 27.03, 27.72, 27.95, 29.26, 29.29, 29.42, 29.49, 29.99, 31.89, 32.90. 38.86, 125.59, 128.58, 130.07, 134.68; MS: 41 (42), 55 (25), 67 (100), 81 (95), 95 (66), 109 (36), 123 (23), 137 (8), 208 (5), 264 (13). Anal. calcd. for $C_{19}H_{36}$ (%): C 86.28, H 13.72, found C 86.34, H 13.77.

*(7E,9Z)-2-Methyl-7,9-octadecadiene (**8**).* Compound **8** (Figure 1, Scheme 2) was synthesized analogous to **4**, starting from 1-decyne (**5**), which was coupled in the presence of tetrakis(triphenylphospine)palladium with (*E*)-5-chloro-1-iodo-1-pentene to produce (*E*)-1-chloropentadeca-4-en-6-yne (**6**). Compound **6** was treated with disiamylborane (as above) to yield (4*E*,6*Z*)-1-chloropentadeca-4,6-diene (**7**) which was converted in two steps to (7*Z*,9*E*)-2-methyl-7,9-octadecadiene (**8**) which was purified by HPLC. ^{1}H NMR (C_6D_6) δ: 0.93 (d and t 9H, J = 6.4, 6.8 Hz); 1.20–1.45 (m, 18H); 1.51 (m, 1H, H-2); 2.13 (td, 2H, H-6, J = 7.1, 6.8 Hz); 2.26 (tdd, 2H, H-11, J = 7.5, 7.5, 1.3 Hz); 5.45 (dt, 1H, H-10, J = 10.8, 7.5 Hz); 5.73 (dt, 1H, H-7, J = 15.0, 7.1 Hz); 6.21 (dd, 1H, H-9, J = 10.8, 10.8 Hz); 6.57 (dddt, 1H, H-8, J = 15.0, 10.8, 1.3, 1.3 Hz). MS: 41 (40), 55 (26), 67 (100), 81 (91), 95 (62), 109 (34), 123 (21), 137 (11), 208 (5), 264 (12).

*(7E,9E)-2-Methyl-7,9-Octadecadiene (**12**).* 2,5-Dihydrothiophene-1,1-dioxide (**9**) (Aldrich; 25.0 g, 210 mmol) in 300 ml of dry THF was placed under argon in a three-neck 1-l flask equipped with two dropping funnels. The mixture was cooled to −78°C and stirred vigorously while lithium bis(trimethylsilyl) amide (70 ml of 1 M solution in THF) and 30 ml of THF were added in two portions via one dropping funnel. Via the second dropping funnel, a mixture of 5-methyl-1-iodohexane [5-methyl-1-chlorohexane (available from previous work) was refluxed with sodium iodide in acetone] (16.0 g, 70 mmol), 45 ml of hexamethylphosphoramide (HMPA), and 140 ml of THF was added in 15 min (Yamada et al., 1983). The reaction mixture was then warmed to room temperature, and the reaction was quenched with 100 ml of water and extracted (3 × 100 ml) with ethyl acetate/hexane (1:1). Extracts were washed with water, dried ($MgSO_4$), and concentrated *in vacuo*. Flash column chromatography of the residue with ether/hexane as eluent [gradually increasing (5–30%) the ether content] afforded the pure mono-substituted sulfone (**10**) (6.10 g, 28.2 mmol, 40% yield based on the iodide). The process was repeated with **10** and excess 1-iodooctane (6.32 ml, 35 mmol), 30 mmol of lithium bis(trimethylsilyl)amide, and 26 ml of HMPA, affording after work-up and separation 2,5-disubstituted sulfone (**11**) (3.30 g, 10 mmol, 35.5% yield based on **10**). Sulfone **11** (1 g, 3.0

mmol) was refluxed in 20 ml of 1-propanol with 0.42 g of K_2CO_3 for 2 hr (Yamada et al., 1983) until desulfonylation was complete and (7*E*,9*E*)-2-methyl-7,9-octadecadiene (**12**) had formed. Compound **12** (0.80 g, 89% yield) (Figure 2, Scheme 3) was extracted with hexane from the reaction mixture and purified by flash chromatography. (*E*,*Z*)- and (*Z*,*E*)-isomer contaminants (5%) were removed by HPLC, affording >99% geometrically pure **12**. ^{1}H NMR (C_6D_6) δ: 0.92 (d, 6H, J = 6.4 Hz); 0.94 (t, 3H, J = 6.8 Hz); 1.20–1.48 (m, 18H); 1.51 (m, 1H, H-2); 2.11 (two td, 4H, H-6 and H-11, J = 7.0, 7.0 Hz); 5.66 (m, 2H, H-7 and H-10); 6.20 (m, 2H, H-8 and H-9). MS: 41 (38), 55 (21), 67 (100), 81 (99), 95 (67), 109 (37), 123 (23), 137 (13), 208 (5), 264 (22).

*(7Z,9Z)-2-Methyl-7,9-octadecadiene (**18**)*. To a solution of **5** (3.68 ml, 20 mmol) in 50 ml of THF under argon at −70°C, 9.2 ml (23 mmol) of 2.5 M BuLi in hexane were added, and 3.0 g of paraformaldehyde 30 min later. The mixture was allowed to warm to room temperature; 5 hr later, the reaction was quenched with water. 2-Undecyn-1-ol (**13**) was extracted (2 × 75 ml) with ether hexane (1:1). Extracts were washed with water and brine, dried, and concentrated. Alcohol **13** (93% pure based on GC) was then hydrogenated with P2-nickel catalyst (Brown and Ahuja, 1973) to yield (*Z*)-2-undecen-1-ol (**14**) (3.0 g, 85% yield, 96% pure based on GC). Alcohol **14** (2.15 g, 12.6 mmol) was epoxidized in 25 ml of dichloromethane with *m*-chloroperbenzoic acid (30 mmol, 8.63 g, 60% pure) at 0°C for 5 hr. The reaction mixture was warmed to room tempeature, water (10 ml) was added, and the product was extracted with ether (50 ml). The extract was washed (2N NaOH, water, brine), dried ($MgSO_4$), and concentrated. Flash chromatography of the crude product afforded *cis*-(2,3)-epoxy-1-undecanol (**15**) (2.30 g, 98% pure based on GC). Alcohol **15** (1 g, 5.37 mmol) was added to a stirred mixture (10 min at −60°C) of oxalyl chloride (0.95 ml, 10.8 mmol) and DMSO (1.53 ml, 21.6 mmol; Mancuso et al., 1978). After 20 min, 12 ml of triethylamine were added. After 30 min, the reaction mixture was allowed to warm to room temperature, quenched with water, and extracted with ether. The organic layer was washed (water, brine), dried ($MgSO_4$), and concentrated *in vacuo* to afford the epoxy-aldehyde (**16**). Without further purification, **16** was placed in 50 ml of dry THF and immediately added dropwise to the ylid freshly prepared from 5.02 g (10 mmol) of 6-methylheptyltriphenylphosphonium iodide [synthesized from 6-methyl-1-chloroheptane (available from previous work) by refluxing it with NaI in acetone, and subsequent reflux of the formed iodide with triphenylphosphine in toluene] in 40 ml of THF and 11 ml (11 mmol, 1 M solution in THF) of sodium bis(trimethylsilyl)amide] at −70°C. The reaction mixture was allowed to gradually warm to room temperature, quenched with 100 ml of water–methanol (1:1), extracted with hexane, and purified by flash chromatography (5% ether in hexane) to afford (*Z*)-2-methyl-*cis*-9,10-epoxy-7-octadecene (**17**) [0.89 g, 3.17 mmol, 59% yield based on **15**, with 10% of the (*E*)-isomer]. (7*Z*,9*Z*)-2-methyl-

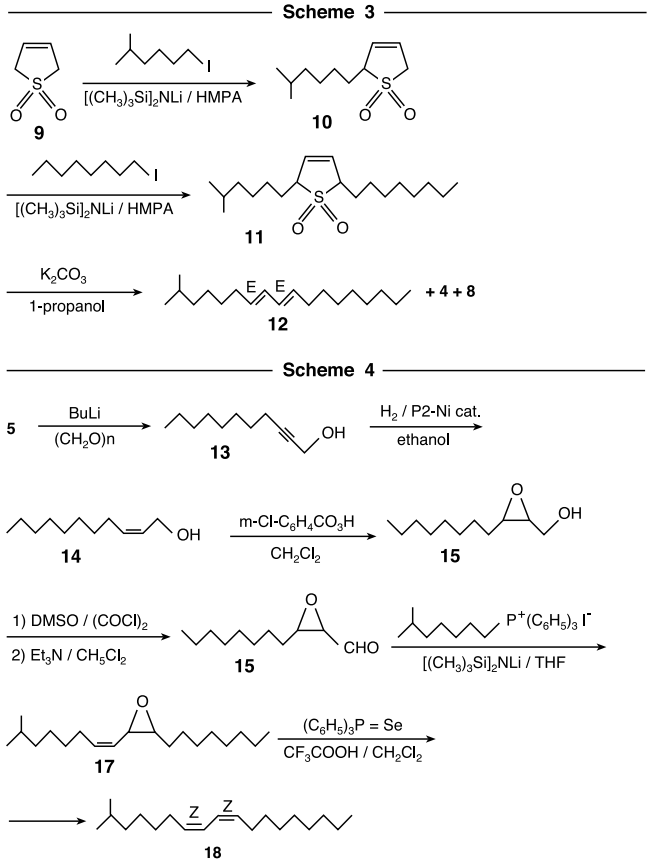

FIG. 2. Syntheses of the (*E*,*E*)-isomer (Scheme 3) and (*Z*,*Z*)-isomer (Scheme 4) of 2-methyl-7,9-octadecadiene.

7,9-octadecadiene (**18**) (Figure 2, Scheme 4) was obtained by treating 0.29 g (1.04 mmol) of **17** with 2.00 mmol of freshly prepared triphenylphosphineselenium in dichloromethane (Clive, 1978) in the presence of trifluoroacetic acid (1 ml) for 20 min. Solvents were removed *in vacuo* from the reaction mixture, and 50 ml of pentane were added. The mixture was filtered through silica (10 g) which was washed with an additional 20 ml of pentane. After concentration, the resulting mixture of **18** (80%, 0.23 g, 83% yield) and some **8** (Scheme 2) was purified by HPLC, affording >99% pure **18**. ^{1}H NMR (C_6D_6) δ: 0.92 (d, 6H, J = 6.4 Hz); 0.92 (t, 3H, J = 6.8 Hz); 1.21–1.45 (m, 18H); 1.50 (m, 1H, H-2); 2.23 (two td, 4H, H-6 and H-11, J = 7.1, 7.1 Hz); 5.56 (m, 2H, H-7 and H-10); 6.50

(m, 2H, H-8 and H-9). MS: 41 (45), 55 (26), 67 (100), 81 (90), 95 (64), 109 (33), 123 (21), 137 (10), 208 (5), 264 (17).

Field Experiments. Candidate pheromone components were field tested in the Iwate University forest (Takizawa Iwate Prefecture; N39°46.5′, E141°9.14′) stocked with mainly oak trees, *Quercus* spp., and some walnut trees, *Juglans* spp., and in the Mizunashi farming valley (N39°53.3′, E141°18.18′) with riparian Siebold walnut trees. At 15- to 20-m intervals, sticky 2-l milk carton traps (Gray et al., 1984) were suspended from trees 1.5 m above ground in complete randomized blocks. Traps were baited with a gray sleeve stopper (The West Co., Lionville, PA, USA) impregnated with test chemicals in HPLC-grade hexane. Experiment 1 tested all four geometrical isomers singly and in quaternary combination, whereas experiment 2 tested the (*E*,*Z*)- and (*Z*,*E*)-isomers of 2-methyl-7,9-octadecadiene singly and combined. Experiment 3 tested the diel periodicity of response by male *L. bantaizana*, by baiting traps ($N = 20$) with synthetic (7*Z*,9*E*)-2-methyl-7,9-octadecadiene (50 μg) and by recording captures of males in 1-hr intervals during the night of July 29, 2001 near Tamayama Village (N39°52.16′, E141°26.16′).

Trap catch data in experiments 1 and 2 were analyzed by nonparametric analyses of variance (Friedman's test) followed by comparison of means by Scheffé test (Zar, 1984; SAS/STAT, 1988). In all analyses, $\alpha = 0.05$.

RESULTS AND DISCUSSION

GC-EAD analyses of pheromone gland extract (Figure 3) on four GC columns revealed a single candidate pheromone component (CPC) that consistently elicited responses from male antennae. CPC occurred at quantities too low to obtain a mass spectrum. Thus, it was identified based on retention time matches and comparative antennal responses of the insect-produced compound and synthetic standards.

Retention indices (RI, relative to alkane standards; van den Dool and Kratz, 1963) of CPC were similar on four nonpolar and polar GC columns (DB-5: RI = 1884; DB-210: RI = 1942; DB-23: RI = 2015; SP-1000: RI = 2002) indicative of a hydrocarbon. Synthetic (*Z*)-2-methyl-7-octadecene (2me-*Z*7-18Hy), a pheromone component of *L. monacha* (Grant et al., 1996; Gries et al., 1996), *L. fumida* (Schaefer et al., 1999), and *L. lucescens* and *L. serva* (Gries et al., 2002b) elicited strong responses from antennae of male *L. bantaizana*, suggesting that it resembled the CPC. The fact that 2me-*Z*7-18Hy elicited the strongest EAD response of all tested 2-methyloctadecenes (Figure 4) substantiated this conclusion. Higher retention indices of CPC than of 2me-*Z*7-18Hy on several GC columns (DB-5: + 42; DB-210: + 82; DB-23: + 138) further

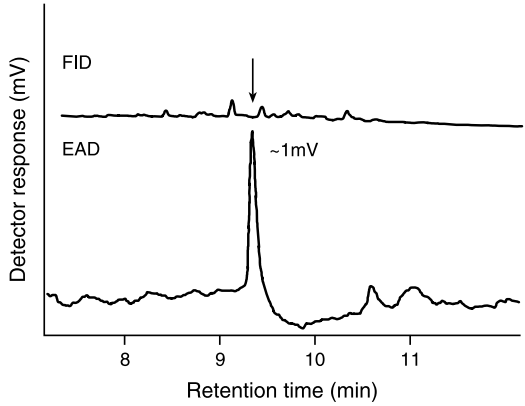

FIG. 3. Flame ionization detector (FID) and electroantennographic detector (EAD: male *L. bantaizana* antenna) responses to one female equivalent (FE) of pheromone extract from female *L. bantaizana*. Chromatography: DB-5 column; splitless injection; injector and FID detector: 240°C; temperature program: 100°C (1 min), then 20°C/min to 190°C (8 min).

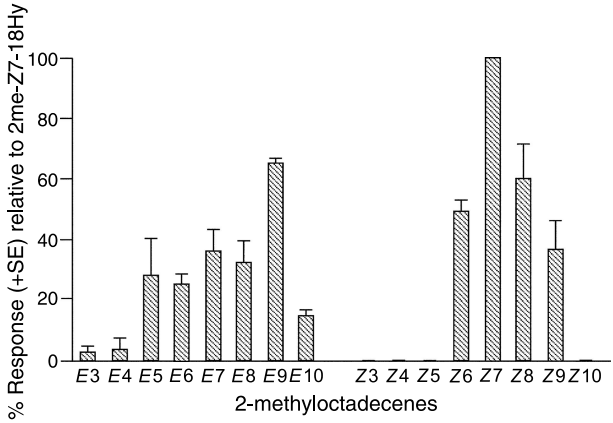

FIG. 4. Relative EAD activity of 2-methyloctadecenes with (*E*)- or (*Z*)-double bond geometry, as determined in replicated ($N = 3$) GC-EAD analyses. For each antennal preparation, four isomers [(*E*3,*E*4,*E*5,*Z*7; (*Z*3,*Z*4,*Z*5,*Z*7); (*E*6,*E*7,*E*8,*Z*7); (*Z*6,*Z*8,*Z*9,*Z*7); or (*E*9,*E*10,*Z*10,*Z*7)] were tested, injected individually in split mode at 30-sec intervals and chromatographed isothermally (200°C; DB-5 column).

suggested that CPC had conjugated double bonds. Taking into account that (*E*)-2-methyl-9-octadecene elicited the second strongest EAD response (Figure 4), we hypothesized that the second double bond was at C9. Strong EAD activity of one geometrical isomer in the 4-isomer (*E*,*E*; *E*,*Z*; *Z*,*E*; *Z*,*Z*) mixture of 2-methyl-7,9-octadecadiene confirmed this hypothesis. In comparative GC-EAD analyses of pheromone gland extract and synthetic 2-methyl-7,9-octadecadienes, the (*E*,*Z*)- and (*Z*,*E*)-isomers had retention times identical with those of the candidate pheromone, but only the latter isomer elicited strong EAD responses.

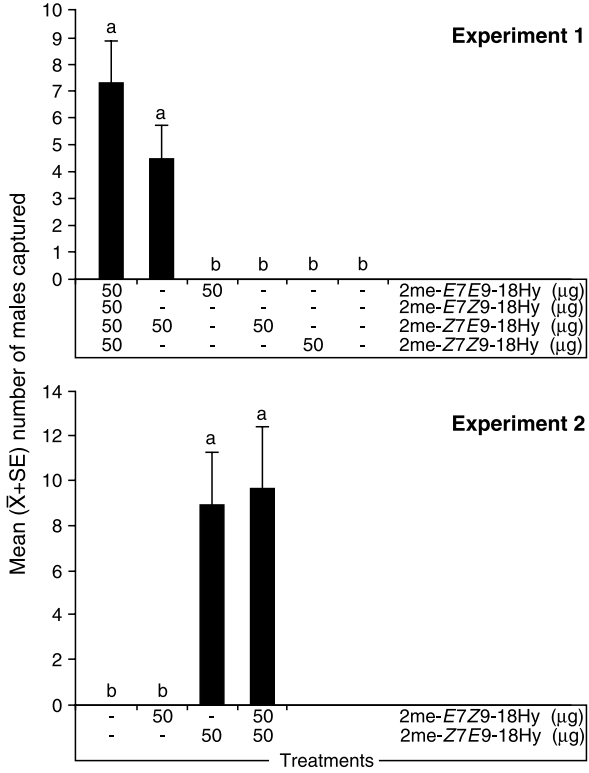

FIG. 5. Mean (+ SE) number of male *L. bantaizana* captured in sticky 2-l milk carton traps baited with geometrical isomers (*E*,*E*; *E*,*Z*; *Z*,*E*; or *Z*,*Z*) of 2-methyl-7,9-octadecadiene. Experiment 1: July 2–August 11, 2003, $N = 6$; Experiment 2: July 5–August 19, 2002, $N = 10$. Bars in each experiment with different letter superscripts are significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means (Scheffé test), $P < 0.05$ (SAS/STAT, 1988).

In field experiments 1 and 2 (Figure 5), only the (*Z*,*E*)-isomer attracted males, whereas other isomers when added to the (*Z*,*E*)-isomer did not inhibit the males' responses. In experiment 3, ten males were captured between 24:00 and 01:00 hr and three males between 01:00 and 02:00 hr. This information about the diel periodicity of sexual communication in *L. bantaizana* will contribute to our ongoing studies as to how communication signals and time of signaling impart specificity to communication channels of five congeners (*Lymantria dispar*, *L. fumida*, *L. lucescens*, *L. mathura*, *L. monacha*) in the complex *Lymantria* communities of Japan.

Following the pioneering discovery of (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane [(+)-disparlure] as the pheromone of gypsy moth, *L. dispar* (Bierl et al., 1970; Klimetzek et al., 1976; Cardé et al., 1977; Plimmer et al., 1977), there is mounting evidence that sex pheromones of *Lymantria* spp. are more diverse than previously realized. (7*R*,8*S*)-*cis*-7,8-Epoxyoctadecane serves as a synergistic pheromone component of nun moth, *L. monacha*, in central Europe (Gries et al., 1996), and as the major pheromone component of *L. monacha* in Japan (Gries et al., 2001). (3*Z*,6*Z*,9*R*,10*S*)-*cis*-9,10-Epoxynonadecadiene and (3*Z*,6*Z*,9*S*,10*R*)-*cis*-9,10-epoxynonadecadiene at a 1:4 ratio are sex pheromone components of pink gypsy moth, *L. mathura*, in Japan (Gries et al., 1999a), and (7*R*,8*S*)-*cis*-7,8-epoxyeicosane is the pheromone of Casuarina moth, *Lymantria xylina*, in Taiwan (Gries et al., 1999b). (*Z*)-2-methyl-7-octadecene is a synergistic pheromone component of both *L. monacha* in Central Europe (Grant et al., 1996; Gries et al., 1996) and Japan (Gries et al., 2001) and of *L. fumida* in Japan (Schaefer et al., 1999). The same compound constitutes the pheromone of *L. lucescens* in Japan and *L. serva* in Taiwan (Gries et al., 2002b). Finally, here we show that a methylated diene hydrocarbon is part of the diverse sex pheromones in *Lymantria* spp.

Considering that *L. bantaizana* larvae defoliate walnut trees (an important nut crop in North America) and that *L. bantaizana* is as likely as Asian *L. mathura*, *L. dispar*, and *L. monacha* to be introduced into North America, traps baited with synthetic *L. bantaizana* pheromone could be included in detection surveys for exotic *Lymantria* spp. Commercial trap lures containing a 1:1 mixture of (*E*,*Z*)- and (*Z*,*E*)-isomers will be cheap because such a mixture is readily obtainable from sulfone **11** by desulfonylation at 110°C in a nonpolar solvent rather than the polar solvent shown in Scheme 3 (Yamada et al., 1983).

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CHARACTERIZATION OF A BEHAVIORALLY ACTIVE, GENDER-SPECIFIC VOLATILE COMPOUND FROM THE MALE ASPARAGUS FLY *Plioreocepta poeciloptera*

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Abstract—Adult male asparagus flies exhibit typical calling behaviors (suggestive of pheromone production) during which they emit a single volatile compound that was identified as isopropyl (*S*)-5-hydroxyhexanoate. In laboratory bioassays, synthetic samples elicited an arrestant response in females, but did not appear to attract females. On the other hand, the synthetic material attracted conspecific males in olfactometer bioassays.

Key Words—*Plioreocepta poeciloptera*, asparagus fly, male-produced volatile, isopropyl (*S*)-5-hydroxyhexanoate.

INTRODUCTION

The asparagus fly, *Plioreocepta (Platyparea) poeciloptera*, is a temperate univoltine monophagous tephritid, the maggots of which are serious pests of asparagus crops in Europe (Lesne, 1913). Adults appear in France in late March to early April (Reulet, 1991). Courtship and mating behaviors of this fly, as observed in both laboratory and field situations (unpublished observations), are similar to those of *Chaestostomella undosa*, wherein males patrol, or remain on the top of the host plant (Steck, 1984). In both of these species, encounters between males lead to agonistic behavior, and encounters between males and

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females occurred without the necessity of lek formation, the females approaching the males perched on their host plant. In the majority of tephritid species (Bateman, 1972; Sivinski and Burk, 1989), the male emits a sex pheromone that attracts females. We earlier described a calling behavior exhibited by adult male *P. poeciloptera*, and described attraction of virgin females to the "calling" males as well as to dissected lateral abdominal pouches, the likely source of pheromone (Seguy, 1951; Thibout and Auger, 1999). We also described experiments with solid-phase microextraction (SPME) fibers, wherein a single volatile compound was collected from calling males and tentatively identified on the basis of its mass and infrared spectra. The structural assignment (Auger et al., 1998) of the presumed pheromone, as demonstrated by synthesis of the proposed structure (Casaña-Giner et al., 2001), turned out to be incorrect, however.

This paper reports our continuing studies of the asparagus fly, including positive identification of the major male-produced volatile. We have determined the optimum age and circadian rhythm of male calling, individually collected male- and female-produced volatiles, and again observed the presence of a major volatile produced exclusively by calling males. Additional gas chromatography-mass spectrometry studies (GC/MS) contributed to a revised structure for this compound, which has now been established as isopropyl (*S*)-5-hydroxyhexanoate. Laboratory behavioral assays and chiral gas chromatography support the assigned structure and stereochemistry of this sex-specific compound.

METHODS AND MATERIALS

Insects. The establishment of laboratory colonies of *P. poeciloptera* (Diptera: Tephritidae) has not been successful (Thibout and Auger, 1999), and test insects were obtained by collecting pupae from asparagus fields in early October. Pupae were collected at the base of spears in the Loire Valley, near Chinon, France, and were maintained in the laboratory at 8 ± 2°C, in a humid atmosphere, until the end of March, after which they were gradually introduced into Petri dishes at a rate of 10–15 pupae per day, in synchronous photoperiod and thermoperiod: 16 hr at 26°C in photophase and eight hr at 17°C in scotophase. About 12 d later, adults (which were isolated by gender) emerged, and were individually maintained in Petri dishes on aqueous sucrose solutions.

Male Specific Volatiles Collection. Collection of volatiles was timed to coincide with the male calling period, determined by hourly observations on 80 isolated males during the 16 hr photophase from day 1 (emergence day) to day 8. The characteristic male calling position (lifted abdomen, protruded abdominal pouches, spread wings) has been previously described (Thibout and

Auger, 1999). SPME-GC was used to trap, transfer, and analyze the collected components of the volatiles. To collect volatiles, a male was placed in a 10-ml glass vial stoppered by a plastic cap with a hole allowing insertion of the SPME fiber. The fiber was positioned a few mm from the abdominal extremity of the immobile male for 1 min. Analysis of the adsorbed sample was carried out within 5 min of sampling by desorption for 2 min in the injection port of the GC. One-, 2-, 4-, and 8-d-old males were used regardless of whether they displayed calling behavior or not. Two- to 4-d-old females were used as controls. Up to seven repetitions were carried out for adults of each sex and age.

Isolation of Male-Specific Volatiles. Volatiles of 4-d-old males and females were trapped on Tenax cartridges (SKC Inc., Eighty-Four, PA). Each adult was introduced into a 20-ml glass vial for aeration. Incoming air (10 ml/min, a rate that did not disturb the flies) was precleaned by passing successively through two Tenax and two activated charcoal cartridges. Exiting air passed through the Tenax cartridge trap (50 mg Tenax). In the morning, a control and a male were aerated for 4 hr. In the afternoon, a female and another male were similarly aerated for 4 hr. This was repeated on 10 consecutive days to obtain four experimental Tenax cartridges, each containing the volatiles from 40 hr of trapping. The entire procedure was repeated a second time with another batch of four cartridges. The two batches of four cartridges were then eluted with pentane at 4°C. Only the two first drops eluting from the cartridge were collected and stored at −20°C until being analyzed by GC/MS.

We first compared the volatiles from females and from a control (vial containing no insects) to those from the two types of male samples (morning and afternoon collections).

Identification of the Male Volatile. ^{1}H NMR spectra were recorded in $CDCl_3$ or C_6D_6 on a Bruker QE-300 spectrometer. In the U.S. laboratory, GC was performed with a Shimadzu Model GC-17A instrument equipped with a 30 m × 0.25 mm SPB-35 capillary column (Supelco, Bellefonte PA) and a flame ionization detector, or on an Agilent 6890N instrument equipped with a 30 m × 0.25 mm HP-5 column (Agilent Avondale PA). Chiral gas chromatography was performed with a 30-m Chiraldex B-DM column (Advanced Separation Technologies, Whippany, NJ). In the French laboratory, solid-phase microextraction-GC (SPME-GC) experiments were conducted with a Varian 3300 instrument with a FID detector equipped with a split–splitless injector, an 80-μl liner for SPME fiber desorption, and a fused-silica HP-1 capillary column (Agilent, 20 m × 0.22 mm I.D., 0.33 μm film thickness). The conditions were as follows: injection temperature 200°C, carrier gas helium, column flow 1.0 ml/min, splitless mode. The column temperature program was 2°C/min from 40 to 200°C.

In the French laboratory, mass spectral experiments were conducted with a benchtop Perkin-Elmer Turbomass system with a split–splitless injector and a 25-m fused-silica HP-1 capillary column similar to that described above. The carrier

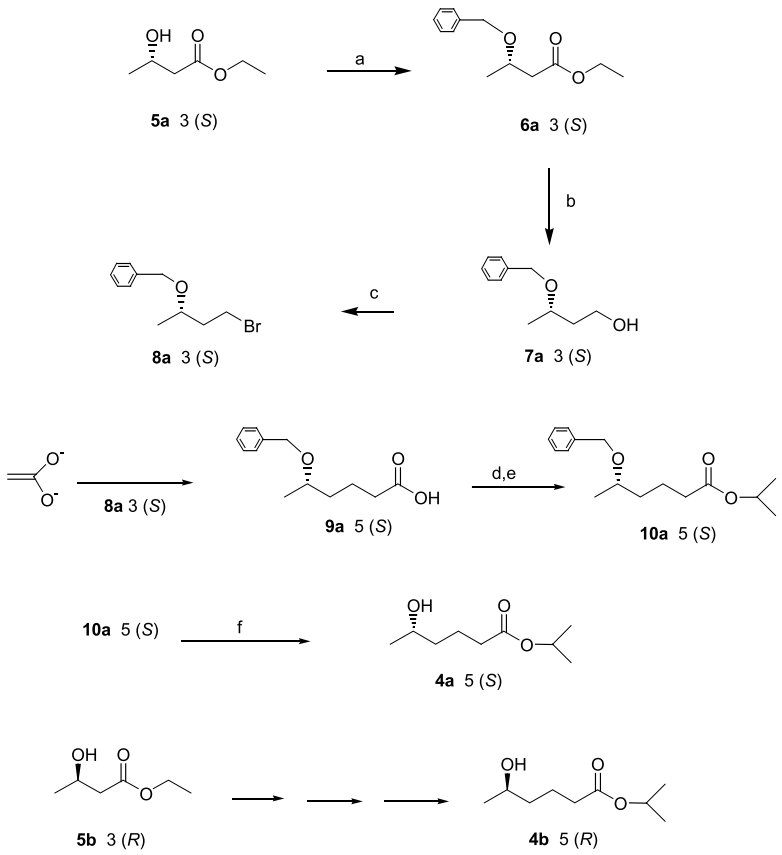
gas was helium at 2 ml/min, and the column temperature program was 5°C/min from 60 to 180°C then 15°C/min from 180 to 280°C. Total ion chromatograms (TICs) and mass spectra were recorded in the electron impact ionization mode at 70 eV. In the U.S. laboratory, mass spectra were obtained from a Shimadzu GCMS-QP 5050A equipped with a 30 m × 0.25 mm DB-5 column (J&W Scientific, Fulsom CA). Chemical ionization spectra were obtained using ammonia as reagent gas. Additional mass spectrometric experiments on the trapped male compound added little to those already published (Auger et al., 1998). Rotations were measured with a Perkin Elmer Model 241 polarimeter.

Syntheses. Described below are a series of reactions leading to the (*S*)-enantiomer of the major volatile, **4a** (Figure 1) An identical series, beginning with the benzyl ether of ethyl (*R*)-3-hydroxybutyrate (Malher et al., 1988), was conducted to prepare the (*R*)-enantiomer **4b**.

*(S)-3-(Benzyloxy)butyric acid ethyl ester **6a*** (Keck and Murry, 1991). To a solution of ethyl (*S*)-3-hydroxybutanoate **5a** (0.9 g, 6.8 mmol) and *O*-benzyltrichloroacetimidate (2.4 g, 7.5 mmol) in 2:1 cyclohexane/dichloromethane (36 ml) was added trifluoromethanesulfonic acid (80 μl). The mixture was stirred at room temperature for 24 hr, then filtered. The filtrate was added to saturated aqueous $NaHCO_3$, and the mixture was extracted with dichloromethane (3 × 8 ml). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. Flash chromatography (9:1 hexanes/EtOAc) afforded **6a** (1.37 g, 91%) as a light yellow liquid.

*(S)-3-(Benzyloxy)butan-1-ol **7a***. Dry ether (11 ml) was cooled with an ice bath, and $LiAlH_4$ (0.15 g, 3.9 mmol) was added. The mixture was warmed to room temperature and stirred for 30 min, then was again cooled with an ice bath, and a solution of **6a** (1.15 g, 5.18 mmol) in dry ether (3 ml) was added dropwise. After stirring for 1 hr, water (0.15 ml), 15% NaOH (0.15 ml), and water (0.45 ml) were added dropwise in that order, and the mixture was stirred at 20°C for 15 min, then was filtered through Celite. The filtrate was washed with 1:1 brine/sat. $NaHCO_3$, dried over $MgSO_4$, and concentrated *in vacuo* to yield **7a** (0.75 g, 84%) as a light yellow liquid. EI-MS, *m/z* (%): 161 (10), 108 (10), 107 (52), 92 (18), 91 (100), 79 (26), 77 (20), 65 (32), 56 (12), 51 (14), 43 (19), 42 (13).

*(S)-3-(Benzyloxy)-1-bromobutane **8a***. Bromine (260 μl, 5 mmol) in dichloromethane (2 ml) was added dropwise to a solution of triphenylphosphine (1.3 g, 5 mmol) in dichloromethane (5 ml) at about −10°C. The solution was warmed to room temperature and stirred 20 min. After cooling to −30°C, a solution of **7a** (0.75 g, 4.2 mmol) in dichloromethane (2 ml) was added dropwise, then the mixture was stirred at room temperature for 16 hr. After concentration *in vacuo* to near dryness, 10 ml pentane was added. The mixture was filtered and the solids were rinsed with several portions of pentane. The combined filtrates were washed with saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated *in*



a. $PhCH_2C(N{=}H)CCl_3$, b. $LiAlH_4$, c. Ph_3PBr_2, d. ClCOCOCl, e. i-PrOH, f. H_2/Pd

FIG. 1. Syntheses of **4a** and **4b** from enantiomers of ethyl 3-hydroxybutyrate.

vacuo. Flash chromatography (9:1 hexanes/EtOAc) afforded **8a** (0.68 g, 67%) as a light yellow liquid. EI-MS, *m/z* (%), 135 (31), 107 (15), 91 (100), 89 (13), 79 (28), 77 (25), 65 (30), 63 (13), 55 (23), 51 (20), 43 (18), 41 (15).

(S)-5-(Benzyloxy)hexanoic Acid ***9a***. Lithium diisopropylamide (LDA) was prepared from diisopropylamine (1.18 ml, 8.46 mmol) and 2.5 M *n*-butyllithium (3.38 ml, 8.46 mmol) in THF (5 ml) at −10°C. The newly prepared LDA was added by syringe to a solution of acetic acid (240 μl, 4.23 mmol) and NaI (2 mg) in THF (5 ml) at −20°C. Hexamethyl phosphoramide (HMPA) (1.47 ml, 8.46 mmol) was added, and after the mixture was stirred at 0°C for 30 min,

it was cooled to −20°C, and a solution of **8a** (0.685 g, 2.82 mmol) in THF (1 ml) was added. The reaction mixture was allowed to warm slowly to room temp and stirred 16 hr, after which time it was added to ice and extracted with ether (2 × 10 ml). The aqueous phase was acidified with 1 N HCl and extracted with 1:1 ether/hexane (3 × 10 ml). The ether/hexane extracts were washed with water and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude liquid was filtered through a plug of silica gel to afford **9a** (0.56 g, 89%) as a yellow liquid. EI-MS (of the methyl ester, formed by diazomethane treatment): 135 (19), 91 (100), 79 (19), 77 (16), 65 (28), 55 (16), 51 (13), 43 (12).

*(S)-5-(Benzyloxy)hexanoic acid isopropyl ester **10a**.* Oxalyl chloride (270 μl, 2.8 mmol) and DMF (2 μl) were added to a solution of **9a** (0.57 g, 2.55 mmol) in benzene. After stirring 1 hr, the reaction mixture was concentrated *in vacuo*, redissolved in benzene, and added to a solution of pyridine (270 μl, 3.2 mmol) and isopropanol (490 μl, 6.4 mmol) in benzene (2 ml). After stirring overnight, the mixture was diluted with ether (15 ml) and extracted with 1 N HCl (10 ml). The organic phase was washed with water, then with saturated aqueous $NaHCO_3$, and finally with brine, dried over $MgSO_4$, and concentrated *in vacuo*. Flash chromatography (20:1 hexanes/EtOAc) afforded **10a** (0.345 g, 51%) as a light yellow liquid. ^{1}H NMR (300 MHz, $CDCl_3$), δ 7.36–7.20 (m, 5H), 5.00 (sept, J = 6.43 Hz, 1H), 4.50 (m, 2H), 3.52 (sex, J = 6.05 Hz, 1H), 2.26 (t, J = 6.81 Hz, 2H), 1.78–1.38 (m, 4H), 1.21 (m, 9H). EI-MS: 158 (39), 117 (11), 116 (49), 115 (20), 113 (20), 107 (29), 97 (16), 91 (100), 87 (24), 79 (15), 77 (13), 73 (26), 69 (21), 65 (32), 55 (21), 43 (39), 42 (20), 41 (35).

*(S)-5-Hydroxyhexanoic acid isopropyl ester **4a**.* To a solution of **10a** (0.345 g, 1.3 mmol) in ethyl acetate (10 ml) was added 10% Pd/C (34 mg). The solution was stirred under H_2 for 16 hr, then filtered through Celite and concentrated to provide **4a** (218 mg, 96%) as a colorless liquid. ^{1}H NMR (300 MHz, $CDCl_3$), 4.99 (sept, J = 6.43 Hz, 1H), 3.79 (sex, J = 6.06 Hz, 1H), 2.29 (t, J = 7 Hz, 2H), 1.68 (m, 2H), 1.57 (br s, 1H), 1.46 (m, 2H), 1.21 (t, J = 6.06 Hz, 3H). EI-MS: 115 (23), 114 (13), 99 (20), 97 (23), 89 (12), 88 (27), 73 (18), 71 (19), 70 (20), 69 (39), 68 (10), 60 (75), 55 (36), 45 (75), 44 (12), 43 (100), 42 (62), 41 (65). Under CI-MS conditions, the compound degraded to form 5-caprolactone. CI-MS m/z (%): 132 (54) ($M + NH_4^+$), 115 (100) ($M + H^+$). $[\alpha]_D^{25} = +7$ (c = 2.9, $CHCl_3$). The trimethylsilyl ether of this compound gave only a single peak on a chiral column, for an estimated enantiomeric excess (ee) of >99%.

*Alternate synthesis (Figure 2). (S)-5-Chloro-2-pentanol tetrahydropyranyl ether **12**.* A solution of (*S*)-5-chloro-2-pentanol (Daicel, Inc.; 9.47 g, 77 mmol) in dry dichloromethane (40 ml) and freshly distilled dihydropyran (8 ml, ca. 88 mmol) was treated with pyridinium *p*-toluenesulfonate (PPTS, 210 mg, 0.9 mmol). An exotherm was observed. The solution was allowed to stand undisturbed for 45 min, then most of the solvent was removed with a rotary evaporator and the residue was partitioned between cold aqueous sodium

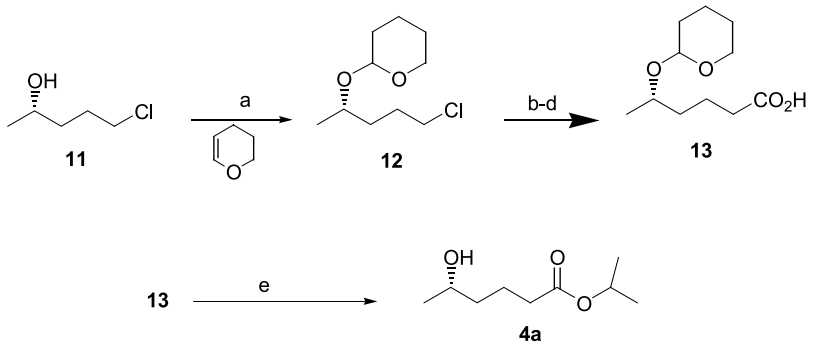


FIG. 2. Alternate synthesis of **4a** from (*S*)-5-chloro-2-pentanol.

carbonate (1 M) and 1:1 ether/hexane. The crude product (17 g) was distilled to give 12.3 g (77%) of **12**, b.p. 56°C, 0.03 Torr. ^{1}H NMR (300 MHz, $CDCl_3$, two diastereomers) 4.67 (m, 1H), 4.61 (m, 1H), 3.83 (m, 2H), 3.54 (m, 4H), 1.99–1.41 (m, ~24H), 1.22 (t, J = 6.43 Hz, 3H), 1.12 (d, J = 6.06 Hz, 3H). This compound has been reported previously (Keinan et al., 1986).

*(S)-5-Hydroxyhexanoic acid tetrahydropyranyl ether **13***. Magnesium (1.56 g, 65 mmol) was covered with dry THF in an argon atmosphere. After activating the metal with a few microliters of 1,2-dibromoethane, THF was heated to reflux and a solution of **12** (6.01 g, 29 mmol) in THF (20 ml) was added dropwise over 0.5 hr. Refluxing was continued an additional 4.5 hr, then the Grignard solution was cooled to room temperature and transferred by cannula onto an excess of powdered dry ice in an argon-flushed flask. After standing about 45 min, ether and water were added, the mixture was acidified with aqueous $KHSO_4$, and the organic products were partitioned into ether. The ether solution was rinsed with water, then extracted with 1 M aqueous sodium carbonate (25 ml, then 2 × 5 ml). The combined extracts were rinsed with ether, then acidified with aqueous $KHSO_4$ and the acidic product was extracted into ether. After rinsing with water and brine, the solution was dried ($MgSO_4$), and concentrated to provide 5.25 g (84%) of **13** as a colorless liquid. ^{1}H NMR (300 MHz, benzene-d_6, mixture of diastereomers) 4.64 (m, 2H), 3.82 (m, 2H), 3.64 (m, 1H), 3.39 (m, 1H), 3.34 (s, 3H), 3.33 (s, 3H), 2.18 (m, 2H), 2.08 (m, 2H), 1.86–1.25 (m, ~22H), 1.22 (d, J = 6.43 Hz, 3H), 0.97 (d, J = 6.05 Hz, 3H). EI-MS: 115 (83), 101 (13), 97 (54), 85 (100), 84 (10), 73 (27), 70 (13), 69 (69), 67 (27), 60 (21), 57 (39), 56 (48), 55 (74), 45 (41), 44 (85), 43 (80), 42 (42), 41 (82).

*Conversion of **13** to **4a***. A 100-ml flask containing a solution of **13** (5.25 g, 24.3 mmol) in 2-propanol (50 ml) plus pyridinium *p*-toluenesulfonate (0.61 g,

2.4 mmol) was fitted with a reflux condenser and introduced into a sonic bath that was operated overnight at room temperature (moderate warming of the water bath occurred during the procedure). The solvent was removed *in vacuo*, and the residue was flash chromatographed on 150 g silica gel, eluting with 10–50% ethyl acetate in hexane. The earlier-eluting fractions provided 1.6 g (38%) of **4a** of 98% purity (GC), identical to **4a** described above. Slightly later-eluting material consisted of mixtures of a little more **4a** and lactone **1** plus some later-eluting (GC) material that appeared to be a dimeric product resulting from intermolecular transesterification of **4a** (or **4a** + **1**) (1.4 g). From chiral GC, the ee of **4a** was estimated to be >98%.

Adult Fly Behavioral Responses to Synthetic Compounds. Behaviors of 4-d-old males and females were observed in an olfactometer similar to that previously described (Thibout and Auger, 1999). Air was passed at 20 cm/sec through a 90 × 9 cm glass cylinder opening into control and test channels. The center of the test channel contained a 4-cm^2 filter paper square treated with 50 μg of a candidate compound dissolved in 5 μl of paraffin oil. One adult was released at the downwind opening of the tube, and its movements and displacements were observed for 2 min. The fly distributions at the end of the observation period were recorded for both sexes, using three classifications: (1) adults moving less than 5 cm; (2) adults moving between 5 and 25 cm; and (3) adults moving more than 25 cm. A second behavior was evaluated in females, that is, the number of flies remaining immobile with spread wings, a posture typically observed when a female is in the proximity of a calling male.

Thirty repetitions were performed, with each fly being used only once. Two similar experiments were carried out, one in 2002 and the other in 2003. In the latter, the *S*-enantiomer **4a** was also tested at a higher concentration (200 μg in paraffin oil). All behavioral data were analyzed, when necessary, with a χ^2 test at $\alpha = 0.05$.

RESULTS

Influence of Male Age on Calling. The study of the number of males that were calling according to the time of day, and of the ages of calling males, showed that 1-d-old males were sometimes able to call, but usually did not (Figure 3). The percentage of 2-d-old males calling was similar to that of older ones (65%). There was a shift in time of calling with age, with young males calling in the middle of the day (mean 8.22 hr in 2-d-old males), earlier than more mature males that called in the early afternoon (9.76 hr in 8-d-old males; $P < 0.001$ using a χ^2 test). All the male distributions were significantly different except between 6 and 8 d.

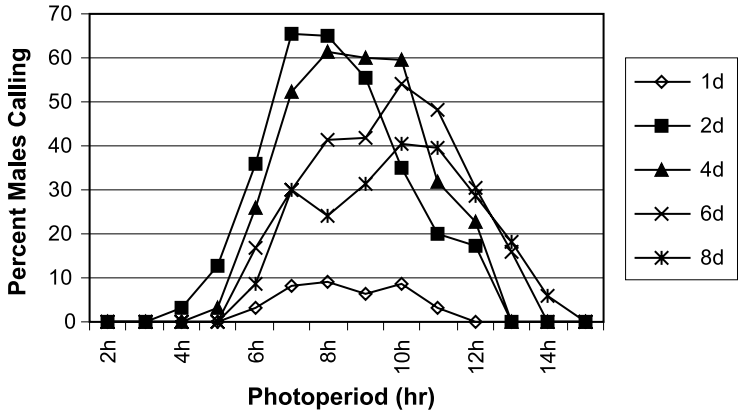


FIG. 3. Calling rhythm of *Plioreocepta poeciloptera* males at various ages. Distributions are statistically different except between 6 and 8 days (χ^2 test).

Identification of the Major Male Specific Volatile. Results obtained by SMPE/GC from individual flies showed that a major GC peak appeared at 13 min only in samples from calling males of 2-d-old or older (Figure 4). This peak was absent from volatiles from females, absent, or nearly so, from samples obtained from 1-d-old males, and absent from volatiles from noncalling males. The GC/MS results of the Tenax trapping of volatiles from males and females paralleled those of the SPME experiments, but reproducibly indicated additional components, particularly one with retention time 7.75 min, present in all extracts including the control (Figure 5). The mass spectra of these components indicated them to be sulfur-containing plant volatiles originating from the asparagus plants, the major being identified as dipropyl disulfide. As had been the case in the SPME/GC study, only one male-specific compound, corresponding to the 13-min eluting peak just discussed, was observed from males aerated in the afternoon, the time at which they were seen calling. In the control, in the female-derived samples, and in the samples from males aerated in the morning (when few or none were calling), no peak with this retention time was observed (Figure 5).

The electron impact ionization mass spectrum of the male-produced unknown was an excellent match for that of δ-caprolactone **1**. A chemical ionization spectrum obtained with methane as reagent gas produced an ion with *m*/*z* 115, consistent with a protonated molecular ion, $M + H^+$, of **1** (MW 114). However, the GC retention time of the unknown was longer than that of **1**, and furthermore, lactone **1** is not consistent with the published infrared spectrum (Auger et al., 1998) that exhibited O–H stretching as well as a carbonyl absorption at 1729 cm^{-1}, more consistent with an aliphatic hydroxy-ester than

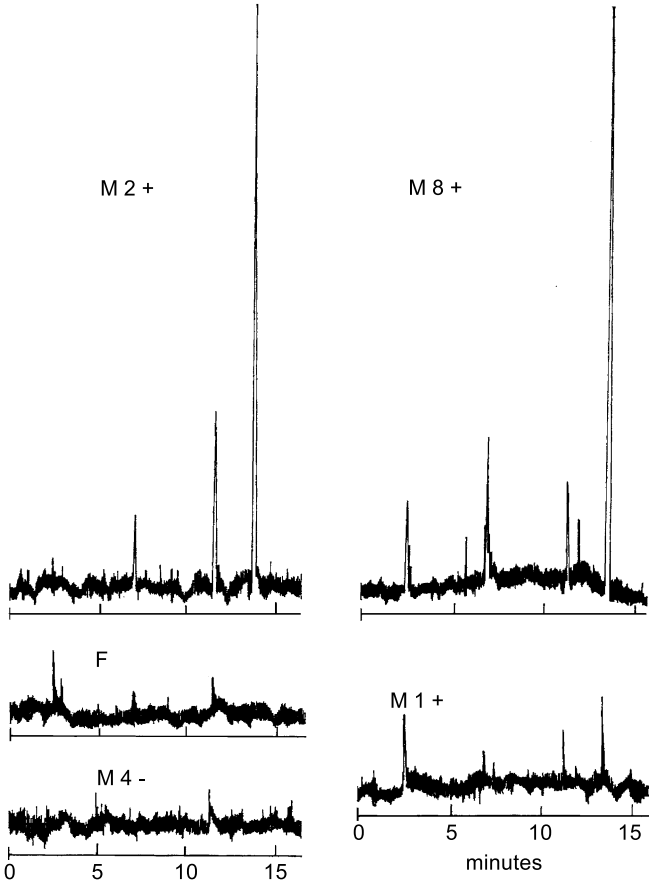


FIG. 4. GC analysis of *Plioreocepta poeciloptera* adult volatiles trapped by SPME at various ages. M1+: 1-d-old calling male; M2+: 2-d-old calling male; M4−: 4-d-old noncalling male; M8+: 8-d-old calling male; F: 4-d-old female.

with a lactone. We postulated that the volatile was possibly a somewhat labile compound that could decompose under MS ionization conditions to provide lactone **1**, but at the same time be stable enough to survive gas chromatography (to produce a retention time longer than that of **1** and a GC–FTIR inconsistent with **1**). We anticipated (incorrectly) that the less energetic CI reagent gas ammonia might improve our chances of determining molecular weight via CI-MS, but the NH_3-CI spectrum consisted cleanly and almost exclusively of ions with *m*/*z* 115 and 132 ($M + H^+$ and $M + NH_4^+$ for **1**, respectively).

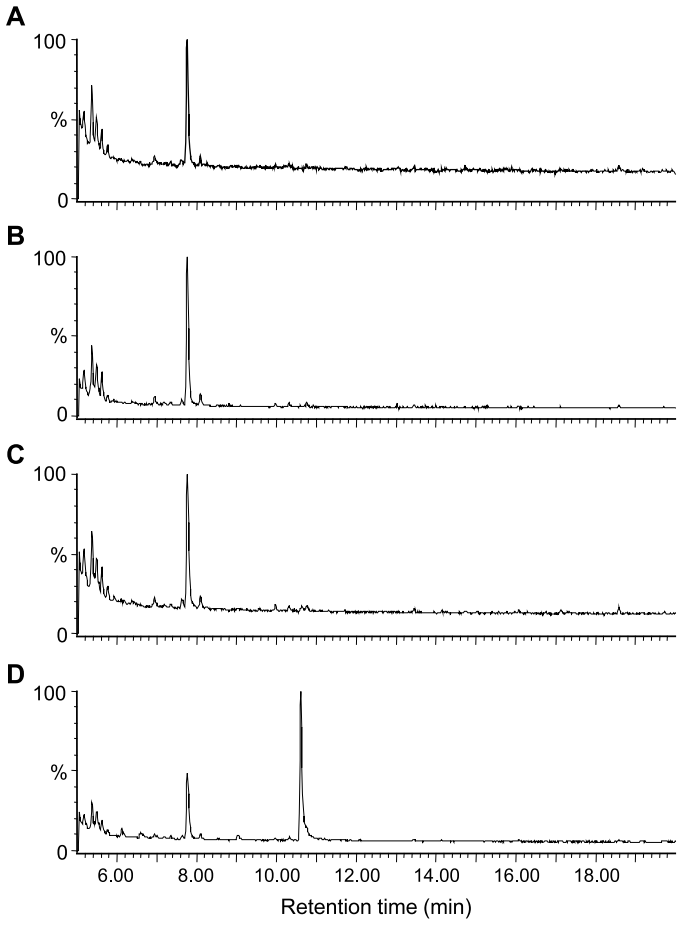


FIG. 5. GC/MS analysis of volatiles from 4-d-old *Plioreocepta poeciloptera* trapped on Tenax cartridges and eluted with pentane. **A** Control without insect; **B** Females, morning collection; **C** Males, morning collection; **D** Males, afternoon collection.

The most conservative explanation seemed to be an ester of 5-hydroxyhexanoic acid that could lose the alcohol component via intramolecular transesterification to form lactone **1**. Accordingly, we prepared ethyl ester **2** (Figure 4) by treatment of **1** with ethanol containing sodium ethoxide (Hernandez et al., 1996). The product **2**, a known compound (Lease and McElvain, 1933), eluted from the GC later than **1**, but still slightly earlier than the unknown; reassuringly, the mass spectra of **2** were also essentially identical to those of **1**. We next converted samples of **1** to the propyl and isopropyl esters **3** and **4**, respectively

(apparently unreported in the literature), and indeed, isopropyl ester **4** proved to be a perfect match for the insect-produced compound.

Racemic **1** was easily prepared by Baeyer–Villiger oxidation of 2-methylcyclopentanone (Hernandez et al., 1996), and opening of the resulting lactone with sodium isopropoxide accordingly produced racemic **4** (Figure 6). By comparing racemate **4** with the insect-produced material by GC on a chiral column (analyses were conveniently conducted on the respective trimethylsilyl ethers, TMS), we established that the latter was a single enantiomer. To assign the absolute configuration of C-5 of the insect-produced compound, we synthesized the enantiomers from precursors in which the stereochemistry was already defined.

*Syntheses of the Isopropyl Ester of (S)-5-Hydroxyhexanoic Acid (**4a**).* Both enantiomers of ethyl 3-hydroxybutanoate are commercially available. The 3-(*S*)-enantiomer **5a** was protected as its benzyl ether **6a** (Keck and Murry, 1991), and **6a** was reduced with $LiAlH_4$ to provide the mono-protected diol **7a** (Figure 1). Treatment with Ph_3PBr_2 converted **7a** to bromide **8a**. Alkylation of the dianion of acetic acid (Pfeffer and Silbert, 1970) with **8a** gave (*S*)-5-hydroxyhexanoic acid benzyl ether **9a**, which was converted to the acid chloride and esterified with isopropanol to afford ester **10a**. Catalytic hydrogenation

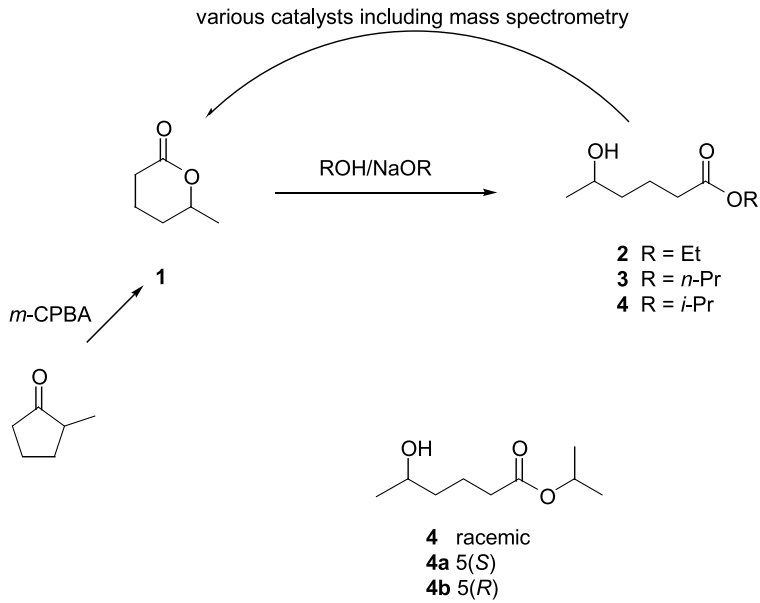


FIG. 6. 5-Hydroxyhexanoates from δ-caprolactone, and the reverse reaction.

removed the benzyl ether and produced the isopropyl ester of (*S*)-5-hydroxyhexanoic acid **4a**.

Repeating the above process beginning with the (*R*)-enantiomer of ethyl 3-hydroxybutanoate **5b**, similarly afforded **4b**, identical to **4a** except for retention time on a chiral GC column and specific rotation ($[\alpha]_D^{25} = -8$ (c = 2.2, $CHCl_3$)).

Comparison by chiral GC proved that the 5-(*S*)-enantiomer **4a** matched the insect-produced compound, and that the insect produced compound gave only a single peak, with no trace of the (*R*)-enantiomer. Better GC results were obtained on the chiral column with TMS ethers of **4a** and **4b** than with the alcohols themselves. The TMS ether of the (*R*)-enantiomer **4b** eluted earlier than the corresponding TMS of the natural (*S*)-enantiomer **4a** from a Chiraldex B-DM column.

At about this time we became aware that (*S*)-5-chloro-2-pentanol **11** was becoming commercially available (Daicel Chemical Industries, Ltd.). Availability of this material suggested a convenient alternative synthesis as outlined in Figure 2. After protection of the secondary alcohol as its tetrahydropyranyl ether **12**, its Grignard reagent was carbonated to give THP acid **13**. Treatment of an isopropanol solution of **13** with Nafion-H™ or with pyridinium *p*-toluenesulfonate resulted in removal of the THP ether and esterification of the carboxylic acid, providing **4a** in a single step.

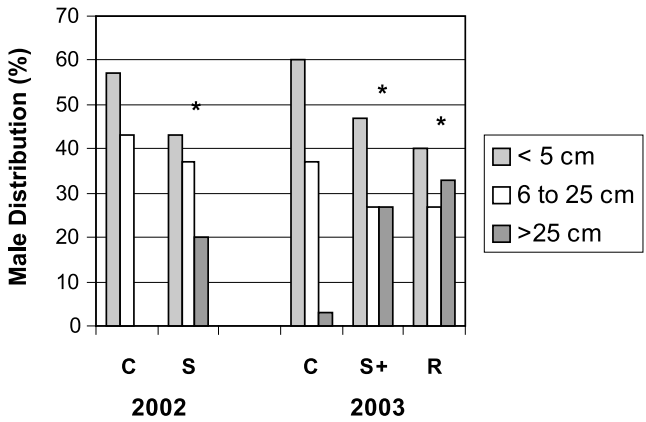


FIG. 7. Distributions in % of *Plioreocepta poeciloptera* males in the olfactometer in the presence of synthetic compounds recorded as distances moved toward the source (in cm). Two years' data are presented. *Distribution is statistically different from the control distribution (χ^2 test). C: Control without volatiles; R: *R*-enantiomer; S: *S*-enantiomer at 50 μg; S+: *S*-enantiomer at 200 μg.

Adult Fly Behavior in the Presence of Synthetic Compounds. The distributions of 4-d-old females and males in the olfactometer, in the presence of a single enantiomer **4a** or **4b**, or the racemate **4**, were compared in 2002 and 2003. The results were similar. Male distributions in response to both the *S*- and *R*-enantiomers were different from the control distributions. Males moved further in the presence of either of the two enantiomers, showing a higher chemo-anemotaxis (Figure 7). Female flies showed no significant upwind movement in response to either enantiomer or the racemate (data not shown).

However, the synthetic compounds were perceived by females and did produce behavioral responses. Females stimulated by the *S*-enantiomer often assumed a position of immobility with spread wings. This behavior was signifi-

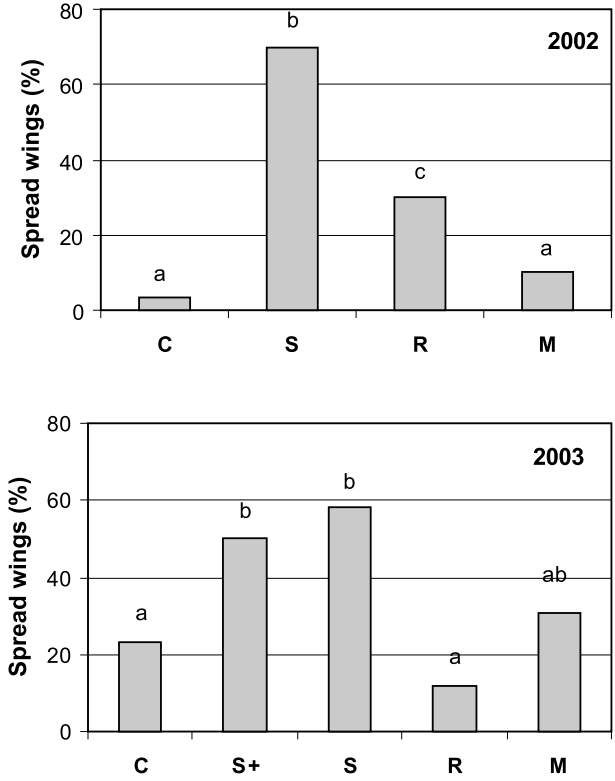


FIG. 8. Percentages of *Plioreocepta poeciloptera* females that spread their wings in the presence of synthetic volatiles in 2002 and 2003. Data with the same letters are not statistically different at $\alpha = 0.05$ (χ^2 test). C: Control without volatiles; M: mixture (racemic); R: *R*-enantiomer; S+: *S*-enantiomer at 200 μg; S: *S*-enantiomer at 50 μg.

cantly more frequent with the *S*-enantiomer than with the control or with the *R*-enantiomer in 2002 (Figure 8). In 2003, females responded more strongly to both doses of the (*S*)-enantiomer than to the control, the (*R*)-enantiomer, or the racemate. Responses to the latter two treatments were no different from responses to the control (Figure 8).

DISCUSSION

As observed previously, male *P. poeciloptera* called in the middle of the day and in the first half of the afternoon (Thibout and Auger, 1999). Calling behavior was rare in 1-d-old males but became frequent from the second d after emergence. The onset of calling and the peak calling time were delayed from age 1 to age 8 d. A time shift in calling corresponding to a time advance has been commonly seen in moth species (Swier et al., 1977; Gemeno and Haynes, 2000), where it was explained as an adaptation of older adults to increase their chances of mating (Swier et al., 1977). The adaptive advantage of delaying calling in mature *P. poeciloptera* males remains unclear.

The major male volatile appeared to be emitted during the afternoon calling period, and release of this compound coincided with eversion of glandular (Dingler, 1934) abdominal pouches (Thibout and Auger, 1999). In contrast to males, females were not attracted by the synthetic compound in the olfactometer. However, females did react behaviorally by spreading their wings, and only in response to the natural (*S*)-enantiomer. The induced wing spreading in females is believed to be a short range arrestant response. This olfactometer result is in contrast to our earlier field and laboratory observations that had indicated that females were attracted to a male pheromone (Thibout and Auger, 1999). The failure to respond in the olfactometer could suggest that factors other than the single compound may be involved in courtship and mating of this fly. We have looked for, but have been unable to find, other noteworthy male-specific volatiles. However, responses may be dose-dependent.

Unexpectedly, males were attracted in the olfactometer by both the *S*-enantiomer and the unnatural *R*-enantiomer. Cases of attraction to a nonnatural enantiomer have been observed in other insects such as cockroaches (Gemeno et al., 2003). However, we have never observed male *P. poeciloptera* attracted to other males or even to females in either the field nor the laboratory (Thibout and Auger, 1999), and the observed attraction of males to the synthetic compounds may result from unnaturally high concentrations, or possibly from some subtlety in the rhythm of emission. One could speculate that wild males could use the emission of invading males to detect their presence and chase them off, or, perhaps more likely, that invading males might exploit the

emission of native males to gain access to already-attracted females. The attraction of males to the male-produced compound, and the absence of attraction by females, can be compared to results previously observed in various tephritid species (Fletcher and Bellas, 1988; Landolt and Heath, 1990; Pike and Meats, 2003).

In summary, in spite of its release as a single, male-specific volatile compound coinciding with typical male calling behavior, and its elicitation of a typical arrestant response in females, isopropyl (*S*)-5-hydroxyhexanoate **4a** has not been established to be a true male sex pheromone of *P. poeciloptera*. Its behavioral effects, at least under laboratory conditions, do not correspond precisely to those observed previously with living males (i.e., lack of demonstrated motility toward a source). However, this volatile compound does attract males, which might be used in management of this insect. Because of the univoltinism of the species, additional studies must be undertaken in future years, particularly to study the activity of isopropyl (*S*)-5-hydroxyhexanoate in asparagus fields.

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IDENTIFICATION OF THE LARVAL AGGREGATION PHEROMONE OF CODLING MOTH, *Cydia pomonella*

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Abstract—Mature larvae of the codling moth, *Cydia pomonella* L. (Lepidoptera: Olethreutidae), exit the fruit and seek sites suitable for pupation. Spinning cocoons in such sites, larvae produce a complex, cocoon-derived blend of volatiles recently shown to attract and/or arrest both conspecific larvae and the prepupal parasitoid *Mastrus ridibundus* Gravenhorst (Hymenoptera: Ichneumonidae). Here we report components of this blend that constitute the pheromone of fifth-instar *C. pomonella* larvae. Thirty-one two-choice olfactometer experiments showed that a blend of synthetic (*E*)-2-octenal, (*E*)-2-nonenal, sulcatone, and geranylacetone, in combination with either 3-carene and/or three saturated aldehydes (octanal, nonanal, decanal), elicited behavioral responses from *C. pomonella* larvae. In on-tree experiments with corrugated cardboard bands as pupation sites for larvae affixed to tree trunks, and with laboratory-reared larvae released onto such trees, more larvae cocooned in those halves of cardboard bands baited with cocoon-spinning conspecific larvae, or with synthetic pheromone components, than in unbaited control halves of the bands. With the larval aggregation pheromone identified in this study, there might be an opportunity to manipulate *C. pomonella* larvae in commercial fruit or nut orchards.

Key Words—Codling moth, larvae, *Cydia pomonella*, *Mastrus ridibundus*, aggregation pheromone, heptanal, octanal, nonanal, decanal, (*E*)-2-octenal, (*E*)-2-nonenal, sulcatone, geranylacetone, (+)-limonene, myrcene, 3-carene.

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INTRODUCTION

When mature larvae of the codling moth, *Cydia pomonella* L. (Lepidoptera: Olethreutidae), complete their development, they exit the fruit and seek sites suitable for pupation. While spinning cocoons in such sites, larvae produce an aggregation pheromone that attracts or arrests conspecific larvae (Duthie et al., 2003; Jumean et al., 2004a). Aggregation of fifth-instars prior to pupation may be part of a mating strategy (Duthie et al., 2003) because in laboratory bioassays, eclosed adult males appeared to be arrested by the sex pheromone (*E*,*E*)-8,10-dodecadienol emanating from mature female pupae, which may allow mating as soon as a female ecloses.

Identification of the cocoon-derived pheromone proved challenging because larval antennae were too small to be used effectively in gas chromatographic–electroantennographic detection (GC–EAD) analyses of cocoon volatiles. Testing the hypothesis that *Mastrus ridibundus* Gravenhorst (Hymenoptera: Ichneumonidae), a parasitoid of late instar/prepupal *C. pomonella*, exploits odors produced by or associated with larvae as a kairomone during host-foraging, Jumean et al. (2004b) demonstrated (1) that 10 cocoon volatiles [3-carene, myrcene, heptanal, octanal, nonanal, decanal, (*E*)-2-octenal, (*E*)-2-nonenal, sulcatone, and geranylacetone] elicited responses from female *M. ridibundus* antennae, and (2) that eight of these components [all except myrcene and (*E*)-2-nonenal] were essential for the attraction of *M. ridibundus* in behavioral bioassays. A blend of the same 10 components and (+)-limonene (an abundant compound in cocoon volatiles) as an 11th component also attracted/arrested foraging fifth-instar *C. pomonella* larvae (Jumean et al., 2004b). Here we report that a blend of (*E*)-2-octenal, (*E*)-2-nonenal, sulcatone, and geranylacetone in combination with either 3-carene and/or three saturated aldehydes (octanal, nonanal, decanal) elicited attraction or arrestment of pupation site-seeking fifth-instar *C. pomonella* larvae.

METHODS AND MATERIALS

Experimental Insects. Larvae were shipped in trays of artificial diet from the Sterile Insect Release Program rearing facility in Osoyoos, British Columbia, Canada. Trays containing 1000 larvae were kept in a glass aquarium ($60 \times 31 \times 31$ cm) and stored at 15°C under a 16L:8D photoperiod. Nondiapausing fifth-instar larvae were removed from the diet as needed for experiments.

Acquisition of Volatiles. To acquire naturally emitted volatiles for olfactometer experiments, 300 cocoon-spinning male and female fifth-instars (1:1 sex ratio) were placed in a cylindrical Pyrex glass chamber (15.5×20 cm). An empty chamber served as control. A water aspirator drew charcoal-filtered

air at ~2 l/min through each chamber and through a glass column (14 × 1.3 cm OD) containing Porapak Q (50–80 mesh; Waters Associates, Inc., Milford, MA, USA). After 72 hr, volatiles were eluted from the Porapak Q trap with 3 ml of pentane and ether (95:5). Extracts were concentrated under a nitrogen stream so that 1 μl was equivalent to 10 cocoon-spinning larvae-hour equivalents (10 CSLHE = volatiles released from 10 cocoon-spinning *C. pomonella* larvae during 1 hr). Extracts were stored in darkness at −15°C, and analyzed by coupled gas chromatography–mass spectrometry (GC–MS) in full-scan electron impact mode, using a Varian Saturn 2000 Ion Trap GC–MS fitted with a DB-5 column (30 m × 0.25 mm i.d., J&W Scientific, Folsom, CA, USA).

Olfactometer Experiments. In two-choice Petri dish olfactometers (detailed drawing in Duthie et al., 2003), test stimuli were randomly assigned to one of two 4-ml vials (Table 1), each with a perforated Eppendorf tube to prevent physical contact of experimental larvae with test stimuli. Stimuli were pipetted onto Whatman no. 1 filter paper disks (1 cm diam.), with treatment and control disks receiving the same amount of solvent. For each replicate, one fifth-instar was placed in the center of the olfactometer, and its pupation site was recorded 18–24 hr later. All experiments were conducted at 21–26°C in complete darkness.

To determine whether storage of cocoon volatile extract diminished its attractiveness, experiments 1 and 2 tested the responses of larvae to 180 CSLHE of fresh (1 d old) and aged (8 d old) extracts. In experiment 3, a synthetic blend (SB) of 11 cocoon volatiles [heptanal, octanal, nonanal, decanal, (*E*)-2-octenal, (*E*)-2-nonenal, (+)-limonene, myrcene, 3-carene, sulcatone, geranylacetone] (Jumean et al., 2004b) was tested at 200 CSLHE to determine whether it had a comparable behavioral effect as Porapak Q extract of natural cocoon volatiles. Testing natural vs. synthetic cocoon volatiles, or two blends of synthetic cocoon volatiles, in the same confined olfactometer was attempted but found to compromise the larva's ability to discriminate between volatile blends (Z. Jumean, unpublished data).

Taking the results of experiment 3 into account, experiments 4–7 tested SB at four doses (1, 10, 100, and 1000 CSLHE) to determine the optimal dose for subsequent experiments. To determine essential pheromone components, experiments 8–16 tested SB vs. blends lacking certain classes of organic chemicals (Byers, 1992), such as ketones (experiment 8), monoterpenes (experiment 9), or aldehydes (experiment 10). Experiments 11–16 took a similar approach by deleting from SB either saturated aldehydes (experiment 11), unsaturated aldehydes (experiment 12), or individual components (experiments 13–16). Considering the results of experiments 8–16, experiments 17–26 tested a four-component rudimentary synthetic blend (RSB) [(*E*)-2-nonenal, (*E*)-2-octenal, sulcatone, geranylacetone] alone (experiment 17) or in combination with one of

TABLE 1. DETAILS ON EXPERIMENTAL INSECTS AND STIMULI TESTED IN LABORATORY OLFACTOMETER AND ON-TREE EXPERIMENTS

Petri dish olfactometer bioassays			
Experiment no.	Vial 1	Vial 2	*N*
1	Porapak Q extract (180 CSLHE,[a] 1 d old)	Solvent[b]	30
2	Porapak Q extract (180 CSLHE, 8 d old)	Solvent	32
3	200 Synthetic Blend[c] (SB)	Solvent	35
4	1 SB	Solvent	30
5	10 SB	Solvent	28
6	100 SB	Solvent	31
7	1000 SB	Solvent	26
8	200 SB *minus* ketones	Solvent	35
9	200 SB *minus* monoterpenes	Solvent	35
10	200 SB *minus* aldehydes	Solvent	31
11	200 SB *minus* saturated aldehydes	Solvent	33
12	200 SB *minus* unsaturated aldehydes	Solvent	31
13	200 SB *minus* (*E*)-2-nonenal	Solvent	30
14	200 SB *minus* (*E*)-2-octenal	Solvent	30
15	200 SB *minus* sulcatone	Solvent	33
16	200 SB *minus* geranylacetone	Solvent	33
17	200 Rudimentary Synthetic Blend[d] (RSB)	Solvent	25
18	200 RSB *plus* (+)-limonene	Solvent	20
19	200 RSB *plus* myrcene	Solvent	30
20	200 RSB *plus* 3-carene	Solvent	32
21	200 RSB *plus* heptanal *plus* octanal *plus* nonanal	Solvent	28
22	200 RSB *plus* octanal *plus* nonanal *plus* decanal	Solvent	27
23	200 RSB *plus* heptanal *plus* nonanal *plus* decanal	Solvent	23
24	200 RSB *plus* heptanal *plus* octanal *plus* decanal	Solvent	25
25	200 RSB *plus* octanal *plus* nonanal	Solvent	25
26	200 RSB plus 3-carene *plus* octanal *plus* nonanal *plus* decanal	Solvent	30
27	10 SB	Solvent	40
28	10 SB (3-carene 10-fold increased)	Solvent	33
29	10 SB [(*E*)-2-octenal 10-fold increased]	Solvent	47
30	10 SB [(*E*)-2-nonenal 10-fold increased]	Solvent	39
31	10 SB [(*E*)-2-octenal 10-fold increased plus (*E*)-2-nonenal 10-fold increased]	Solvent	36
On-tree experiments			
	Treatment	Control	
32	CB + 25 ♀ larvae (1 d old)[e,f]	CB	12
33	1000 SB	Solvent	18
34	100 SB	Solvent	18
35	10,000 SB	Solvent	12
36	100 SB [(*E*)-2-octenal 10-fold increased plus (*E*)-2-nonenal 10-fold increased]	Solvent	12

three monoterpenes [(+)-limonene, myrcene, or 3-carene; experiments 18–20], or with one of four 3-component blends of saturated aldehydes [heptanal, octanal, nonanal, decanal; experiments 21–24]. Considering that only the blend of saturated aldehydes that contained octanal, nonanal, and decanal enhanced the effectiveness of RSB (experiment 22), experiment 25 explored whether decanal could be deleted from this blend without affecting the blend's behavioral activity. Experiment 26 then was designed to confirm that the RSB plus four essential components [3-carene (experiment 20); octanal, nonanal, and decanal (experiments 22, 25)] was attractive to larvae seeking pupation sites. Final laboratory experiments 27–31 explored whether SB at the low and behaviorally inactive dose of 10 CSLHE would become stimulatory upon increasing the amount of 3-carene (experiment 28), or either one or both of (*E*)-2-octenal and (*E*)-2-nonenal (experiments 28–31).

The on-tree experiments (32–36) were conducted at Simon Fraser University (May to October 2003) and employed 4-cm wide corrugated cardboard bands (cut from stock of 0.46 × 76 m single-face corrugated cardboard; Shippers Supply Inc., British Columbia, Canada). Cardboard bands were affixed with metal wire 45 cm above ground to trunks of maple (*Acer* spp.) trees that were 10–16 cm in diam at that height. Bands were divided into two halves, with test stimuli applied to the waxed center (4 cm^2) of each half.

For each replicate in experiments 32–36, 20 fifth-instars were released from a thin circular collar affixed to the tree's main branch crotch (~1.50 m above ground). Experiments were started at 22:00 hr and terminated 10–12 hr later by recording the number of larvae cocooning in treatment or control halves of the cardboard bands.

Experiments 32 and 33 tested whether cardboard band halves baited with 25 1-d-old *C. pomonella* cocoons containing larvae or prepupae (experiment 32), or baited with a synthetic blend at 1000 CSLHE (experiment 33), attracted or arrested more *C. pomonella* larvae than did unbaited cardboard band halves. In

Footnotes to Table 1

[a] CSLHE = cocoon-spinning larvae hour equivalents.

[b] Solvent consisted of redistilled pentane (20–100 μl).

[c] 10 SB = synthetic blend of 11 components: decanal (1.4 ng) [Aldrich], nonanal (4.1 ng) [Aldrich], octanal (0.94 ng) [Aldrich], heptanal (0.85 ng) [Aldrich], (*E*)-2-nonenal (1.00 ng) [Bedoukian], (*E*)-2-octenal (0.41 ng) [Bedoukian], geranylacetone (0.50 ng) [Aldrich], sulcatone (0.81 ng) [Aldrich], 3-carene (0.95 ng) [Aldrich], myrcene (0.84 ng) [Aldrich], (+)-limonene (10.00 ng) [Aldrich].

[d] 10 RSB = rudimentary synthetic blend of four components: (*E*)-2-nonenal (1.00 ng), (*E*)-2-octenal (0.41 ng), geranylacetone (0.50 ng), sulcatone (0.81 ng).

[e] Fifth-instar larvae were allowed to cocoon in an open-fluted cardboard (CB) strip (4 cm^2).

[f] Female larvae were used as test stimuli, and male larvae were bioassayed to allow recognition and recording of all those larvae that had responded to test stimuli.

experiment 32, female larvae served as test stimuli and male larvae (as determined by testes visible through the dorsal integument) were bioassayed to allow recognition and recording of those larvae that had responded to test stimuli. This experimental design was justified because both male and female cocoon-spinning larvae produce and respond to the same volatile components (Jumean et al., 2004a). Experiments 34–35 determined whether a 10-fold decrease (experiment 34) or a 10-fold increase (experiment 35) in the dose of the synthetic blend affected the response of *C. pomonella* larvae in the field. Finally, experiment 36 explored whether the synthetic blend at the low and behaviorally inactive dose of 100 CSLHE (see experiment 34) would become active by increasing the amounts of (*E*)-2-octenal and (*E*)-2-nonenal as essential blend components.

Statistical Analyses. Numbers of larvae responding to treatment and control stimuli in laboratory olfactometer experiments were analyzed with the χ^2 goodness-of-fit test, using Yates correction for continuity ($\alpha = 0.05$) (Zar, 1999). Numbers of larvae responding to treatment and control stimuli in on-tree bioassays were analyzed with the Wilcoxon paired-sample test ($\alpha = 0.05$) (Zar, 1999).

RESULTS

In laboratory olfactometer experiments, both fresh and aged Porapak extracts of cocoon volatiles at 180 CSLHE attracted larvae (Figure 1; experiments 1, 2), as did a synthetic blend (SB) of 11 candidate pheromone components at 200 CSLHE (Figure 1, experiment 3). SB elicited a behavioral response also at 100 CSLHE (Figure 1; experiment 6), but not at 1, 10, or 1000 CSLHE (Figure 1; experiments 4, 5, 7). SBs lacking ketone or monoterpene components remained moderately attractive (Figure 2; experiments 8, 9), whereas an SB lacking aldehydes was inactive (Figure 2; experiment 10). An SB lacking saturated aldehydes was still active (Figure 2; experiment 11), whereas SBs lacking one or both unsaturated aldehydes [(*E*)-2-nonenal or (*E*)-octenal], or ketones [sulcatone or geranylacetone] elicited no significant responses from larvae (Figure 2; experiments 12–16). A rudimentary synthetic blend [RSB: (*E*)-2-octenal, (*E*)-2-nonenal, sulcatone, and geranylacetone] was not attractive (Figure 3; experiment 17), but the addition of 3-carene, unlike myrcene or (+)-limonene, rendered RSB attractive (Figure 3; experiments 18–20). Addition of the three saturated aldehydes octanal, nonanal, and decanal, unlike other three-component blends of saturated aldehydes, also rendered RSB attractive (Figure 3; experiments 21–24) to a level comparable with that of RSB plus all four saturated aldehydes (= SB minus monoterpenes; experiment 9).

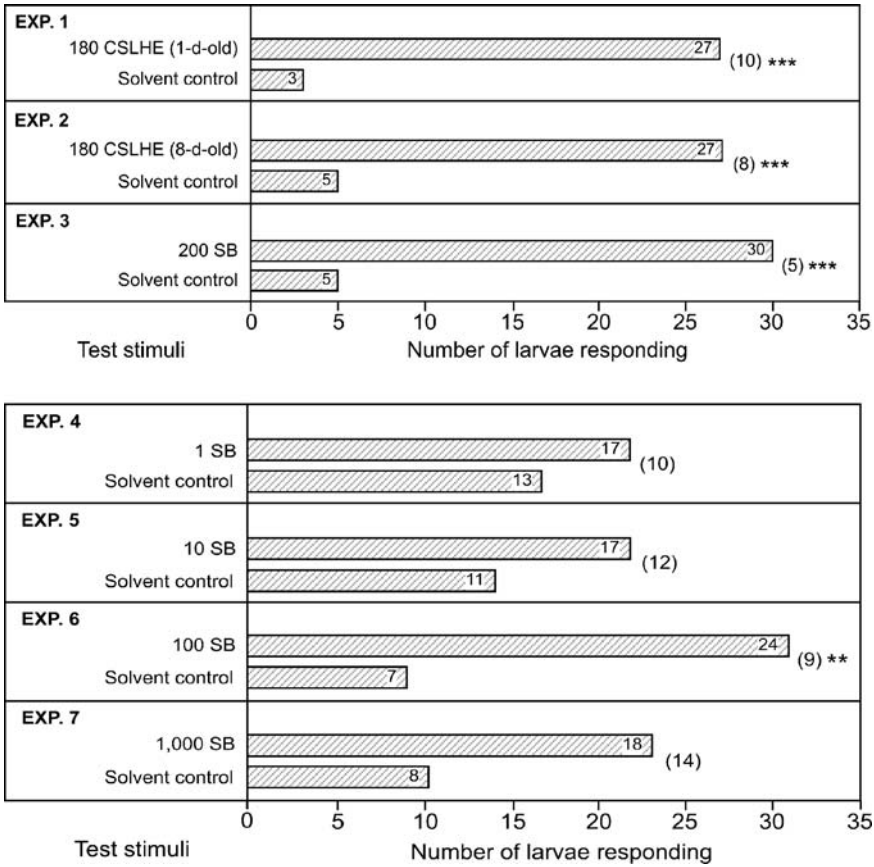
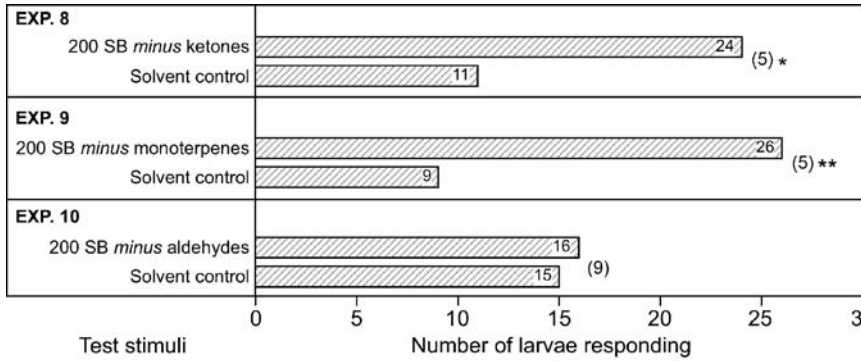


FIG. 1. Response of fifth-instar *Cydia pomonella* larvae in Petri dish olfactometers to extracts of cocoon-derived volatiles and to a synthetic blend (SB) of 11 candidate pheromone components (experiments 1–3), or to varying doses of SB (experiments 4–7). Number of larvae responding to each stimulus is given within bars; number of larvae not responding in each experiment given within parentheses; asterisks indicate a significant response to a particular treatment; χ^2 test with Yates correction for continuity; $^{**}P < 0.005$; $^{***}P < 0.001$. Ten SB consisted of three monoterpenes [(+)-limonene (10.00 ng), 3-carene (0.95 ng), myrcene (0.84 ng)], four saturated aldehydes (heptanal, octanal, nonanal, decanal), two unsaturated aldehydes [(*E*)-2-octenal (0.41 ng), (*E*)-2-nonenal (1.00 ng)], and two ketones [sulcatone (0.81 ng), geranylacetone (0.50 ng)]. Cocoons were 1–3 d old at the time of aeration but Porapak Q extracts were tested before and after aging to determine stability of semiochemicals. Aliquots of 180 or 200 CSLHE (cocoon-spinning larvae hour equivalents) were tested in experiments 1–3. Aliquots of 1, 10, 100, or 1000 CSLHE were tested in experiments 4–7; the same amount (20–25 μl) of pentane was applied to treatment and control stimuli in experiments 1–7.



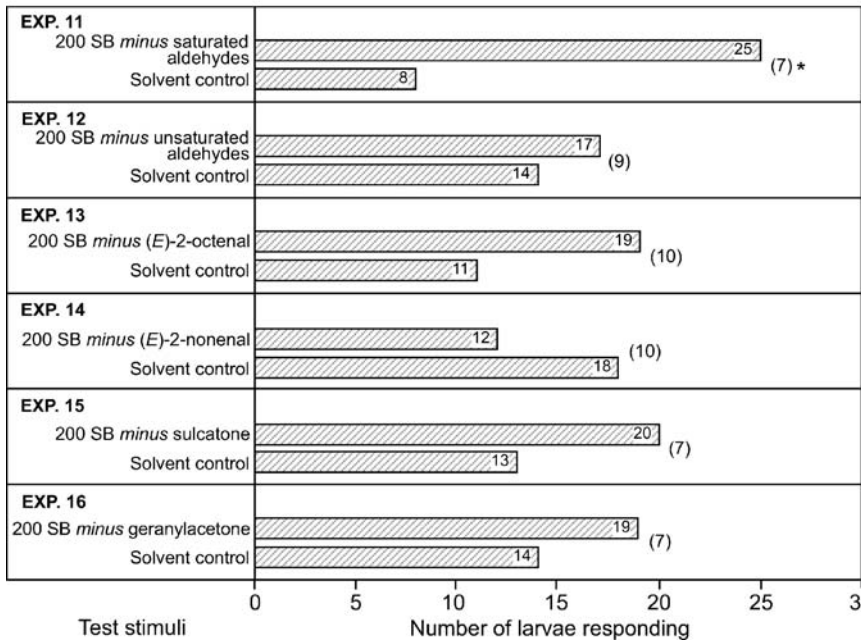


FIG. 2. Response of fifth-instar *Cydia pomonella* larvae in Petri dish olfactometers to synthetic blends (SB) lacking one or more candidate pheromone components. Number of larvae responding to each stimulus is given within bars; number of larvae not responding given in parentheses; asterisks indicate a significant response to a particular treatment; χ^2 test with Yates correction for continuity; $^*P < 0.05$; $^{**}P < 0.01$. Aliquots of 200 CSLHE (see caption of Figure 1) were tested. The same amount (20 µl) of pentane was applied to treatment and control stimuli.

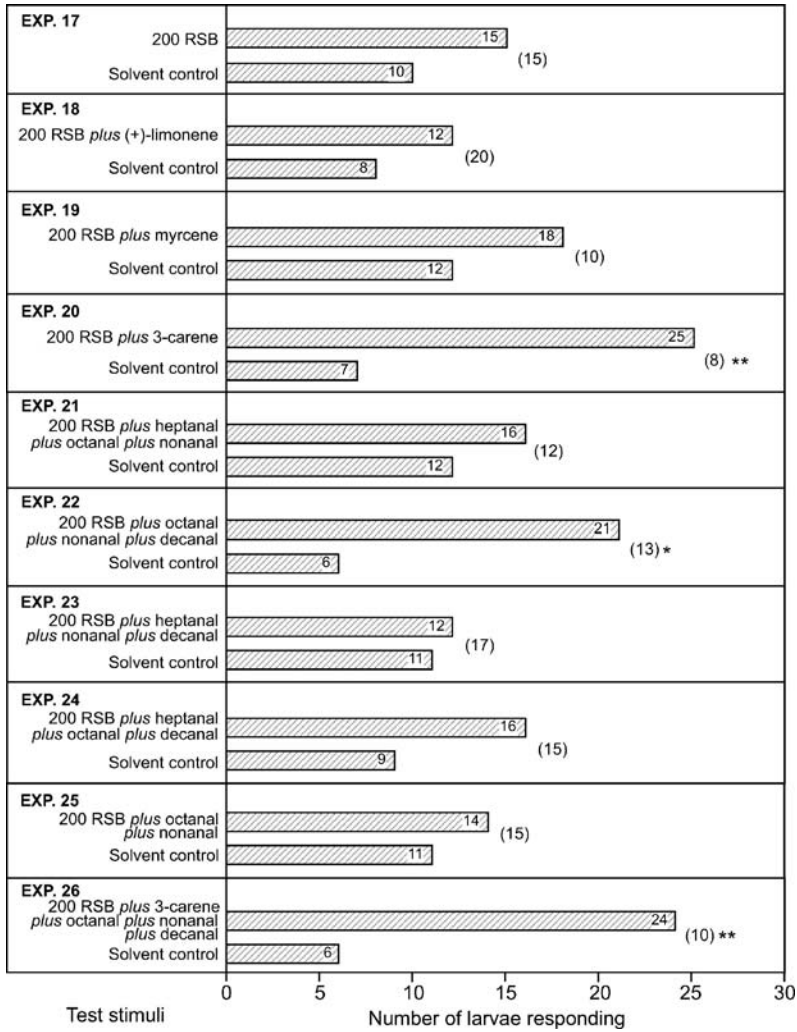


FIG. 3. Response of fifth-instar *Cydia pomonella* larvae in Petri dish olfactometer experiments 17–26 to a rudimentary synthetic blend (RSB) of pheromone components and to RSB plus individual or groups of candidate pheromone components. Number of larvae responding to each stimulus is given within bars; number of larvae not responding given in parentheses; asterisks indicate a significant response to a particular treatment; χ^2 test with Yates correction for continuity; $^*P < 0.01$; $^{**}P < 0.005$. Ten RSB consisted of two unsaturated aldehydes [(*E*)-2-octenal (0.41 ng), (*E*)-2-nonenal (1.00 ng)] and two ketones [sulcatone (0.81 ng), geranylacetone (0.50 ng)]. Aliquots of 200 CSLHE (see caption of Figure 1) were tested. The same amount (20 μl) of pentane was applied to treatment and control stimuli.

Addition of only octanal and nonanal to RSB failed to elicit a response from pupation site-seeking larvae (Figure 3; experiment 25), but RSB plus 3-carene, and octanal, nonanal, and decanal did elicit a behavioral response (Figure 3; experiment 26). SB at the low dose of 10 CSLHE had no effect on larval behavior (Figure 4; experiment 27). Ten-fold increases of either 3-carene, (*E*)-2-octenal, or (*E*)-2-nonenal in that low-dose blend did not modify its attractiveness (Figure 4; experiments 28–30) but a 10-fold increase of both (*E*)-2-octenal and (*E*)-2-nonenal in that blend stimulated a positive response from larvae (Figure 4; experiment 31).

In on-tree experiments, cocoons from conspecifics (experiment 32), and SB at 1000 CSLHE (experiment 33), attracted or arrested *C. pomonella* larvae foraging for pupation sites (Figure 5). In contrast, SB at 100 or 10,000 CSLHE was not active (Figure 5; experiments 34–35). Although the SB had no behavioral effect at 100 CSLHE (experiment 34), a 10-fold increase of both (*E*)-

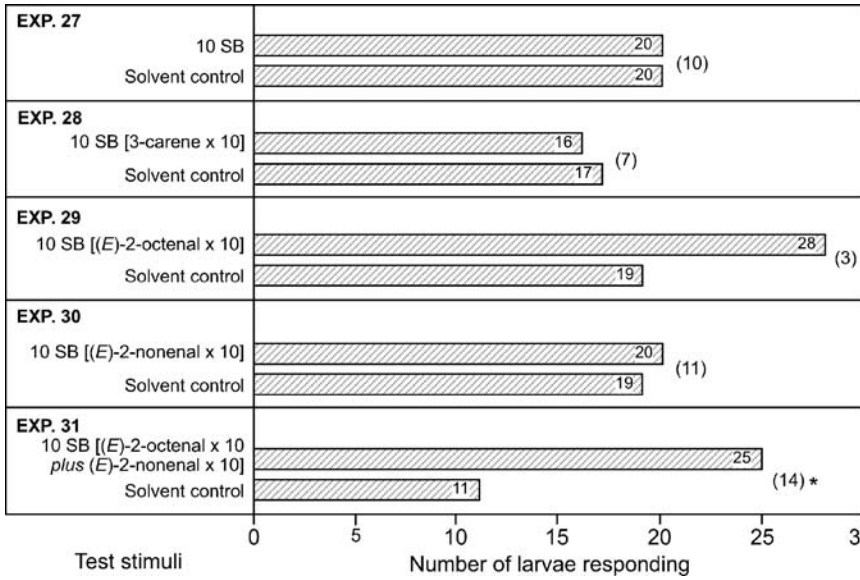


FIG. 4. Response of fifth-instar *Cydia pomonella* larvae in Petri dish olfactometer experiments 27–31 to a synthetic blend (SB; see caption of Figure 1) of 11 components with the relative proportion of 3-carene or unsaturated aldehydes [(*E*)-2-octenal, (*E*)-2-nonenal] increased by 10-fold. Number of larvae responding to each stimulus is given within bars; number of larvae not responding given in parentheses; asterisks indicate a significant response to a particular treatment; χ^2 test with Yates correction for continuity; $^*P < 0.05$. Aliquots of 10 CSLHE (see caption of Figure 1) were tested. The same amount (20 μl) of pentane was applied to treatment and control stimuli.

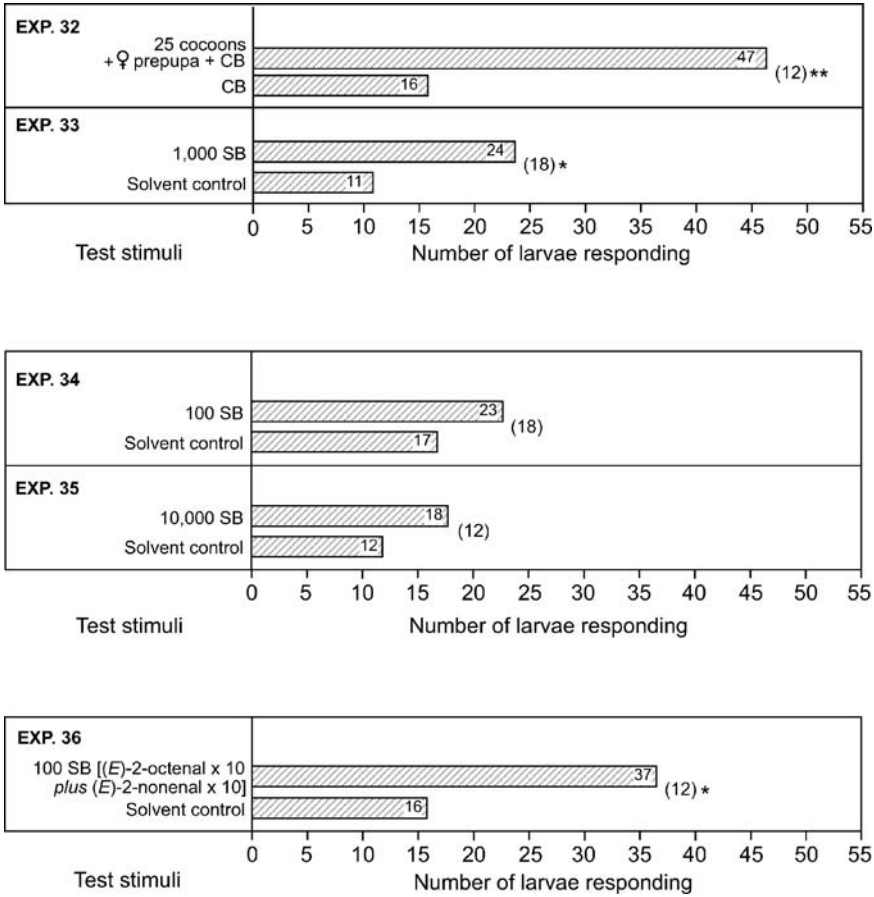


FIG. 5. Response of male (experiment 32), or male and female (experiments 33–36), fifth-instar *Cydia pomonella* larvae in on-tree experiments to stimuli consisting of either 25 cocoons with female larvae (experiment 32), or synthetic blends (SB) of 11 components (see caption of Figure 1) at varying doses and component ratios. Strips of corrugated cardboard (CB) served as a pupation site. Number of larvae responding to each stimulus is given within bars; number of replicates given in parentheses; asterisks indicate a significant response to a particular treatment; Wilcoxon paired-sample test; $*P < 0.01$; $**P < 0.005$. Aliquots of 100, 1,000, or 10,000 CSLHE (see caption of Figure 1) were tested. The same amount (100 µl) of pentane was applied to treatment and control stimuli. In experiment 36 the proportion of (*E*)-2-octenal and (*E*)-2-nonenal in the blend was increased by 10-fold.

2-octenal and (*E*)-2-nonenal in that blend produced responses from larvae (experiment 36).

DISCUSSION

Our laboratory and field data provide evidence that cocoon-spinning *C. pomonella* larvae produce a pheromone that attracts and/or arrests conspecific larvae seeking pupation sites (Duthie et al., 2003; Jumean et al., 2004a).

The cocoon-derived 11 candidate pheromone components that were bioassayed in olfactometer experiments 1–31 were selected based on evidence that they attracted not only *M. ridibundus* parasitoids but also *C. pomonella* larvae (Jumean et al., 2004b). The comparable biological activity of the 11-component synthetic blend and the Porapak Q extract of cocoon volatiles (Figure 1, experiments 1–3) suggested that all essential pheromone components were present in the synthetic blend.

The aggregation pheromone of *C. pomonella* larvae is surprisingly complex and responses were critically dependent on dose and blend composition. Low- or high-dose blends were ineffective (Figure 1), as were blends lacking either (*E*)-2-octenal, (*E*)-2-nonenal, sulcatone, or geranylacetone (Figure 2; experiments 13–16). Synergism between components was also evident when the four-component rudimentary blend of (*E*)-2-octenal, (*E*)-2-nonenal, sulcatone, and geranylacetone failed to affect larval behavior (Figure 3, experiment 17), but addition of either 3-carene (experiment 20) or three saturated aldehydes (octanal, nonanal, decanal) (experiment 23) resulted in attractive blends. Similar positive effects caused by 3-carene, or by saturated aldehydes, suggested redundancy in the blend composition. With five components needed for *C. pomonella* larvae to respond (Figure 3; experiment 20), and eight components needed for *M. ridibundus* parasitoids to respond (Jumean et al., 2004b), it appears that *M. ridibundus* requires a more complex signal to locate *C. pomonella* host prepupae than *C. pomonella* larvae require to communicate among themselves. The fact that the pheromone at low dose but with 10-fold increase of (*E*)-2-octenal and (*E*)-2-nonenal elicited a behavioral response from larvae (Figure 4; experiment 31) suggests that both of these unsaturated aldehydes are major components of the pheromone blend.

Pheromone components are perceived by *C. pomonella* larvae as airborne signals, because baffles in olfactometer experiments prevented physical contact with natural or synthetic pheromone. However, whether the pheromone serves primarily to attract or arrest conspecific larvae is not yet known.

The possible adaptive significance of pheromone-based larval aggregation will be intriguing to investigate. Duthie et al. (2003) proposed that

aggregations of *C. pomonella* larvae are part of a reproductive strategy that facilitates the earliest possible mating of eclosed adults. The proposed fitness advantage, however, may be offset by costs associated with larval aggregations. Host-derived pheromones are reliable indicators of host presence (Wiskerke et al., 1993; Wertheim et al., 2003), and are exploited by foraging parasitoids as illicit receivers of such signals (Stowe et al., 1995; Haynes and Yeargan, 1999; Jumean et al., 2004b). If, however, foraging parasitoids are egg-limited (Bezemer and Mills, 2001), individual *C. pomonella* larvae or prepupae in aggregations may be at a lower risk of parasitism than larvae that cocoon in isolation.

Aggregation of *C. pomonella* larvae as part of a proposed reproductive strategy (Duthie et al., 2003) might explain localized fruit damage in orchards treated with synthetic sex pheromone for *C. pomonella* control. Female *C. pomonella* eclosing from larval aggregations might be mated irrespective of otherwise functional pheromone-based tactics to disorient or attract and kill mate-foraging males. With the larval aggregation pheromone identified in this study, and shown to attract or arrest larvae in on-tree experiments (Figure 5; experiments 32–36), there may be a new opportunity to manipulate *C. pomonella* larvae in commercial fruit or nut orchards. Larval manipulation would be compatible with other biorational tactics of *C. pomonella* control including pheromone-mediated mating disruption and postharvest fruit removal (Judd et al., 1997).

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THE CHAFER PHEROMONE BUIBUILACTONE AND ANT PYRAZINES ARE ALSO PRODUCED BY MARINE BACTERIA

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Abstract—Headspace extracts obtained from agar plate cultures of two marine bacteria from the North Sea (Germany), *Loktanella* strain BIO-204 and *Dinoroseobacter shibae* strain DFL-27, were analyzed by GC-MS. Several γ-lactones and one δ-lactone were identified, besides pyrazines and some sulfur compounds. The absolute configuration of the major lactone (*R*,*Z*)-dodec-5-en-4-olide, known as buibuilactone, a pheromone of several scarab beetles, was determined by a new catalytic enantioselective synthesis and GC on a chiral stationary phase. Unsaturated lactones in the extracts included (*E*)-dodec-5-en-4-olide and the regioisomer (*Z*)-dodec-6-en-4-olide, previously identified as a component of black-tailed deer urine. The pyrazines 2-butyl-3,6-dimethylpyrazine and 2-isopentyl-3,6-dimethylpyrazine were identified by comparison with synthesized material. The latter compound is a known ant pheromone, as is another identified pyrazine, 2-ethyl-3,6-dimethylpyrazine. The striking similarity between insect pheromones and these bacterial volatiles is discussed, suggesting the possibility of more widespread occurrence of symbiosis between microorganisms and insects than previously thought.

Key Words—Alphaproteobacteria, buibuilactone, coleoptera, headspace analysis, marine bacteria, pyrazines, lactones, *Roseobacter* clade, symbiosis.

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INTRODUCTION

Considerable knowledge of intra- and interspecific chemical communication exists for insects, whereas other orders have received less attention. During the last decade, interest in bacterial communication has increased, and it has been shown that many effects, such as formation of biofilms, production of antibiotics, aggregation, expression of virulence factors, and bioluminescence, are mediated through bacterial chemical signals. Nevertheless, much less is known about such signals than about insect semiochemicals (Schulz, 2004, 2005). Whereas some groups of compounds have been intensively studied, such as the *N*-acylhomoserine lactones (e.g., Taga and Bassler, 2003; Daniels et al., 2004), overall, not many compounds have been identified. We, therefore, became interested in the volatile compounds emitted by marine bacteria which occur in biofilms or live associated with dinoflagellates. When these bacteria are cultivated on agar plates with a marine medium, many produce volatile compounds. These compounds have been rarely investigated compared with other bacterial metabolites, predominantly because of their normally low pharmacological activity and the special procedures required for their identification. We report here on the striking structural similarity of some bacterial volatiles to insect pheromones. Our results from the study of agar plate cultures of two marine Alphaproteobacteria from the North Sea (Germany) are reported.

METHODS AND MATERIALS

Bacteria. BIO-204 was isolated from a biofilm grown in the North Sea. Sterilized glass plates were mounted in a biofilm collector and immersed at a depth of 3 m in the channel between the island of Helgoland and the so-called Dune. After 1 wk, biofilms were aseptically scraped off, serially diluted, plated onto seawater agar (autoclaved North Sea water, 15 g/l agar, Difco), and incubated at room temperature for 3 wk. Routine cultivation of BIO-204 was performed on LBSS [25 g/l Luria Bertani broth (Sigma), 14 g/l sea salts (Sigma) in distilled water] at room temperature under ambient day/night cycle. DFL-27 was isolated from cells of the dinoflagellate *Alexandium ostenfeldii*, which were washed several times using artificial seawater and then placed on a solid medium containing 0.04 g/l peptone and 0.008 g/l yeast extract in filtered (0.3 μm) autoclaved North Sea water and incubated at 18°C in the dark. The dinoflagellates were sampled from the culture collection of the Biologische Anstalt Helgoland, culture KO287, after 72-hr growth in the late stationary growth phase. Routine cultivation of DFL-27 used marine broth (MB 2216, Difco); incubation was performed at room temperature. See Allgaier et al. (2003) for

details on sampling and cultivation. For analysis of volatile compounds, strains were incubated on a rotary shaker in liquid culture in LBSS or MB until good growth was obtained. An aliquot of 100 μl was distributed evenly on solid marine media [LBSS or MB; 15 g/l agar (Difco)] in a glass Petri dish using a drygalski spatel and incubated at room temperature at the ambient day/night cycle until the surface was completely covered by the bacteria (between 7 and 10 d).

Sampling of Volatiles. Volatile organic compounds released by cultures of the strains BIO-204 and DFL-27 on agar plates were collected using the closed loop stripping analysis (CLSA) technique as described previously (Dickschat et al., 2004; Schulz et al., 2004).

GC-MS. GC-MS analyses were carried out on an HP 6890 Series GC connected to an HP 5973 mass selective detector (Hewlett-Packard Company, Wilmington, DE, USA) fitted with a BPX5 fused-silica capillary column (25 m $\times$ 0.22 mm i.d., 0.25-μm film, SGE Inc., Melbourne, Australia). Conditions were as follows: inlet pressure, 77.1 kPa; injection volume, 1 μl; transfer line, 300°C; electron energy, 70 eV. The GC was programmed as follows: 5 min at 50°C, 5°C/min to 300°C, and operated in splitless mode (60-s valve time). The carrier gas was He at 1 ml/min. Retention indices (I) were determined relative to a homologous series of *n*-alkanes (C_8–C_{25}). Identification of compounds was performed by comparison of mass spectra to the Wiley 6 Library and the Essential Oils Library (Massfinder) as well as by comparison with synthetic compounds.

Chiral GC. The determination of the absolute configuration of natural **13** was carried out on an HP 6890 Series GC connected to an HP 5973 mass selective detector fitted with a heptakis-(6-*O*-TBDMS-2,3-di-*O*-acetyl)-β-cyclodextrin fused-silica capillary column (15 m $\times$ 0.25 mm i.d., Machery-Nagel, Düren, Germany). The GC was programmed from 100°C, increasing at 1.5°C/min to 180°C, and operated in splitless mode (60-s valve time). The carrier gas was He at 1 ml/min, and the injection volume was 1 μl.

Synthesis (General Methods). Chemicals were purchased from Fluka Chemie GmbH (Buchs, Switzerland) or Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and used without further purification. NMR spectra were obtained using a Bruker AMX400 (^{1}H NMR, 400 MHz; ^{13}C NMR, 100 MHz) spectrometer with tetramethylsilane as internal standard. Optical rotations were determined on a Dr. Kernchen Propol Digital Automatic Polarimeter. Column chroma-tography was carried out using Merck Kieselgel 60. Thin-layer chromatography was carried out using Polygram Sil G/UV_{254} 0.2-mm precoated plastic sheets (Machery-Nagel, Düren, Germany). Solvents were purified by distillation and dried according to standard methods.

*Preparation of 4-(2-Tetrahydropyranyloxy)butan-1-ol (**24**).* A solution of 3,4-dihydro-2*H*-pyran (DHP, 8.40 g, 100 mmol), 1,4-butanediol [**23**] (22.5 g,

250 mmol), and *p*-toluenesulfonic acid (*p*-TsOH, 950 mg, 5.0 mmol) in dry acetonitrile (250 ml) was stirred for 1 hr. Diethyl ether (500 ml) was added. The organic layer was washed with saturated $NaHCO_3$. The aqueous layer was separated and extracted ×3 with diethyl ether. The combined organic layers were dried ($MgSO_4$) and concentrated. The residue was purified by column chromatography on silica gel with pentane/diethyl ether (1:1) to yield **24** (13.1 g, 75 mmol, 75% based on DHP) and 1,4-bis-(2-tetrahydropyranyloxy)butane (2.22 g, 8.6 mmol, 9%) as colorless liquids. 4-(2-Tetrahydropyranyloxy)butan-1-ol **24**. TLC (pentane/diethyl ether, 1:1): R_F = 0.15. GC: I = 1395. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 1.47–1.62 (4H, m, 2 × CH_2), 1.63–1.77 (5H, m, 3 × CH_2), 1.77–1.88 (1H, m, CH_2), 3.28–3.30 (1H, br s, OH), 3.38–3.47 (1H, m, CH_2), 3.47–3.55 (1H, m, CH_2), 3.62–3.68 (2H, m, CH_2), 3.73–3.83 (1H, m, CH_2), 3.83–3.90 (1H, m, CH_2), 4.57–4.63 (1H, m, CH). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) = 19.4 (CH_2), 25.3, 25.4 (CH_2), 26.4, 26.4 (CH_2), 29.7, 29.8 (CH_2), 30.5, 30.6 (CH_2), 62.1, 62.2 (CH_2), 62.4 (CH_2), 67.2, 67.4 (CH_2), 98.7, 98.9 (CH). EI-MS (70 eV): *m/z* (%) = 101 (21) $[C_5H_9O_2]^+$, 85 (100) $[C_5H_9O]^+$, 73 (90), 67 (15), 55 (79), 41 (52).

*Preparation of 4-(2-Tetrahydropyranyloxy)butanal (**25**).* A solution of **24** (6.25 g, 35.9 mmol) in dry CH_2Cl_2 (20 ml) was added in one portion to a suspension of pyridinium dichromate (PDC, 20.2 g, 53.9 mmol) in dry CH_2Cl_2 (150 ml). The reaction mixture was stirred at room temperature for 24 hr, concentrated, and then filtered through silica gel. The pure **25** (4.39 g, 25.5 mmol, 71%) was obtained by column chromatography on silica gel with pentane/diethyl ether (1:1) as a colorless liquid. TLC (pentane/diethyl ether, 1:1): R_F = 0.48. GC: I = 1322. ^{1}H NMR ($CDCl_3$): δ 1.45–1.87 (6H, m, 3 × CH_2), 1.95 (2H, quin, J = 6.6 Hz, CH_2), 2.54 (2H, dt, J = 1.6 Hz, J = 7.0 Hz, CH_2), 3.36–3.55 (2H, m, CH_2), 3.72–3.88 (2H, m, CH_2), 4.55–4.62 (1H, m, CH), 9.79 (1H, t, J = 1.6 Hz, CHO). ^{13}C NMR ($CDCl_3$): δ 19.4 (CH_2), 22.6 (CH_2), 25.4 (CH_2), 30.5 (CH_2), 41.0 (CH_2), 62.2 (CH_2), 66.3 (CH_2), 98.8 (CH), 202.2 (CHO). EI-MS (70 eV): *m/z* (%) = 101 (13) $[C_5H_9O_2]^+$, 85 (68) $[C_5H_9O]^+$, 71 (100) $[C_4H_7O]^+$, 57 (17), 41 (53).

*Preparation of 4-Hydroxy-1-(2-tetrahydropyranyloxy)dodec-5-yne (rac-**26**).* A solution of ethylmagnesium bromide (~19.3 mmol) in dry diethyl ether (20 ml) was prepared from magnesium (470 mg, 19.3 mmol) and ethyl bromide (2.10 g, 19.3 mmol). Then a solution of 1-octyne (2.12 and 19.3 mmol) in dry diethyl ether (5 ml) was added dropwise. The reaction mixture was stirred until no more ethane evolved (ca. 1 hr). The resulting solution was added dropwise to a solution of **25** (2.77 g, 16.1 mmol) in dry diethyl ether (100 ml), heated to reflux for 2 hr, and then quenched by the addition of saturated NH_4Cl. The aqueous layer was separated and extracted ×3 with diethyl ether. The combined organic layers were dried with $MgSO_4$ and concentrated *in vacuo*. Purification by column chromatography on silica gel with pentane/diethyl ether afforded *rac*-**26**

(3.86 g, 13.7 mmol, 85%) as a colorless liquid. TLC (pentane/diethyl ether, 1:1): R_F = 0.44. GC: I = 2141. ^{1}H NMR ($CDCl_3$): δ 0.89 (3H, t, J = 6.6 Hz, CH_3), 1.22–1.41 (6H, m, 3 × CH_2), 1.46–1.65 (6H, m, 3 × CH_2), 1.67–1.87 (6H, m, 3 × CH_2), 2.20 (2H, dt, J = 7.1 Hz, J = 1.9 Hz, CH_2), 2.77 (1H, br s, OH), 3.41–3.55 (2H, m, CH_2), 3.75–3.90 (2H, m, CH_2), 4.41 (1H, br s, CH), 4.60–4.63 (1H, m, CH). ^{13}C NMR ($CDCl_3$): δ 14.0 (CH_3), 18.6 (CH_2), 19.3 (CH_2), 22.5 (CH_2), 25.3 (CH_2), 25.5, 25.5 (CH_2), 28.5 (CH_2), 28.6 (CH_2), 30.5 (CH_2), 31.2 (CH_2), 35.4, 35.4 (CH_2), 62.1 (CH_2), 62.3, 62.3 (CH), 67.1, 67.2 (CH_2), 81.1 (C), 85.3, 85.3 (C), 98.5, 98.6 (CH). EI-MS (70 eV): m/z (%) = 223 (1), 195 (1), 179 (4), 151 (3), 121 (8), 101 (8) $[C_5H_9O_2]^+$, 95 (17), 85 (100) $[C_5H_9O]^+$, 67 (25), 55 (25), 41 (35).

*Enantioselective Preparation of (R)-4-Hydroxy-1-(2-tetrahydropyranyloxy) dodec-5-yne [(R)-**26**].* As described by Anand and Carreira (2001), $Zn(OTf)_2$ (700 mg, 1.93 mmol, 0.2 equiv.) was heated to 130°C *in vacuo* for 12 hr to remove traces of water. (+)-*N*-Methylephedrine (380 mg, 2.12 mmol, 0.22 equiv.) was added after cooling to room temperature. The mixture was dried *in vacuo* for an additional 30 min. Dry toluene (10 ml) and triethylamine (0.49 g, 4.83 mmol, 0.5 equiv.) were added. The reaction mixture was heated to 50°C for 2 hr. Then 1-octyne (1.27 g, 11.6 mmol, 1.2 equiv.) was added at room temperature. After stirring for 15 min, a solution of **25** (1.66 g, 9.65 mmol, 1.0 equiv.) in dry toluene (10 ml) was added dropwise by a syringe pump over 2.5 hr. During the first 30 min, the mixture was stirred at room temperature and then heated to 80°C. After the solution of the aldehyde **25** was completely added, the reaction mixture was stirred for additional 15 hr at 80°C and then quenched by addition of saturated NH_4Cl. The aqueous layer was separated, extracted ×3 with diethyl ether, and the combined organic layers were dried with $MgSO_4$ and concentrated. The crude product was purified by column chromatography on silica gel with pentane/diethyl ether (1:1) to yield (*R*)-**26** (1.32 g, 4.67 mmol, 49%) as a colorless liquid. Spectroscopic and physical data corresponded to those of *rac*-**26**. $[\alpha]_D^{21.1}$ = +7.9 (c = 2.1, pentane).

*Preparation of Dodec-5-yne-1,4-diol (rac-**27**).* The alcohol *rac*-**26** (3.12 g, 11.1 mmol) was dissolved in dry methanol (25 ml), and *p*-TsOH (209 mg, 1.1 mmol) was added. The reaction mixture was stirred for 1 hr at room temperature, diluted with diethyl ether (200 ml), and washed with saturated $NaHCO_3$. The aqueous layer was separated and extracted ×3 with diethyl ether. The combined organic phases were dried with $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with diethyl ether to give *rac*-**27** (1.96 g, 9.90 mmol, 89%) as a colorless liquid. TLC (diethyl ether): R_F = 0.50. GC: I = 1699. ^{1}H NMR ($CDCl_3$): δ 0.89 (3H, t, J = 6.6 Hz, CH_3), 1.23–1.41 (6H, m, 3 × CH_2), 1.45–1.54 (2H, m, CH_2), 1.68–1.85 (4H, m, 2 × CH_2), 2.20 (2H, t, J = 7.1 Hz, CH_2), 2.93 (2H, br s, 2 × OH), 3.65–3.75 (2H, m, CH_2), 4.40–4.47 (1H, m, CH). ^{13}C NMR ($CDCl_3$):

δ 14.0 (CH_3), 18.6 (CH_2), 22.5 (CH_2), 28.4 (CH_2), 28.5 (CH_2), 28.6 (CH_2), 31.3 (CH_2), 35.1 (CH_2), 62.3 (CH), 62.5 (CH_2), 80.9 (C), 85.7 (C). EI-MS (70 eV): *m/z* (%) = 167 (1), 151 (3), 139 (19), 123 (23), 110 (70), 95 (97), 79 (59), 69 (67), 55 (82), 41 (100).

*Preparation of (R)-Dodec-5-yne-1,4-diol [(R)-**27**].* As described for *rac*-**27**, enantiopure (*R*)-**26** was transformed into the corresponding diol (*R*)-**27**. Physical and spectroscopic data as well as the yield corresponded to those of *rac*-**27**. $[\alpha]_D^{22.2} = +11.1$ (c = 3.5, CH_2Cl_2).

*Preparation of Dodec-5-yn-4-olide (rac-**28**).* As described by Bloch and Brillet (1991) and Ley et al. (1994), a mixture of *rac*-**27** (396 mg, 2.0 mmol), *N*-methylmorpholine-*N*-oxide (405 mg, 3.0 mmol), powdered molecular sieve (4 Å, 1.0 g), and tetrapropylammonium perruthenate (TPAP, 35 mg, 5 mol%) in dry CH_2Cl_2 (6 ml) was stirred for 1 hr. Solids were removed by filtration, and the filtrate was concentrated. The residue was purified by column chromatography on silica gel with pentane/diethyl ether (3:1) to obtain *rac*-**28** (264 mg, 1.36 mmol, 68%) as a colorless liquid. TLC (pentane/diethyl ether, 3:1): R_F = 0.18. GC: I = 1714. ^{1}H NMR ($CDCl_3$): δ 0.89 (3H, t, J = 6.9 Hz, CH_3), 1.22–1.43 (6H, m, 3 × CH_2), 1.47–1.54 (2H, m, CH_2), 2.20–2.28 (3H, m, 2 × CH_2), 2.45–2.55 (2H, m, CH_2), 2.59–2.70 (1H, m, CH_2), 5.11–5.14 (1H, m, CH). ^{13}C NMR ($CDCl_3$): δ 13.9 (CH_3), 18.6 (CH_2), 22.4 (CH_2), 27.9 (CH_2), 28.1 (CH_2), 28.4 (CH_2), 30.1 (CH_2), 31.2 (CH_2), 69.6 (CH), 76.4 (C), 88.7 (C), 176.2 (CO). EI-MS (70 eV): *m/z* (%) = 165 (23), 147 (10), 137 (16), 124 (18), 109 (36), 95 (45), 85 (51) $[C_4H_5O_2]^+$, 79 (100), 67 (57), 55 (52), 41 (73).

*Preparation of (R)-Dodec-5-yn-4-olide [(R)-**28**].* This enantiopure compound was prepared as described for *rac*-**28** from (*R*)-**27**. Spectroscopic and physical data as well as the yield corresponded to those of *rac*-**28**. $[\alpha]_D^{23.6} = -20.7$ (c = 3.7, pentane).

*Preparation of Dodec-5-en-4-olide (rac-**13**).* Dodec-5-yn-4-olide (*rac*-**28**) (62 mg, 0.32 mmol) was dissolved in dry methanol (10 ml). The mixture was stirred in a H_2 atmosphere (1 bar) for 30 min after the addition of a catalytic amount of Lindlar's catalyst ($Pd/CaCO_3$/Pb, 10 mg, 10% Pd). Solids were filtered off, and the filtrate was concentrated. Dodec-5-en-4-olide (*rac*-**13**) (57 mg, 0.29 mmol, 94%) was obtained after column chromatography on silica gel with pentane/diethyl ether (3:1) as a colorless liquid. The *Z*/*E*-diastereomers were not separable by column chromatography, and were obtained in a ratio of 8:1. (*Z*)-Isomer (**13**). TLC (pentane/diethyl ether, 3:1): R_F = 0.25. GC: I = 1677. NMR and MS data correspond to those reported by Leal (1991).

(*E*)-Isomer (**14**). TLC (pentane/diethyl ether, 3:1): R_F = 0.25. GC: I = 1691.

*Preparation of (R)-Dodec-5-en-4-olide [(R)-**13**, Buibuilactone].* Buibuilactone was prepared in the same manner as described for *rac*-**13** using (*R*)-**28** as starting material. Spectroscopic and physical data as well as the yield corresponded to those of *rac*-**13**. $[\alpha]_D^{24.0} = -52.2$ (c = 2.0, hexane). The ee of

84% was determined by chiral GC: $t_R(S)$ = 32.4 min, $t_R(R)$ = 33.2 min. Leal (1991) reported an $[\alpha]_D^{24.0} = -61.2$ (c = 0.85, hexane) with 98.4% ee.

*Preparation of 2,5-Dimethylpyrazine-N-oxide (**30**).* As described by Ohta and Ohta (1985), a mixture of 2,5-dimethylpyrazine (**29**) (25.0 g, 231 mmol) and $NaBO_2 \cdot H_2O_2 \cdot 3H_2O$ (42.7 g, 277 mmol) in glacial acetic acid (250 ml) was heated to 80°C for 8 hr. Acetic acid was removed under reduced pressure, and the residue was diluted with 2N NaOH (250 ml). The aqueous layer was extracted ×3 with diethyl ether. The combined organic layers were dried with $MgSO_4$ and concentrated. Crude product was purified by column chromatography on silica gel with diethyl ether/methanol (10:1) to give 2,5-dimethylpyrazine-*N*-oxide **30** (24.3 g, 196 mmol, 85%) as a colorless solid. TLC (diethyl ether/methanol, 10:1): R_F = 0.29. GC: I = 1279. ^{1}H NMR ($CDCl_3$): δ 2.43 (3H, s, CH_3), 2.50 (3H, s, CH_3), 8.07 (1H, s, CH), 8.36 (1H, s, CH). ^{13}C NMR ($CDCl_3$): δ 13.9 (CH_3), 21.1 (CH_3), 132.3 (CH), 141.2 (C), 146.4 (CH), 154.7 (C). EI-MS (70 eV): m/z (%) = 124 (90) $[M]^+$, 107 (53) $[M\text{-OH}]^+$, 93 (2), 80 (95), 66 (30), 52 (20), 42 (43), 39 (100).

*Preparation of 2-Chloro-3,6-dimethylpyrazine (**31**).* As described previously (Inoue et al., 1985; Sato and Fujii, 1994), a solution of 2,5-dimethylpyrazine-*N*-oxide **30** (13.1 g, 106 mmol) in $POCl_3$ (70 ml) was heated to reflux for 3 hr. The reaction mixture was poured onto ice (1 kg), and the resulting aqueous layer was neutralized with NaOH and then extracted ×3 with chloroform. The combined organic layers were dried with $MgSO_4$ and concentrated. Pure 2-chloro-3,6-dimethylpyrazine **31** (12.0 g, 84 mmol, 79%) was obtained as a colorless liquid by column chromatography on silica gel with pentane/diethyl ether (3:1). TLC (pentane/diethyl ether, 3:1): R_F = 0.43. GC: I = 1128. ^{1}H NMR ($CDCl_3$): δ 2.52 (3H, s, CH_3), 2.62 (3H, s, CH_3), 8.25 (1H, s, CH). ^{13}C NMR ($CDCl_3$): δ 20.2 (CH_3), 21.2 (CH_3), 141.0 (CH), 147.5 (C), 149.1 (C), 150.9 (C). EI-MS (70 eV): m/z (%) = 144 (28), 142 (85) $[M]^+$, 107 (100) $[M\text{-Cl}]^+$, 80 (50), 66 (16), 52 (19), 39 (52).

*Preparation of 2-Alkyl-3,6-Dimethylpyrazines (**21** and **22**).* According to the method of Fürstner et al. (2002), a solution of the respective alkylmagnesium bromide in dry THF (10 ml) prepared from magnesium (292 mg, 12 mmol) and the alkyl bromide (12 mmol) was added to an ice-cooled solution of **31** (1.42 g, 10 mmol) and $Fe(acac)_3$ (182 mg, 0.5 mmol) in dry THF (10 ml) and *N*-methyl-2-pyrrolidinone (2 ml). The reaction mixture was stirred at 0°C for 30 min and then quenched by the addition of water. The aqueous layer was extracted ×3 with diethyl ether. The combined organic layers were dried with $MgSO_4$ and concentrated. Purification of the residue by column chromatography on silica gel with pentane/diethyl ether (5:1) yielded the alkylated pyrazines as colorless liquids. 2-Butyl-3,6-dimethylpyrazine (**21**). Yield: 1.12 g (6.83 mmol, 68%). TLC (pentane/diethyl ether, 5:1): R_F = 0.18. GC: I = 1263. ^{1}H NMR ($CDCl_3$): δ 0.96 (3H, t, J = 7.3 Hz, CH_3), 1.43 (2H, sext, J = 7.4 Hz, CH_2),

1.62–1.70 (2H, m, CH_2), 2.49 (3H, s, CH_3), 2.53 (3H, s, CH_3), 2.74–2.79 (2H, m, CH_2), 8.15 (1H, s, CH). ^{13}C NMR ($CDCl_3$): δ 13.8 (CH_3), 21.0 (CH_3), 21.1 (CH_3), 22.7 (CH_2), 30.7 (CH_2), 34.8 (CH_2), 140.5 (CH), 148.3 (C), 149.9 (C), 154.8 (C). EI-MS (70 eV): *m/z* (%) = 163 (1) $[M\text{-H}]^+$, 149 (7) $[M\text{-}CH_3]^+$, 135 (11) $[M\text{-}C_2H_5]^+$, 122 (100) $[M\text{-}C_3H_6]^+$, 107 (4), 96 (2), 80 (4), 66 (2), 53 (7), 42 (11), 39 (12).

2-Isopentyl-3,6-dimethylpyrazine (**22**). Yield: 1.21 g (6.80 mmol, 68%). TLC (pentane/diethyl ether, 5:1): R_F = 0.18. GC: I = 1321. ^1H NMR ($CDCl_3$): δ 0.98 (6H, d, J = 6.6 Hz, 2 × CH_3), 1.51–1.58 (2H, m, CH_2), 1.68 (1H, sept, CH), 2.48 (3H, s, CH_3), 2.52 (3H, s, CH_3), 2.74–2.79 (2H, m, CH_2), 8.14 (1H, s, CH). ^{13}C NMR ($CDCl_3$): δ 21.0 (CH_3), 21.0 (CH_3), 22.4 (2 × CH_3), 28.3 (CH), 33.2 (CH_2), 37.6 (CH_2), 140.5 (CH), 148.3 (C), 149.9 (C), 155.0 (C). EI-MS (70 eV): *m/z* (%) = 177 (1) $[M\text{-H}]^+$, 163 (9) $[M\text{-}CH_3]^+$, 149 (1) $[M\text{-}C_2H_5]$, 135 (15) $[M\text{-}C_3H_7]^+$, 122 (100) $[M\text{-}C_4H_8]^+$, 107 (3), 96 (1), 80 (5), 66 (2), 53 (6), 42 (9), 39 (11).

RESULTS

As part of our studies on volatiles emitted by different microorganisms, we investigated a number of marine bacteria from the North Sea by GC-MS using the CLSA headspace method adapted for agar plates (Dickschat et al., 2004; Schulz et al., 2004). The marine bacteria BIO-204 and DFL-27 have been isolated from biofilms and a toxin-producing dinoflagellate growing in the North Sea, respectively (Allgaier et al., 2003). They both belong to the *Roseobacter* clade of the Alphaproteobacteria subclass of the Proteobacteria. DFL-27 is capable of aerobic anoxygenic photosynthesis. Based on its 16S rRNA sequence, it is distantly related to *Rhodobacter sphaeroides* (92.8% similarity over 1318 nucleotides) and *Jannaschia helgolandensis* (Wagner-Döbler et al., 2003) and is currently being described as *Dinoroseobacter shibae* (Biebl et al., unpublished). BIO-204 is a strain of the recently described genus *Loktanella* (van Trappen et al., 2004).

The results of the analyses of the two species, *Loktanella* strain BIO-204 and *D. shibae* strain DFL-27, are summarized in Table 1. Total ion chromatograms of two characteristic analyses are shown in Figure 1. Both prokaryotic species produce a diverse array of volatiles. The different samples of the bacteria showed some differences in the number of compounds as well as the total amounts of the emitted volatiles. One of the predominant substance classes present in the extracts were lactones (Figure 2). DFL-27 produced a homologous series of unbranched γ-lactones, ranging from 4-hexanolide (**2**) to 4-dodecanolide (**8**), which were identified by comparison of mass spectra and retention times with

TABLE 1. COMPOUNDS IDENTIFIED IN THE HEADSPACE OF THE NORTH SEA BACTERIA *Loktanella* STRAIN BIO-204 AND *D. shibae* STRAIN DFL-27

GC[a]	Compound[b]	*I*[c]	Identification[d]	BIO-204[e,f]	DFL-27[f,g]
	S-Methyl methylthiopropionate (**17**)	867	ms	0, 0, 1	0, 0, 0, 0
	4-Pentanolide (**1**)	978	ms	1, 1, 1	0, 0, 0, 0
a	Dimethyl trisulfide (**18**)	982	ms	1, 1, 1	1, 0, 1, 1
	3-Methyl-4-butanolide (**9**)	986	com	0, 0, 1	0, 0, 0, 0
	6-Methyl-5-hepten-2-one	999	ms, ri	0, 1, 0	0, 0, 1, 0
b	4-Methylthio-2-butanone (**19**)	1005	syn	1, 1, 0	0, 0, 0, 0
c	3-Methyl-4-pentanolide (**10**)	1023	ms	0, 0, 0	1, 1, 0, 1
	2-Methyl-4-pentanolide (**11**)	1058	ms	0, 0, 0	0, 1, 0, 1
d	4-Hexanolide (**2**)	1074	ms	1, 0, 0	1, 0, 0, 0
e	*S*-Methyl methanethiosulfonate (**20**)	1088	ms	3, 2, 1	3, 3, 1, 1
	2-Ethyl-3,6-dimethylpyrazine	1088	ms	1, 0, 0	1, 0, 0, 1
	Tetramethylpyrazine	1096	ms, ri	1, 0, 0	1, 0, 0, 1
	Methyl 2-furancarboxylate	1099	ms	1, 1, 0	0, 0, 0, 0
f	2-Phenylethanol	1130	com	1, 0, 0	1, 0, 0, 1
	Methyl methylthiomethyl disulfide	1144	ms	1, 1, 1	0, 0, 1, 1
g	Benzyl cyanide	1161	com	1, 0, 1	1, 0, 0, 1
	4-Heptanolide (**3**)	1172	ms	0, 0, 0	1, 0, 1, 1
h	1-Nonanol	1181	com	0, 0, 0	1, 0, 1, 1
i	Tropone	1182	ms	1, 1, 1	0, 0, 0, 0
j	1-Phenyl-1,2-propandione	1186	ms	1, 1, 0	0, 0, 0, 0
k	2-Butyl-3,6-dimethylpyrazine (**21**)	1263	syn	1, 0, 0	0, 1, 0, 0
l	4-Octanolide (**4**)	1276	com	0, 0, 0	1, 1, 1, 1
m	Undecanal	1319	com	1, 1, 0	1, 0, 1, 1
n	Indole	1319	com	1, 1, 1	0, 0, 0, 0
o	2-Isopentyl-3,6-dimethylpyrazine (**22**)	1321	syn	1, 0, 0	0, 0, 0, 0
	4-Methylquinazoline	1364	ms	1, 1, 1	1, 0, 0, 1
p	4-Nonanolide (**5**)	1380	ms	1, 0, 0	1, 1, 0, 0
	Butyl benzoate	1389	ms	0, 0, 0	0, 0, 0, 1
q	5-Nonanolide (**12**)	1404	ms	0, 0, 0	1, 1, 0, 0
	Dodecanal	1421	com	0, 0, 0	1, 0, 1, 1
	Geranylacetone	1456	com	1, 1, 1	1, 0, 1, 1
r	4-Decanolide (**6**)	1486	com	1, 0, 0	2, 2, 1, 1
	4-Undecanolide (**7**)	1590	ms	0, 0, 0	0, 0, 1, 1
	(*Z*)-Dodec-6-en-4-olide (**15**)	1676	syn	0, 0, 0	0, 0, 1, 1
s	(*R*,*Z*)-Dodec-5-en-4-olide (**13**)	1677	syn	3, 3, 1	3, 2, 1, 1
t	1-Tetradecanol	1686	com	1, 0, 0	0, 0, 0, 0
u	(*E*)-Dodec-5-en-4-olide (**14**)	1691	syn	1, 0, 0	1, 0, 1, 1
v	4-Dodecanolide (**8**)	1699	com	2, 2, 1	2, 2, 1, 1

[a] Marker in TIC (see Figure 1).
[b] Artifacts that were also present in blank runs are not mentioned.
[c] Retention index.
[d] Identification of compounds based on ms: mass spectrum, ri: retention index literature data (Adams, 1995), syn/com: comparison of retention index and ms data to those of a synthetic (syn) or commercial (com) available compound.
[e] Three different samples of *Loktanella* strain BIO-204.
[f] 0: Not detected, 1: 0–2%, 2: 2–8%, 3: >8% of total area in GC.
[g] Four different samples of *D. shibae* strain DFL-27.

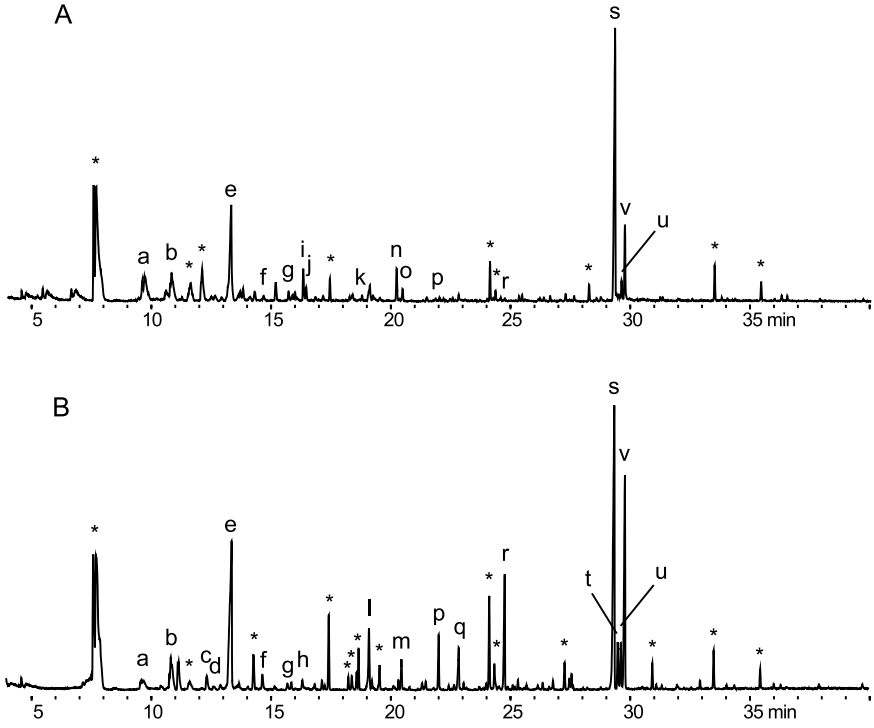


FIG. 1. Total ion chromatograms of the headspace extracts of (A) *Loktanella* strain BIO-204 and (B) *D. shibae* strain DFL-27. Artifacts are marked by an asterisk.

those of authentic reference compounds or mass spectral databases, whereas BIO-204 emitted **1**, **2**, **5**, **6**, and larger amounts of **8**. The branched 3-methyl-4-butanolide (**9**) was only found in one sample of BIO-204, whereas 3-methyl-4-pentanolide (**10**) as well as 2-methyl-4-pentanolide (**11**) was restricted to DFL-27. Additionally, this species produced the δ-lactone 5-nonanolide (**12**). One of the major compounds present in the extracts of both species eluted slightly earlier (I = 1677, I: retention index after van den Dool and Kratz, 1963) than 4-dodecanolide (I = 1699), and its mass spectrum showed a molecular ion at m/z = 196 (Figure 3A). From these data, we inferred the compound to be an unsaturated analog of 4-dodecanolide, which has a molecular weight of 198. Furthermore, the mass spectrum was characterized by a base peak at m/z = 111, as would be expected for dodec-5-en-4-olide. Another compound eluting slightly later (I = 1691) showed a similar mass spectrum (Figure 3B) with the same molecular ion (m/z = 196) and base peak (m/z = 111) and was thus suggested to be the other

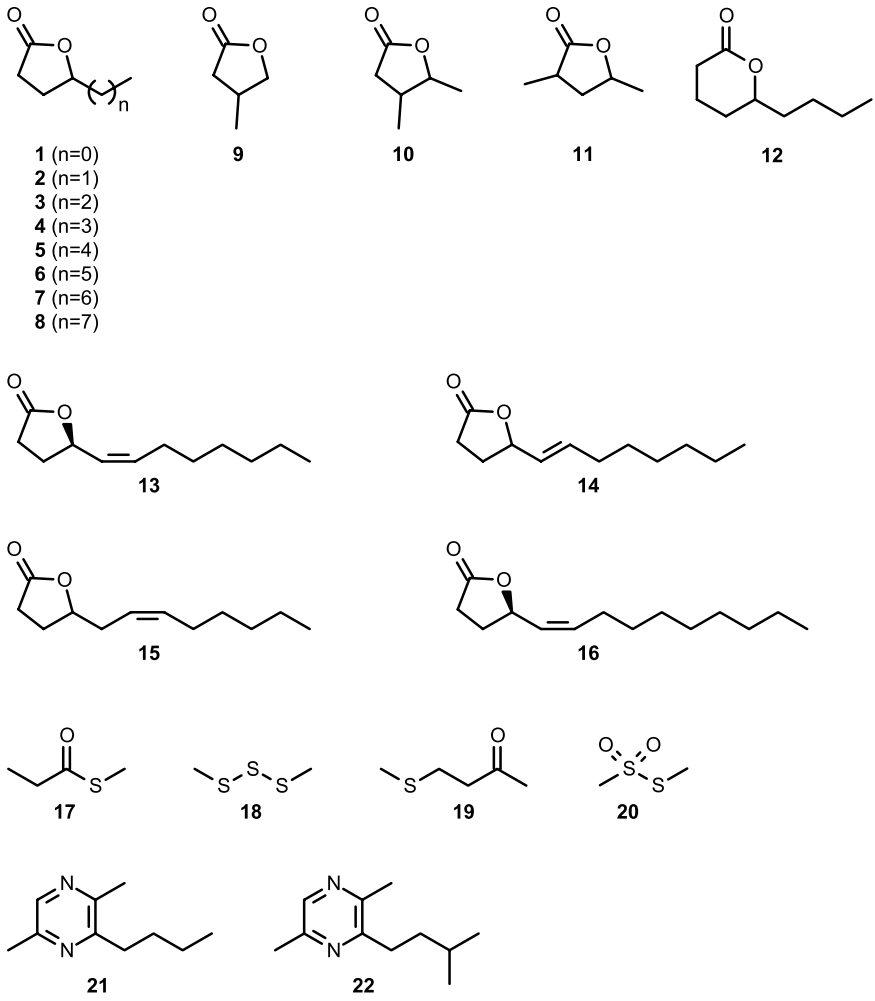


FIG. 2. Structures of some of the volatiles released by the North Sea bacteria strain BIO-204 and strain DFL-27.

diastereomer. The lactone (*R*,*Z*)-dodec-5-en-4-olide is known as an insect sex pheromone, first identified in the scarab beetle *Anomala cuprea* (Coleoptera: Scarabaeidae) (Leal, 1991), and its published mass spectrum (Leal et al., 1993, 1999) was similar to the one obtained by us. To clarify the identity of these two bacterial volatiles, we carried out a total synthesis according to Scheme 1,

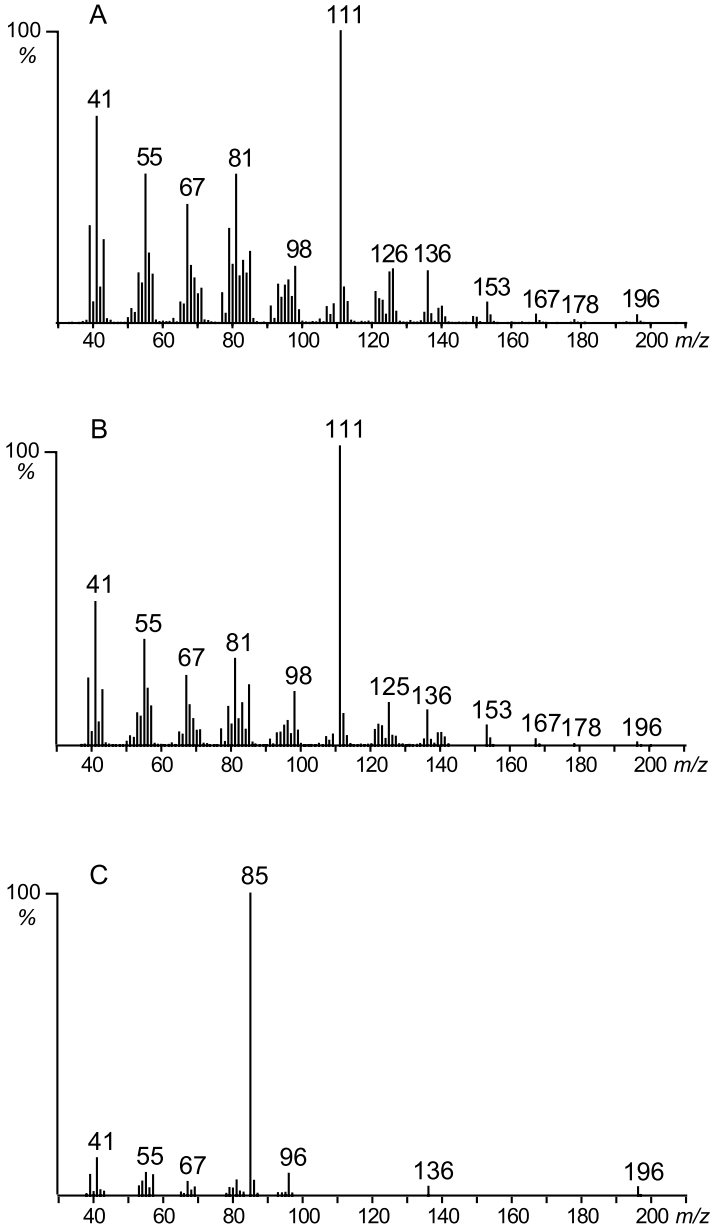
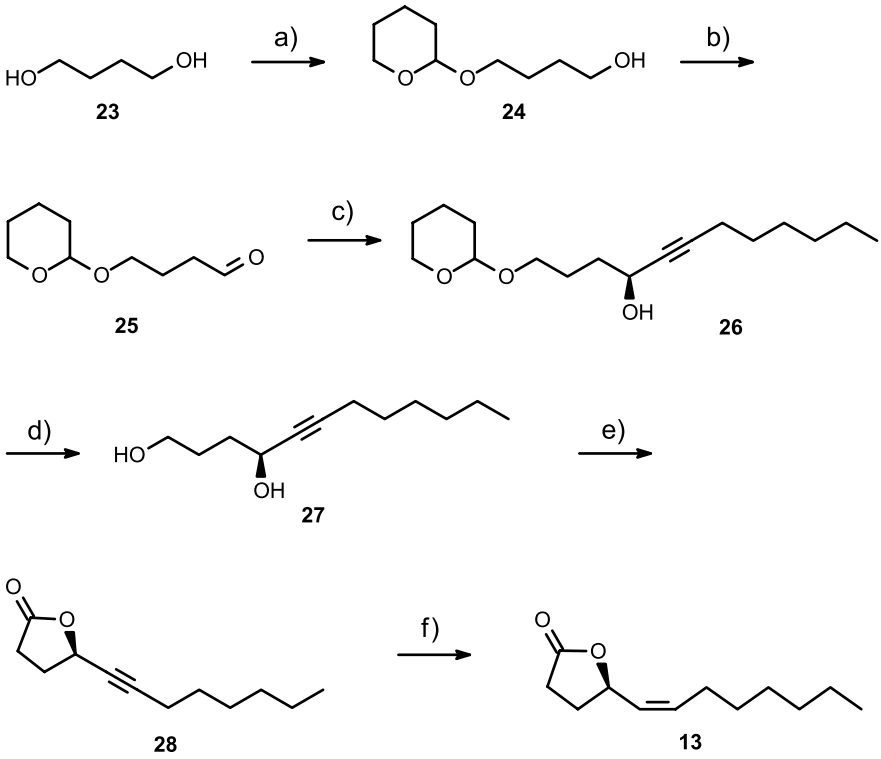


FIG. 3. Mass spectra of (A) (*R*,*Z*)-dodec-5-en-4-olide (**13**), (B) (*E*)-dodec-5-en-4-olide (**14**), and (C) (*Z*)-dodec-6-en-4-olide (**15**).



SCHEME 1. Total synthesis of (*R*,*Z*)-dodec-5-en-4-olide (*R*)-**13**. (a) 3,4-Dihydro-2*H*-pyran, *p*-TsOH, CH_3CN, 75%; (b) PDC, CH_2Cl_2, 71%; (c) racemic: octynylmagnesium bromide, Et_2O, 85%, enantioselective: $Zn(OTf)_2$, (+)-*N*-methylephedrine, Et_3N, 1-octyne, toluene, 80°C, 49%, 84% ee; (d) *p*-TsOH, MeOH, 89%; (e) TPAP, NMO, CH_2Cl_2, 68%; (f) H_2, Lindlar's catalyst, MeOH, 94%.

representing a new entry into the enantioselective synthesis of vinyllactones. The synthesis started with 1,4-butanediol (**23**), which was first transformed into its monoprotected THP ether **24** by using an excess of the diol (2.5 equiv.) to reduce the formation of the doubly protected diol. The aldehyde **25** was prepared from **24** by standard oxidation with PDC and used in a Grignard reaction with octynylmagnesium bromide to obtain 4-hydroxy-1-(2-tetrahydropyranyloxy)dodec-5-yne (*rac*-**26**). Deprotection afforded dodec-5-yne-1,4-diol (*rac*-**27**), which was oxidized with tetrapropylammonium perruthenate/*N*-methylmorpholine-*N*-oxide, furnishing dodec-5-yn-4-olide (*rac*-**28**). In this step, we successfully relied on the higher reactivity of the primary compared with the secondary alcohol function and on the formation of an intermediate lactol that would be further oxidized to

give the lactone (Bloch and Brillet, 1991; Ley et al., 1994). The alkyne was then partially hydrogenated using Lindlar's catalyst ($Pd/CaCO_3/Pb$). The product *rac*-dodec-5-en-4-olide (*rac*-**13**/*rac*-**14**) was obtained with a *Z*/*E* ratio of 8:1. The two synthetic compounds confirmed the identification of (*Z*)-dodec-5-en-4-olide as the main component from the bacterial extract and (*E*)-dodec-5-en-4-olide as the minor one. To clarify the absolute configuration of (*Z*)-dodec-5-en-4-olide, we carried out a stereoselective synthesis (Scheme 1) using the Carreira enantioselective alkynylation as the key step (Anand and Carreira, 2001). The stereogenic center was introduced by the enantioselective addition of 1-octyne to the *Si* face of the aldehyde **25** using $Zn(OTf)_2$ and (+)-*N*-methylephedrine as chiral ligand. The yield of this reaction was moderate (49%), and the enantioselectivity (>84% ee) was adequate. The ee of this step was determined by chiral GC of the final product **13**, assuming that no racemization took place during the final steps of the synthesis. Subsequent deprotection, oxidative cyclization, and partial hydrogenation as described for *rac*-**13** yielded predominantly (*R*,*Z*)-dodec-5-en-4-olide [buibuilactone, (*R*)-**13**]. The absolute configuration of natural **13** was detemined by GC on a chiral cyclodextrin stationary phase and found to be exclusively *R* for both bacterial species, identical to the configuration of the scarab beetle pheromone (Leal, 1991). Interestingly, the saturated analog 4-dodecanolide (**8**), present in both strains, showed the same configuration (*S* in this case because of the change of ligand priorities according to the Cahn, Ingold, and Prelog rules), but the other enantiomer was also present in minor amounts. The investigated samples gave *S*/*R* ratios of 92:8 for DFL-27 and 78:22 for BIO-204 (Figure 4). The absolute configuration of the minor compound (*E*)-dodec-5-en-4-olide (**14**) was not clarified because of overlapping peaks under different separation conditions.

Furthermore, strain DFL-27 produced another unsaturated lactone, dodec-6-en-4-olide (**15**), that was tentatively identified from its mass spectrum (Figure 3C). To elucidate the double-bond configuration, we carried out a total synthesis according to the route of Burger et al. (1977). The natural lactone matched both the GC and MS data of synthesized **15**.

Besides the lactones, we identified some sulfur compounds in the headspace extracts. Whereas *S*-methyl methylthiopropionate (**17**) and 4-methylthio-2-butanone (**19**) were only produced by BIO-204 in trace amounts, dimethyl trisulfide (**18**) and *S*-methyl methanethiosulfonate (**20**), one of the major compounds, were emitted by both strains under investigation. The structure of **19** was deduced from its mass spectrum and confirmed by total synthesis from 3-methylthiopropanal which will be reported elsewhere.

Additionally, trace amounts of pyrazines were found in the extracts of the bacteria. Whereas one sample of BIO-204 contained 2-butyl-3,6-dimethylpyrazine (**21**) and 2-isopentyl-3,6-dimethylpyrazine (**22**), only **21** was emitted by one culture of DFL-27. These tentative structures were derived from the mass

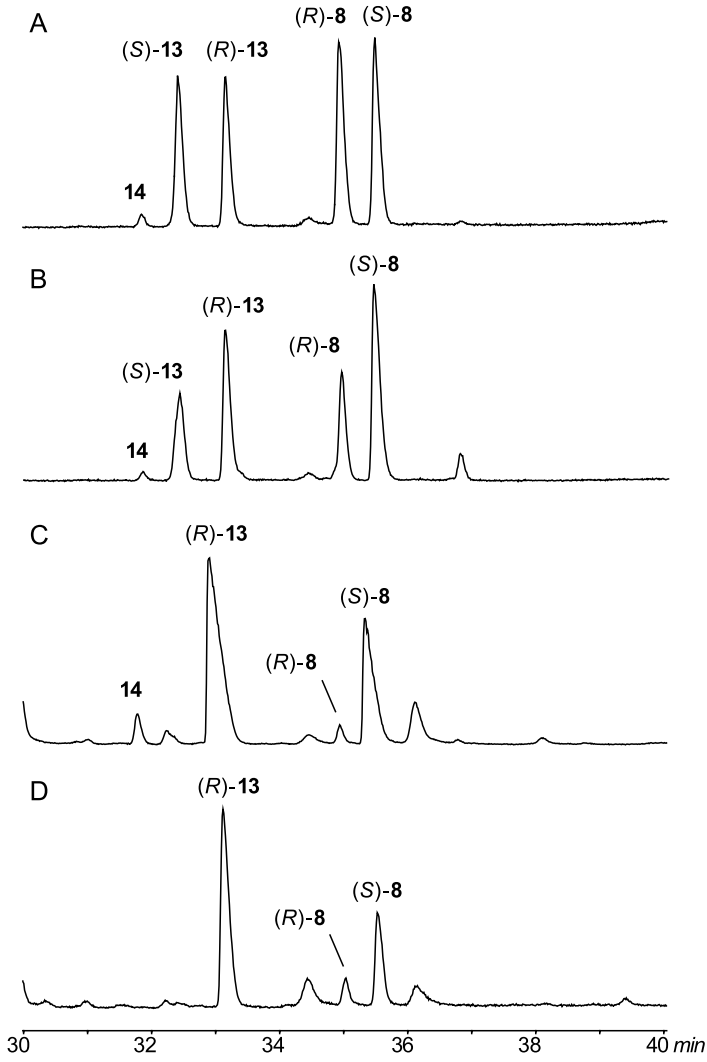
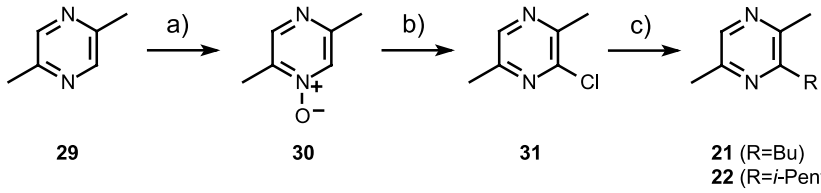


FIG. 4. Total ion chromatogram of (*Z*)-dodec-5-en-4-olide (**13**) and 4-dodecanolide (**8**) on a chiral 15-m heptakis-(6-*O*-TBDMS-2,3-di-*O*-acetyl)-β-cyclodextrin fused-silica capillary column, programmed from 100 to 180°C with 1.5°C/min. (A) Racemate; (B) racemate spiked with (*R*)-**13** and (*S*)-**8**; (C) strain BIO-204; (D) strain DFL-27.



SCHEME 2. Total synthesis of pyrazines **21** and **22**. (a) $NaBO_2 \cdot H_2O_2 \cdot 3H_2O$, AcOH, 80°C, 85%; (b) $POCl_3$, reflux, 79%; (c) RMgBr, $Fe(acac)_3$, THF, NMP, 0°C, 68%.

spectra. The mass spectrum of **21** (I = 1263) showed a molecular ion of m/z = 164 and a base peak at m/z = 122 that originated from the loss of a neutral C_3H_6 fragment by McLafferty rearrangement, showing the presence of a butyl or isobutyl moiety attached to the aromatic ring. The appropriate ions at m/z = 178 (molecular ion) and m/z = 122 (loss of C_4H_8 by McLafferty rearrangement) in the case of **22** (I = 1321) suggested a pentyl, isopentyl, or 2-methylbutyl side chain. Because of the difference between the retention indices of the two compounds (ΔI = 58), we assumed that **21** should contain a butyl group, whereas **22** was likely to be the higher isopentyl homologue. These suggestions were verified by synthesis (Scheme 2). Thus 2,5-dimethylpyrazine (**29**) was oxidized to its *N*-oxide **30** (Ohta and Ohta, 1985), which was transformed into 2-chloro-3,6-dimethylpyrazine (**31**) in boiling $POCl_3$ (Inoue et al., 1985; Sato and Fujii, 1994). This pyrazine was alkylated with butyl and isopentyl magnesium bromides, respectively, with $Fe(acac)_3$ as catalysis (Fürstner et al., 2002). The pyrazines **21** and **22** were identical to the natural compounds. Furthermore, tetramethylpyrazine and 2-ethyl-3,6-dimethylpyrazine were tentatively identified in the headspace extracts. Fürstner's Grignard-iron catalyst method represents an easy access to such substituted alkylpyrazines.

DISCUSSION

The major lactone in the odor bouquet of *Loktanella* strain BIO-204 and *Dinoroseobacter shibae* strain DFL-27 was identified as enantiomerically pure buibuilactone, (*R*,*Z*)-dodec-5-en-4-olide (**13**), accompanied by its diastereomer (*E*)-dodec-5-en-4-olide (**14**), and the regioisomer (*Z*)-dodec-6-en-4-olide (**15**). Buibuilactone was first identified as the sex pheromone of the cupreous chafer beetle, *Anomala cuprea* (Coleoptera: Scarabaeidae) (Leal, 1991). Other scarab beetle species using **13** as a pheromone component, sometimes in combination with other compounds such as (*E*)-2-nonen-1-ol and (*R*,*Z*)-tetradec-5-en-4-olide (**16**, japonilure), include *Anomala daimiana* (Leal, 1993), *A. octiecostata* (Leal

et al., 1994a), *A. albopilosa sakishimana* (Leal et al., 1994b), and *A. solida* (Tóth et al., 2003). Furthermore, (*R*,*E*)-dodec-5-en-4-olide (**14**) was identified as a female-specific component of *A. albopilosa sakishimana*. This compound was also present in the investigated bacterial headspace samples.

Several enantioselective syntheses of buibuilactone have been reported, but the control of the stereochemistry with high enantiomeric purity always proved to be challenging. Different approaches, such as the use of chiral reducing agents and further purification by enantiomeric resolution (Leal, 1991), kinetic resolution by application of enzymes (Fukusaki et al., 1992, 1998), or synthetic routes using chiral pool synthons (Koseki et al., 1993), have been used. Furthermore, structurally related (*R*)-japonilure has been synthesized in high enantiomeric purity (>99% ee) from (*R*)-glutamic acid (Tumlinson et al., 1977). Other syntheses of japonilure using chiral precursors started from D-ribose (Ebata et al., 1992; Koseki et al., 1993), D-glucose (Salas-Reyes, 1995), or (*S*)-glycerol-2,3-acetonide (Chattopadhyay et al., 1990). Several synthetic approaches to japonilure using asymmetric reducing agents for the preparation of chiral propargylic alcohol intermediates have been developed, e.g., BINAL-H (Nishizawa et al., 1981; Senda and Mori, 1983; Noyori et al., 1984; Mori, 1996) or 9-(pinan-3-yl)-9-borabicyclo[3.3.1]nonane (Midland and Tramontano, 1980; Midland and Nguyen, 1981; Baker and Rao, 1982). The optical yields in these reduction steps varied widely and have rarely been satisfactory (74–98% ee). Furthermore, in these procedures, up to three equivalents of the chiral reducing agents were necessary. Other synthetic routes to japonilure used chiral auxiliaries (Nemoto et al., 1990; Hiscox and Matteson, 2000; Papillon and Taylor, 2002). The separation of the enantiomers of a racemic intermediate in the synthesis of (*R*)-japonilure was carried out by kinetic resolution using enzymes (Sugai et al., 1990; Fukusaki et al., 1991) or chiral derivatization reagents for the formation of easily separable diastereomers (Pirkle and Adams, 1979; Sato et al., 1979), thus limiting the yield in these key steps to 50%. In contrast to all these synthetic routes leading to (*R*)-**13** or (*R*)-**16**, respectively, our enantioselective synthesis of (*R*)-**13** was carried out with only catalytic amounts of (+)-*N*-methylephedrine and $Zn(OTf)_2$ in the key step, the enantioselective Carreira alkynylation. The enantiomeric excess of (*R*)-**13** was 84%.

The biosynthesis of buibuilactone in *A. cuprea* has been elucidated (Leal et al., 1999). Hexadecanoic acid is transformed to (*Z*)-9-hexadecenoic acid by the action of a Δ^9-desaturase. The stereogenic center of natural buibuilactone is introduced by an 8-hydroxylase furnishing (*R*,*Z*)-8-hydroxyhexadec-9-enoic acid with high chain length specificity. Two β-oxidation steps and subsequent cyclization complete the biosynthesis of **13** by *A. cuprea*. Analogous enzymes are also known from the Alphaproteobacteria. The Δ^9-desaturase (stearoyl-CoA-desaturase, E.C. 1.14.19.1) is, besides Eukarya, also present in Prokarya such as the fully sequenced bacteria *Rhodospirillum baltica* and *Rickettsia typhi*, which

belong to the Alphaproteobacteria subclass such as the strains analyzed here (KEGG database, http://www.genome.jp/dbget-bin/www_bget?ec:1.14.19.1). The stereospecific enzyme palmitoleic acid hydroxylase belongs to the fatty acid hydroxylase family of enzymes (http://www.genome.jp/dbget-bin/www_bget?ec:1.14.15.3). Although they have originally been described as eukaryotic enzymes, these enzymes have been recently found in some bacteria, especially in *Bacillus* sp. and in Alpha- and Gammaproteobacteria, including an uncultured marine Alphaproteobacterium (de la Torre et al., 2003; accession number Q6UCQ5 in the EMBL database). Thus, buibuilactone may be formed by the bacteria by a similar pathway as present in the beetles.

The isomeric (*Z*)-dodec-6-en-4-olide (**15**), found in the headspace of DFL-27, was previously identified as a constituent of gland secretions of several mammals, including the tarsal gland secretions of the black-tailed deer *Odocoileus hemionus columbianus* (Brownlee et al., 1969; Müller-Schwarze, 1969) and the interdigital gland secretions of the bontebok *Damaliscus dorcas dorcas* (Mammalia: Bovidae) and the blesbok *Damaliscus dorcas phillipsi* (Burger et al., 1977, 1999). It was later shown that this compound does not originate from the tarsal glands but from the animal's urine and is extracted by the tarsal hair tuft (Müller-Schwarze et al., 1977). Interestingly, the aerobic bacterium *Planococcus citreus*, isolated from the secretion of both species, produces **15** when cultured (Burger et al., 1999). Furthermore, **15** was identified in plant extracts of *Polianthes tuberosa* (Maurer and Hauser, 1982), *Morinda citrifolia* (Farine et al., 1996), and in the fungus *Penicillium roqueforti* (Chalier and Crouzet, 1998).

Alkylated pyrazines are known from a number of Hymenoptera (Wheeler et al., 1982), and the occurrence of pyrazines in insects has been reviewed (Brophy, 1989). The two pyrazines, 2-butyl-3,6-dimethylpyrazine (**21**) and 2-isopentyl-3,6-dimethylpyrazine (**22**), respectively, were minor constituents of the headspace volatiles of the bacteria. Compound **22** is an alarm pheromone of the ponerine ants *Odontomachus hastatus* and *O. clarus* (Wheeler and Blum, 1973), and **21** and **22** both occur in the mandibular gland secretions of the ant *Dinoponera australis* (Oldham and Morgan, 1993; Oldham et al., 1994). In more recent investigations on other species of the Ponerinae subfamily, **21** and **22** were found in mandibular and abdominal gland secretions (Morgan et al., 1999, 2003), and **22** was identified in cephalic extracts of two ponerine *Mesoponera* spp. (Fales et al., 1988) and two *Anochetus* spp., respectively (Jones et al., 1999). The isopentyl derivative **22** was also present in the heads of the argentine ant *Iridomyrmex humilis*, now called *Linepithema humile* (Cavill and Houghton, 1974a, b). Furthermore, this pyrazine was found as a trace compound in cephalic secretions of female *Epeolus cruciger* bees (Tengö et al., 1982). The related pyrazine 2-ethyl-3,6-dimethylpyrazine is a trail pheromone of several ant species belonging to the Myrmicinae (Billen and Morgan, 1998).

The structural similarities between the insect pheromones and bacterial volatiles are striking. The co-occurrence of single unsaturated lactones and a range of saturated analogs parallels the situation in some insect pheromone glands in which an active unsaturated compound is accompanied by several inactive analogs. The different enantiomeric purities of the lactones may reflect enzyme specificity targeted toward **13**. Furthermore, we found lactones in only relatively few of the many strains we investigated (Dickschat and Schulz, unpublished). However, we do not know yet the biological function of the lactones in the ecology of these marine bacteria.

Finding identical compounds in two totally independent habitats (marine and terrestrial) raises the question as to whether symbiotic bacteria or other microorganisms might be involved in the formation of the chafer or ant pheromones, as has been shown to be the case for phenol in another scarabaeid, the grass grub chafer, *Costelytra zealandica* (Hoyt et al., 1971). Other examples in which bacteria or other microorganisms appear to be involved in insect pheromone biosynthesis include guaiacol in the locust *Schistocerca gregaria* (Dillon et al., 2000), oxidized terpenes in bark beetles (Brand et al., 1975, 1976; Leufvén et al., 1984), and mellein in a male butterfly (Kunesch et al., 1987). The existence of elaborate symbiotic insect–bacterial relationships has been shown (Boursaux-Eude and Gross, 2000), and, therefore, the possibility exists that the normally tightly regulated pheromone production may nevertheless be performed by symbiotic bacteria, as seems to be the case in the mammal *Damaliscus dorcas* mentioned above.

Contrary to lactones, pyrazines represent one of the major compound classes of bacterial volatiles (Dickschat and Schulz, unpublished), and the same or similar compounds are also relatively widespread as pheromones in ants (Brophy, 1989; Billen and Morgan, 1998). Ants are well known for the presence of multiple endosymbionts (Schröder et al., 1996; van Borm et al., 2002), and, therefore, a bacterial involvement in the production of these compounds may be possible.

Nevertheless, other explanations for the structural similarities exist. The compounds under discussion might be structurally well suited for their tasks, so that convergent evolution in different organisms may have resulted in the formation of identical compounds, which might not interfere because of biological separation. Another explanation might be nonsymbiotic relationships between bacteria and insects, as well as the exchange of genes during evolution, leading to similar biosynthetic options. More research on the biosynthesis and biology of insect pheromones and bacterial volatiles is needed to address some of these questions.

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RAPID COMMUNICATION

SURVEY OF THE CHEMICAL DEFENCE POTENTIAL OF DIATOMS: SCREENING OF FIFTY ONE SPECIES FOR α,β,γ,δ–UNSATURATED ALDEHYDES

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Abstract—In recent years a negative influence of diatom-derived α,β,γ,δ-unsaturated aldehydes (PUA) on the reproductive success of copepods and invertebrates has been suggested. Since adverse chemical properties of diatoms would question the traditional view of the marine food web, this defense mechanism has been investigated in detail, but the PUA-release by test organisms has only been determined in a few cases. The observed effects were nevertheless frequently discussed from a general point of view often leading to contradictory conclusions. We have examined the PUA-production of 51 diatom species (71 isolates) in order to provide a basis for the interpretation of laboratory and field results on the influence of diatom food on the reproductive success of their consumers. PUA-production is species and strain dependent. Thirty-six percent of the investigated species (38% of the cultivated isolates) release α,β,γ,δ-unsaturated aldehydes upon cell disruption in concentrations from 0.01 to 9.8 fmol per cell. *Thalassiosira rotula* and *Thalassiosira pacifica*, major spring-bloom forming diatoms

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isolated from Roscoff (Bretagne, English Channel, France) and Puget Sound (Washington, USA) were among the PUA-producing strains.

Key Words—Alga/herbivore interactions, plankton, pentafluorobenzylhydroxylamin, copepod, reproductive success

INTRODUCTION

Diatom-derived α,β,γ,δ-unsaturated aldehydes (polyunsaturated aldehydes, PUA) can interfere with the hatching success and larval development of copepods as well as with the embryonic development of invertebrates (see Ianora et al., 2003 and Pohnert, 2005 for reviews). Diatoms are unicellular algae that contribute substantially to the phytoplankton and are thus an important base of the marine food web. A generally negative impact of diatoms on herbivores would have major implications for the classical concept of plankton ecosystem functioning. Therefore, numerous studies, mainly based on laboratory experiments, have been carried out to clarify this predator-prey relationship. While some authors have proposed a deleterious effect of diatoms (Ianora et al., 2003), others have not found any adverse effects (e.g., Jonasdottir et al., 1998). Moreover, no general negative relationship between copepod egg hatching and diatom biomass was detected in the global field survey by Irigoien et al. (2002). Most of these studies correlated hatching parameters to the presence or absence of diatoms rather than monitoring PUA production of the diet, even if this is the postulated determining factor in these interactions. This is surprising, since only a few marine and fresh water diatoms have been investigated for PUA-production (Pohnert, 2005). Indeed, some of the controversially discussed results on reproductive parameters (Paffenhöfer et al., 2005) might be explained by variability of PUA production in different diatom species. To overcome this limitation and to provide a basis for further ecological and modelling studies, we conducted a survey of volatile PUA-production from representative diatoms of the major classes by using a protocol based on in situ trapping of the reactive metabolites (Wichard et al., 2005).

METHODS AND MATERIALS

Cultivation. Seventy one diatom-isolates from different sources were investigated (Table 1). All isolates were grown in stationary cultures using 250 ml jars containing 100 ml artificial medium (Table 1). Sylvania (Germany) "Cool white deluxe (F36W 840, 4000K)" tubes provided illumination. A light regime of 16:8 (light/dark) with 30–40 $\mu E/m^2/sec$ light intensity was used. Generally, the growth temperature was 15.5°C, except for *Thalassiosira*

TABLE 1. OVERVIEW OF THE ISOLATES INVESTIGATED BY SOLID PAHSE MICROEXTRACTION AND PFBHA-DERIVATISATION

Species	SPME/ PFBHA[a]	Detected PUA	Medium[b]	Source or culture no.
Actinocyclus subtilis	•/•	–	f_2	CCAP 1000/1
Amphiphora paludosa	–/•	+[c]	f_2	SAG 15.83
Asterionella formosa	•/•	–[k]	WC	SAG 8.95
Asterionellopsis glacialis	•/•	+	f_2	PLY 607
Chaetoceros calcitrans	•/•	–	f_2	CCAP 1010/11
Chaetoceros compressus	•/•	+	f_2	PLY 550
Chaetoceros muelleri	•/•	–	f_2	CCMP 1316
Coscinodiscus granii	•/•	–	f_2	Unknown
Coscinodiscus sp.	–/•	–	f_2	RCC 773[d]
Cyclotella meneghiniana	•/•	–	WC	SAG 1020-1a
Ditylum brightwellii	•/•	–	f_2	PLY 609
Ditylum brightwellii	•/–	–	f_2	Friday Harbor[e]
Ditylum brightwellii	–/•	–	f_2	RCC 775[d]
Fragilaria capucina	•/•	–	WC	Lake Constance[f]
Fragilaria sp.	•/•	+	WC	Lake Constance[f]
Gomphonema parvulum	•/–	–[k]	WC	SAG 1032-1
Guinardia deliculata	–/•	+	K	Roscoff[d]
Guinardia striata	–/•	–	K	Roscoff[d]
Melosira nummuloides	•/•	+	f_2	CCAP 1048/6
Melosira sulcata	•/•	+	f_2	Jiaozhou Bay, China
Navicula pelliculosa	•/•	–	WC	SAG 1050-3
Navicula sallinicola	–/•	–	f_2	SAG 40.96
Navicula sp.	•/•	–	f_2	RCC 781[d]
Navicula sp.	•/–	–	K	RCC 457
Navicula transitans	•/•	–	f_2	RCC 80
Nitzschia sp.	•/•	–	f_2	RCC 782[d]
Nitzschia closteridium	–/•	–	f_2	RCC 81
Nitzschia curvilineata	–/•	–	f_2	SAG 48.91
Nitzschia frustulum	–/•	–	br	SAG 1052-2
Odontella regia	•/•	+[c]	f_2	RCC 772[d]
Odontella sinensis	•/–	–	f_2	PLY 606
Paralia sulcata	•/•	–	f_2	DML[g]
Phaeodactylum tricornutum	•/•	–	br	UTEX 646
Phaeodactylum tricornutum	•/•	–	br	SAG 1090-1a
Phaeodactylum tricornutum	–/•	–	br	SAG 1090-1b
Phaeodactylum tricornutum	•/•	–	f_2	PLY 100
Pleurosigma normanii	•/–	–	f_2	MBA[g]
Pseudonitzschia sp.	•/•	–	f_2	PLY 611
Rhizosolenia setigera	–/•	–	K	Roscoff[d]
Rhizosolenia setigera	–/•	–	f_2	CCMP 1820
Skeletonema costatum	•/•	+	f_2	RCC 75
Skeletonema costatum	–/•	+	f_2	SAG 19.99
Skeletonema costatum	•/–	+	f_2	CCMP 781
Skeletonema costatum	•/–	+	f_2	CCMP 784

TABLE 1. CONTINUED

Species	SPME/ PFBHA[a]	Detected PUA	Medium[b]	Source or culture no.
Skeletonema costatum	•/–	+	f_2	CCMP 2092
Skeletonema pseudocostatum	•/•	+	f_2	See reference[h]
Skeletonema subsalsum	–/•	+	WC	SAG 8.94
Stephanodiscus hantzschii	•/•	–	WC	Lake Constance[f]
Stephanodiscus minutulus	•/•	–	WC	SAG 49.91
Stephanophyxis turris	•/•	–[k]	f_2	DML[g]
Thalassionema nitzschioides	–/•	–	K	RCC 785[d]
Thalassiosira aestivalis	•/•	+	f_2	Dabob Bay (779)[e]
Thalassiosira anguste-lineata	•/•	+	f_2	Dabob Bay (779)[e]
Thalassiosira eccentrica	•/•	–	f_2	CCAP 1085/6
Thalassiosira minima	•/•	+	f_2	CCAP 1085/8
Thalassiosira nordenskioeldii	•/•	+	f_2	Dabob Bay (748)[e]
Thalassiosira pacifica	•/•	+	f_2	Dabob Bay (779)[e]
Thalassiosira pseudonana	•/•	–	f_2	CCAP 1085/12
Thalassiosira pseudonana	•/•	–	f_2	CCMP 1335
Thalassiosira pseudonana	–/•	–	br	SAG 1020-1b
Thalassiosira punctigera	•/–	–	f_2	Point Wells (748)[e]
Thalassiosira rotula	•/•	+	f_2	CCMP 1018
Thalassiosira rotula	•/•	+	f_2	CCMP 1647
Thalassiosira rotula	•/•	+	f_2	CCMP 1812
Thalassiosira rotula	–/•	+	f_2	RCC 776[d]
Thalassiosira rotula	–/•	+	f_2	Point Wells (805)[e]
Thalassiosira rotula	•/–	+	K	RCC 290
Thalassiosira sp.	•/–	+	K	RCC 349
Thalassiosira weissflogii	•/•	–	f_2	Unknown
Thalassiosira weissflogii	–/•	–	br	SAG 122.79
Thalassiosira weissflogii	–/•	–	f_2	RCC 76
Prorocentrum micans[i]	•/–	–	f_2	Unknown
Prorocentrum minimum[i]	•/•	–	f_2	RCC 291

aestivalis and *Skeletonema costatum* (CCMP 784), which were grown at 13°C and 23°C, respectively. Cultures reached the stationary phase after 2–3 wk, when 60–90 ml (10^4-10^6 cells/ml culture medium, depending on species) were harvested. The cell morphology of each culture was checked prior to harvest with light microscopy. Cells were counted with a Neubauer-improved haemocytometer in four replicates. The counting variance ranged from 10–35%.

Quantitative PUA Analysis. The cultures were harvested by filtration as described in Wichard et al. (2005). For a first rapid screening of the cultivated isolates, solid phase microextraction was performed with a polydimethylsiloxan fiber after wounding by sonication as described in Pohnert et al. (2002). To quantify PUA release upon cell damage, a protocol based on derivatization of PUA with O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamin hydrochloride

(PFBHA·HCl) and subsequent GC/MS (EI) analysis was applied (Wichard et al., 2005). The limit of quantification for PUA in concentrated diatom cultures was 5 ng/ml. Each analysis was performed in triplicate.

Quantitative Chlorophyll a + c analysis. In cases where PUA were determined, the chlorophyll content of the diatom isolates was quantified as well. The extraction in 90% acetone and quantification was performed in triplicate according to the standardized method No 446.0 (U.S. Environmental protection agency: Microbiological and Chemical Exposure Research and references herein). Jeffrey and Humphrey's trichromatic equations were applied: Chlorophyll a (mg/l) = 11.85 E_{664} – 1.54 E_{647} – 0.08 E_{630} and Chlorophylls $c_1 + c_2$ (mg/l) = −1.67 E_{664} – 7.60 E_{647} + 24.52 E_{630}. Each value was corrected by the absorbance at 750 nm. The ratio of PUA to Chl a + c of identical culture batches was calculated as PUA/Chl (ppm) = PUA (μg/ml)/Chl (μg/ml) × 1,000,000.

Determination of Cell Volume. Living cells were measured microscopically in planar view (minimum: 20 cells). Linear measurements were converted to cell volume using different geometric approximations: a cylinder for *Chaetoceros compressus*, *Guinardia deliculata*, *Melosira* spp., *Skeletonema subsala-*

Footnotes to Table 1

Note. A cross (+) identifies a diatom as PUA-producer. Among those, several strains were selected for quantification (see Table 2).

[a] Applied analytical method.

[b] f_2 = marine enriched medium (artificial seawater) and K = K medium (filtered seawater), see for references Pohnert et al., 2002; WC = fresh water medium; br = brackish water: f_2 medium diluted with Chu-12 medium (2 + 1), see for references Carotenuto and Lampert, 2004.

[c] Only traces of PUA were detected after PFBHA derivatisation.

[d] Strains were isolated from coastal waters off Roscoff in 2004.

[e] Strains were isolated (cruise number of research vessel "C.A. Barnes" in parenthesis) from Dabob Bay, Point Wells or Friday Harbour (Puget Sound and San Juan Island, respectively, Washington, USA) in 2001–2003.

[f] See Carotenuto and Lampert, 2004.

[g] No strain number is available.

[h] Genetically described in Pohnert et al., 2002.

[j] Dinoflagellates often used as a control diet for copepods.

[k] Acidic polyunsaturated aldehydes were detected: 12-oxo-dodeca-5,8,10-trienoic acid (*A. formosa*, *S. turris*) and 9-oxo-nona-5,7-dienoic acid (*G. parvulum*).

Abbreviations: CCAP (DML) Culture Collection of Algae and Protozoa (Dunstaffnage Marine Laboratory, Scotland); CCMP = Centre for Culture of Marine Phytoplankton Main, USA; PLY (MBA) = Marine Biological Association Plymouth, England; RCC = Roscoff Culture Collection, France; SAG = Culture Collection Göttingen, Germany; UTEX = The Culture Collection of Algae, University of Texas at Austin, TX, USA.

tum, and *Thalassiosira* spp.; a cylinder + 2 half spheres for *Skeletonema costatum* and *Skeletonema pseudocostatum*, and a cone for *Asterionellopsis glacialis*. The carbon content was determined by the carbon to volume relationship based on the equation C (pg/cell) = 0.288 × volume$^{0.811}$ (Menden-Deuer and Lessard, 2000). The PUA to carbon ratio was calculated as PUA/C (ppm) = PUA (fg/cell)/C (fg/cell) × 1,000,000.

RESULTS AND DISCUSSION

Seventy one diatom-isolates were analyzed for PUA-formation upon cell damage by sonication. The diatoms were either obtained from algal collections or freshly isolated from coastal waters off Roscoff (48°45′ N and 3°58′ W, Bretagne, France) and during several cruises to Dabob Bay, Point Wells and Friday Harbour (47° 46.14′N and 122° 50.10′W/47° 44.63′ N and 122° 25.34′ W/48.535° N and 123.005° W, Washington, USA). A total of 50 different species was investigated, with an emphasis on the family Thalassiosiraceae and the species *Phaeodactylum tricornutum*, because these diatoms are widely used in bioassays on the reproductive success of copepods (Miralto et al., 1999; Pohnert et al., 2002; Paffenhöfer et al., 2005). Under defined culture conditions (see Method section), 27 PUA-producers were identified among the 71 isolates investigated (Table 1). Out of the PUA producers, two released the unsaturated aldehydes only in trace amounts. The PUA-production upon wounding of 20 selected isolates (18 marine and 2 freshwater) was quantified during the stationary growth phase. PUA-production ranged from 0.01 fmol PUA/cell (*Thalassiosira nordenskioeldii*) to 9.8 fmol PUA/cell (*Thalassiosira pacifica*) (Table 2). This wide range over four orders of magnitude, as well as the isolate-dependent variability of structurally different unsaturated aldehydes, reflects a high plasticity within the Bacillariophyceae.

Since the calculation of PUA per cell underestimates the aldehyde contribution of species with low cell volume but probably high cell abundance in a typical herbivore diet, the PUA to carbon (PUA/C) and the PUA to chlorophyll a + c (PUA/Chl) ratio were also calculated. With respect to the PUA/Chl and PUA/C ratios, other dominant producers, such as *Skeletonema pseudocostatum* (PUA/Chl = 40,650 ppm) or *Skeletonema costatum* (SAG 19.99, PUA/C = 488 ppm), come to the fore.

Within the Bacillariophyceae, more than half of the investigated species do not produce PUA upon wounding in the stationary growth phase. In the light of the ongoing discussion about the influence of diatoms on herbivores, this result stresses that a general PUA-mediated effect can not be assumed for any given phytoplankton bloom, but that a species and strain-specific analysis is required.

TABLE 2. QUANTIFICATION OF PUA PER CELL, PER CHLOROPHYLL a + c AND PER CARBON CONTENT (STATIONARY PHASE)

Producer of polyunsaturated aldehydes	Analysis of PUA						Analysis of Chl a + c		Analysis of carbon		
	PUA	C7:2	C8:2	C8:3	C10:2	C10:3	Chl a + c	PUA/Chl a + c	cell vol.	C/cell	PUA/C
	fmol/cell	% of total PUA					$\mu g/10^6$ cells	ppm	μm^3	pg/cell	ppm
Thalassiosira pacifica	9.81 ± 0.069	70	19	11	0	0[a]	6.58 ± 0.20	113,368	1,256	94	11,859
Melosira nummuloides	8.68 ± 0.424	7	72	0	2	19	11.7 ± 1.52	95,584	5,011	288	3,717
Thalassiosira rotula (CCMP 1647)	6.35 ± 0.289	5	32	11	2	50	3.36 ± 0.23	255,331	1,574	113	7,364
Thalassiosira rotula (origin: Roscoff)	5.69 ± 0.472	7	16	18	0	58	36.4 ± 0.16	21,573	12,972	624	1,348
Chaetoceros compressus	2.82 ± 0.635	31	12	0	8	49	3.67 ± 0.17	103,668	1,704	120	2,395
Thalassiosira aestivalis	1.54 ± 0.266	78	16	6	0	0	70.6 ± 6.96	2,462	4,630	270	525
Thalassiosira anguste-lineata	1.53 ± 0.079	69	17	14	0	0	48.5 ± 7.38	3,597	2,245	150	913
Thalassiosira rotula (origin: Point Wells)	1.27 ± 0.108	24	8	41	0	27	46.6 ± 4.50	3,459	16,406	755	212
Thalassiosira rotula (CCMP 1812)	1.04 ± 0.030	24	28	23	0	24	7.69 ± 0.55	9,672	3,497	215	588
Skeletonema pseudocostatum	0.38 ± 0.041	50	49	1	0	0	1.10 ± 0.31	40,650	186	20	2,343
Thalassiosira rotula (CCMP 1018)[c]	0.22 ± 0.048	31	45	24	0	0	8.10 ± 0.28	3,179	1,593	114	278
Guinardia deliculata	0.18 ± 0.015	100	0	0	0	0	17.4 ± 1.01	1,122	3,712	226	94
Skeletonema costatum (RCC 75)	0.13 ± 0.016	58	38	3	0	0	2.21 ± 0.11	6,623	286	28	578
Fragilaria sp.	0.10 ± 0.010	65	32	0	3	0	n.d.	n.d.	452[b]	41	252
Asterionellopsis glacialis	0.05 ± 0.005	68	28	4	0	0	6.09 ± 0.03	960	176	19	343
Thalassiosira minima	0.05 ± 0.002	23	10	0	0	61	1.16 ± 0.18	5,960	172	19	393
Skeletonema subsalsum	0.04 ± 0.014	0[a]	80	0	20	0	n.d.	n.d.	227	23	303

TABLE 2. CONTINUED

Producer of polyunsaturated aldehydes	Analysis of PUA						Analysis of Chl a + c		Analysis of carbon		
	PUA	C7:2	C8:2	C8:3	C10:2	C10:3	Chl a + c	PUA/Chl a + c	cell vol.	C/cell	PUA/C
	fmol/cell	% of total PUA					µg/10^6 cells	ppm	µm^3	pg/cell	ppm
Thalassiosira nordenskioeldii	0.01 ± 0.004	0[a]	100	0	0	0	35.2 ± 2.19	121	3,776	229	14
Melosira sulcata	0.01 ± 0.001	0	100	0	0	0	n.d.	n.d.	369	35	36
Skeletonema costatum (SAG 19.99)	0.01 ± 0.001	81	19	0	0	0	3.45 ± 0.13	260	11	2	488

Note. Species sorted by descending total amount of PUA released (fmol/cell). Valuse: mean ± SD.

[a] Traces were detected;

[b] See reference (Carotenuto and Lampert, 2004);

[c] While Pohnert et al. (2002) did not detect any PUA in this strain using solid phase microextraction, the more sensitive PFBHA-derivatisation reveals that this species has to be considered as a weak PUA-producer. Isomeric mixtures of C7:2 = 2,4-heptadienal, C8:2 = 2,4-octadienal, C8:3 = 2,4,7-octatrienal, C10:2 = 2,4-decadienal and C10:3 = 2,4,7-decatrienal were detected.

Recently, the hypothesis that PUA-production could be the reason for poor copepod reproductive success during spring blooms of diatoms was proposed (Ianora et al., 2004). In this context, it is interesting to note that some of the most abundant spring-bloom forming species like *Thalassiosira* spp. (e.g. *Th. rotula* and *Th. pacifica*) release high amounts of PUA. These species were isolated from different habitats, such as the Adriatic Sea (Miralto et al., 1999), the coastal waters off Roscoff (NE Atlantic) and Dabob Bay (NE Pacific).
Because the ability to produce PUA is distributed heterogeneously in the major classes of Bacillariophyceae, one cannot predict the defensive potential of certain species. Moreover, PUA-production within different isolates of one species ranges widely, and thus case-specific chemical investigations accompanying bioassays are required. For example, the different *Thalassiosira rotula* isolates investigated release PUA in a wide range of concentrations from 0.15 to 6.34 fmol/cell. In this study, only cultures in the stationary growth phase were investigated. This culture condition was selected since it is also used in most laboratory investigations. Additional variation of PUA-production during different phases of diatom blooms or growth phases of cultures might have to be taken into account as well.

Based on this survey, we not only recommend performing future bioassays along with chemical analyses, but also urge for a reconsideration of the general conclusions drawn in the past. It is likely, that the observed reduction of hatching success in several studies/regions may not be due to the formation of deleterious PUA, but may have other causes. On the other hand, in regions where major PUA producers are the main constituents of blooms, there might be effects on the reproduction of herbivorous grazers and their population dynamics (Ianora et al. 2004; Halsband-Lenk et al. unpublished). Whether secondary production can be significantly affected by this chemically mediated interaction in such ecosystems requires further investigation.

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RAPID COMMUNICATION

DIFFERENTIAL UTILIZATION OF PYRROLIZIDINE ALKALOIDS BY MALES OF A DANAID BUTTERFLY, *Parantica sita*, FOR THE PRODUCTION OF DANAIDONE IN THE ALAR SCENT ORGAN

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Abstract—Males of the chestnut tiger butterfly, *Parantica sita*, secrete danaidone as a major component from the alar androconial organ (sex brand). Since danaidone has been postulated to be derived from various pyrrolizidine alkaloids (PAs), which males ingest as adults from PA-containing plants, we conducted oral administration tests of several PAs to examine their availability for danaidone production by *P. sita* males. Males fed with a mixture of intermedine (80%) and lycopsamine (20%) produced danaidone at an average of 25.7 μg per individual, which was comparable to that found in field-caught males. In contrast, a smaller amount of danaidone (5.7 to 7.0 μg/male) was formed when males ingested retronecine or heliotrine, and those fed with an HCl salt of monocrotaline or retrorsine produced only traces of danaidone (<0.5 μg/male). In addition, males showed a strong feeding response to intermedine/lycopsamine, whereas the other PAs elicited no positive feeding behavior. These results indicate that, unlike the arctiid moths, *P. sita* males can only successfully convert limited chemical types of PAs into danaidone, and further suggest that in the field, males selectively ingest particular PAs that are readily transformable into danaidone.

Key Words—Androconial secretion, sex brand, *Parantica sita*, danaidae, pyrrolizidine alkaloids, biosynthesis, danaidone, dihydropyrrolizine.

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INTRODUCTION

Males of most danaid butterflies possess characteristic androconial organs, viz. abdominal hairbrushes (hairpencils) and alar patch- or pouch-like glands (sex brand), which secrete a variety of compounds that are thought to serve as pheromones during the courtship behavior (Boppré and Vane-Wright, 1989). Dihydropyrrolizine derivatives such as danaidone, hydroxydanaidal, and danaidal are the components most frequently encountered in danaid secretions (Schulz, 1998). Adults of danaids, particularly males, often gather on withering or dead plants containing pyrrolizidine alkaloids (PAs) to ingest the alkaloids. Plant-acquired PAs are sequestered in the body tissue and used as precursors for pheromone production and also for chemical defense against predators (Eisner and Meinwald, 1987). Similar pharmacophagous acquisition and utilization of PAs have also been reported for many ithomiid butterflies (Schulz et al., 2004) and larvae of some arctiid moths (Hartmann et al., 2003).

Males of *Parantica sita*, an Asian danaid butterfly, have been reported to secrete two dihydropyrrolizines, danaidone (major) and hydroxydanaidal (minor), from both the sex brand and hairpencils (Komae et al., 1983). In some arctiid moths, the biosynthetic pathway of hydroxydanaidal from PA precursors has hitherto been studied intensively (Schulz et al., 1993; Hartmann et al., 2003), while little is known about the mechanisms by which danaid butterflies produce dihydropyrrolizines. Since the finding by Schneider et al. (1975) that danaidone is derived from PAs in *Danaus* butterflies, danaid butterflies are also believed to make use of diverse PAs for the production of dihydropyrrolizines. This study investigated whether PAs containing various structures of necic acids were equally utilized by *P. sita* males for danaidone formation in the sex brand.

METHODS AND MATERIALS

Insects. Males of *P. sita* originated from the population of Hiroshima and its neighbor prefectures of Japan. Larvae were reared on potted *Cynanchum caudatum*, *Tylophora tanakae,* or *Marsdenia tomentosa* plants (Asclepiadaceae) under standard laboratory conditions (16L-8D, 23–24°C). Newly emerged male adults were kept in transparent plastic chambers (25 × 35 cm; height, 21 cm) at 25°C under a 16L-8D regime and fed with 15% aq. sucrose solution once daily throughout the experiments.

PAs. Monocrotaline (Aldrich, purity: 99%), retrorsine (Aldrich, 97%), and heliotrine (Latoxan, 98%) were commercially purchased. Since monocrotaline and retrorsine were sparingly soluble in water, their HCl salts were prepared. Retronecine (98% by GC and NMR) was prepared from monocrotaline

(Bogner and Boppré, 1989). A PA mixture composed of intermedine (80%) and lycopsamine (20%) was isolated from a methanol extract of *Eupatorium chinense* (Compositae) roots collected in Hiroshima prefecture, and purified by ion-exchange chromatography (Accell Plus CM and QMA, Waters). Their structures and purity (95%) were determined by GC-MS and NMR (Roeder, 1990). The chemical structures of these PAs are shown in Figure 1.

Feeding Response to PAs. Ten 7-d-old males were tested. An individual, which had been given 15% aq. sucrose to satiety before the bioassay, was placed on a filter paper strip (20 × 20 mm) impregnated with a 2.5% w/v (net concentration of PA) aq. solution of a PA. One μl of the same solution was applied with a microsyringe to the coiled proboscis. Manifestation of a sequential behavior consisting of proboscis extension and sucking for more than 30 sec was appraised as positive. The response was expressed as the percentage of individuals that responded positively.

Oral Administration of PAs and Quantitative Analysis of Danaidone. After 7 d of eclosion, individual males were fed daily with 0.2 mg (net weight of PA) of any one of the PA samples for 7 d (1.4 mg/male). Several PA samples that

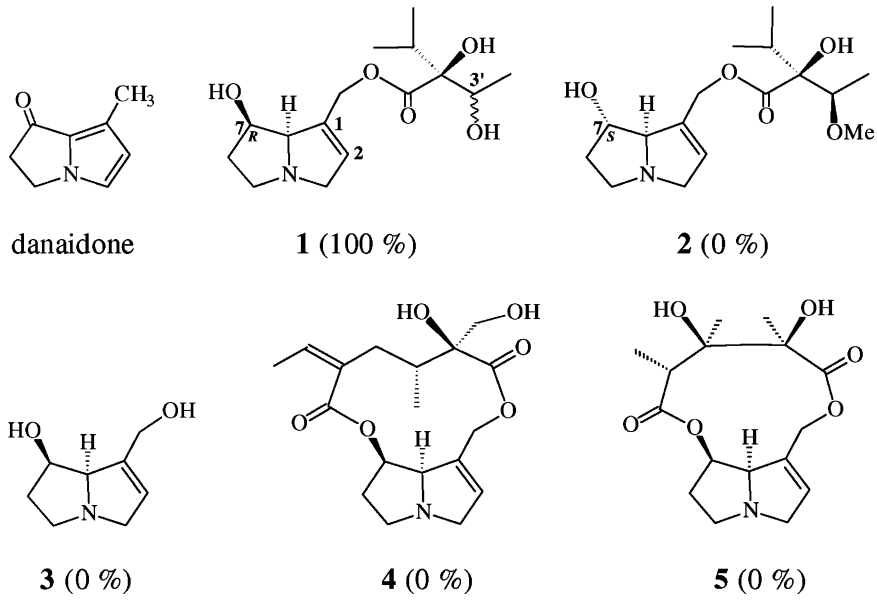

FIG. 1. Chemical structures of danaidone and pyrrolizidine alkaloids administered to *P. sita* males. Their feeding responses to individual compounds are given in parentheses. 1: intermedine (3′*R*)/lycopsamine (3′*S*), 2: heliotrine, 3: retronecine, 4: retrorsine, 5: monocrotaline.

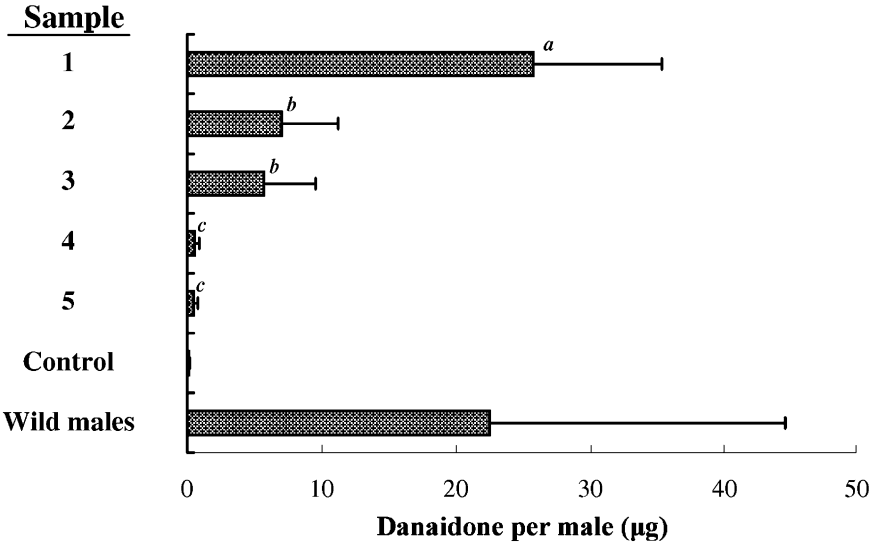

FIG. 2. Amount of danaidone (mean + SD) in the sex brand of *P. sita* males subjected to PA-administration tests or captured in the field. Sample numbers correspond to those of PAs shown in Figure 1. $N = 10$. Different letters represent statistically significant Mann–Whitney rank sum comparisons ($P < 0.001$).

did not stimulate spontaneous feeding were compulsorily given to males together with 15% sucrose solution. Control males were fed with plain sucrose solution only. Ten individuals were used for each treatment. On d 14 of emergence, a pair of sex brands on the hindwings were dissected individually, extracted with 100 μl of purified dichloromethane, and stored at −20°C until use. Quantitative determination of danaidone was performed as described previously (Honda et al., 1995) using benzyl alcohol as the internal standard. Ten wild males collected in the field of Hiroshima prefecture in July, 2001 were also examined for danaidone content by the same method.

RESULTS AND DISCUSSION

Male adults of *P. sita* showed strong positive feeding responses to intermedine/lycopsamine, whereas none of the other PAs stimulated feeding (Figure 1). These results indicate that the males have definite preferences for particular PAs, and also suggest that they selectively ingest certain PAs in the field.

Males that were given intermedine/lycopsamine (**1**) produced the largest amount (25.7 μg/male) of danaidone, which was comparable to that found in wild males (Figure 2). However, uptake of retronecine (**3**) or heliotrine (**2**) resulted in the formation of smaller amounts (5.7 and 7.0 μg/male, respectively) of danaidone, while macrocyclic diester-type PAs, i.e., retrorsine (**4**) and monocrotaline (**5**), were almost ineffective for danaidone formation. Although the number of PA molecules administered differed among the samples depending on their molecular weights, we think that this matters little, because males were given a large excess of PA; the incorporation of compound **1** into danaidone amounted to a low 4% molar proportion.

PAs are undoubtedly the biosynthetic precursors of danaidone, however, it is evident that not all types of PAs are equally utilized for its production. This means that the efficiency of biosynthesis of danaidone, which is apparently derived from a necine base of PAs, depends largely on the chemical structure of the necic acid moieties. At present, however, we cannot determine whether both intermedine and lycopsamine serve as precursors for danaidone.

In contrast, in polyphagous arctiid moths, hydroxydanaidal, which is also a necine base-derived compound, is considered to be formed from diverse plant-acquired PAs, including macrocyclic PAs through their hydrolysis by an unspecific esterase, followed by several characteristic reactions (Hartmann et al., 2003). Therefore, *P. sita*, unlike arctiid moths, may lack a similar esterase that catalyzes the hydrolysis of cyclic or diester PAs and, thus, have a poor ability for processing retronecine. The configuration of a hydroxy group at C-7, however, does not seem to exert a decisive influence on danaidone formation, because heliotrine (7*S*) also afforded danaidone in a small but significant quantity, similar to that produced from retronecine (7*R*). A similar instance has been reported for the arctiid moth, *Creatonotos transiens*, which can utilize 7*S*-heliotrine for the production of 7*R*-hydroxydanaidal (Schulz et al., 1993).

The precursors that *P. sita* males can successfully transform into danaidone seem to be limited to particular PAs, and only those with high availability appear to evoke strong feeding responses. The present findings provide important information on the mechanisms involved in the attraction to and ingestion and utilization of PAs by danaid butterflies in regard to dihydropyrrolizine biosynthesis.

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RAPID COMMUNICATION

SHORT AND SIMPLE SYNTHESES OF 4-OXO-(*E*)-2-HEXENAL AND HOMOLOGS: PHEROMONE COMPONENTS AND DEFENSIVE COMPOUNDS OF HEMIPTERA

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Abstract—One-step syntheses of 4-oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal from commercially available 2-ethyl- and 2-butylfuran are described. A two-step synthesis of the homolog 4-oxo-(*E*)-2-decenal from furan is also reported. These compounds are common components of true bug defensive secretions, and recently have been identified as pheromone components for several species. The simple syntheses reported here will make these compounds readily available for further research.

Key Words—4-Oxo-(*E*)-2-alkenal, true bug, Hemiptera, Heteroptera, allomone, attractant

INTRODUCTION

4-Oxo-(*E*)-2-alkenals of 6, 8, and 10 carbon chain lengths are common components of the defensive secretions of true bugs (Hemiptera) (Aldrich, 1988, 1995; Millar, 2004). More recently, these compounds also have been identified as pheromone components of nymphal (Fucarino et al., 2004) and adult bugs (Innocenzi et al., 2004; Millar, 2004). Several syntheses of these compounds have been reported (e.g., Ward and van Dorp, 1969; Marques et al., 2000; Zarbin et al., 2000), but most have required at least several steps. Because of the increasing interest in these compounds, and in an effort to make them readily available to other researchers, we report here simple, one step syntheses of 4-oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal from commercially available 2-

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ethyl- and 2-butylfurans respectively, and the two step synthesis of the ten carbon analog from furan. The ready availability of these compounds should also enhance their potential for exploitation for insect pest management.

METHODS AND MATERIALS

Tetrahydrofuran (THF) was distilled from sodium/benzophenone ketyl under argon. ^{1}H- and ^{13}C-NMR spectra (400 and 100 MHz, respectively) were obtained in $CDCl_3$ solutions with a Varian INOVA-400 spectrometer. Mass spectra were obtained with an Hewlett-Packard 5970 (70 eV) mass selective detector interfaced to an H-P 5890 gas chromatograph fitted with a DB5-MS column (25 m × 0.20 mm id × 0.33 μm film) with helium as carrier gas (100 kPa). Products were purified by vacuum flash chromatography on silica gel (E. Merck, 230-400 mesh).

Synthesis of 2-n-Hexylfuran. Butyl lithium (2.5 M in hexanes, 5.9 ml, 14.7 mmol) was added dropwise to a stirred solution of furan (1.00 g, 14.7 mmol) in dry THF (60 ml) at −20°C under argon atmosphere. The resulting mixture was stirred for 3 hr, then 1-iodohexane (3.12 g, 14.7 mmol) in dry THF (8 ml) was added dropwise. The solution was stirred at −20°C for 2 hr and room temperature for 1 hr, then quenched with water. The mixture was extracted with 1:1 ether-pentane (3 × 20 ml). The combined organic layers were washed with aqueous 5% sodium bisulfite solution (2 × 30 ml), water (2 × 50 ml), and brine (2 × 50 ml), then dried over Na_2SO_4 and concentrated. The crude product was purified by vacuum flash chromatography over silica gel eluting with pentane to afford 2.01 g of the product (89.8% yield). ^{1}H NMR δ 0.89 (*t*, 3H, J = 7.0 Hz), 1.24–1.44 (*m*, 6H), 1.63 (quint, 2H, J = 7.6 Hz), 2.62 (*t*, 2H, J = 7.6 Hz), 5.95–5.98 (*m*, 1H), 6.27 (dd, 1H, J = 2.0 and 3.1 Hz), 7.29 (dd, 1H, J = 0.8 and 2.0 Hz). ^{13}C NMR δ 14.31, 22.81, 28.22, 28.24, 29.10, 31.82, 104.71, 110.24, 140.84, 156.87 ppm. MS (*m/z*, rel. intensity): 152 (M^+, 13), 123 (5), 109 (3), 95 (14), 81 (100), 67 (6), 53 (20), 43 (8).

Synthesis of 4-Oxo-(E)-2-hexenal. N-bromosuccinimide (2.72 g, 15.3 mmol, 1.5 eq.) and pyridine (1.61 g, 20.4 mmol, 2 eq.) were sequentially added to a solution of 2-ethylfuran (1.00 g, 10.2 mmol, 1 eq.; Lancaster Synthesis, Pelham, NH, USA) in THF/acetone/water (5:4:2, 22.0 ml) at −15°C. The resulting mixture was stirred for 3 hr at −15° and then warmed to room temperature and stirred overnight. The mixture was poured into aqueous HCl (0.5 M, 20 ml) and extracted with ether (3 × 20 ml). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, and the solvent was removed by distillation. The crude product was purified by vacuum flash chromatography over silica gel (ether: pentane, 15:85) to afford 4-oxo-(*E*)-2-hexenal (0.55 g, 48%). ^{1}H NMR δ 1.15 (*t*, 3H, J = 7.2 Hz), 2.73 (*q*, 2H, J = 7.2 Hz), 6.77 (dd, 1H, J = 7.2 and 16.2

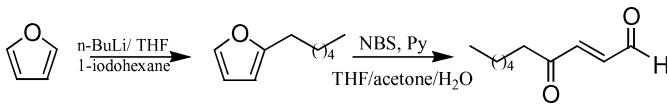
Hz), 6.88 (d, 1H, J = 16.2 Hz), 9.77 (d, 1H, J = 7.2 Hz). ^{13}C NMR δ 7.76, 34.75, 137.51, 144.98, 193.65, 200.59 ppm. MS: *m/z* 112 (M^+, 16), 97 (2), 84 (15), 83 (100), 57 (18), 55 (77), 53 (10).

Syntheses of 4-Oxo-(E)-2-octenal and 4-Oxo-(E)-2-decenal. In the same manner, 2-*n*-butylfuran (0.50 g, 4.0 mmol, Lancaster Synthesis) was converted to 4-oxo-(*E*)-octenal (0.28 g, 50% yield), and 2-*n*-hexylfuran (1.00 g, 6.6 mmol) gave 4-oxo-(*E*)-2-decenal in 56% yield (0.62 g). 4-Oxo-(*E*)-2-octenal: ^{1}H NMR δ 0.92 (*t*, 3H, J = 7.2 Hz), 1.30–1.40 (*m*, 2H), 1.58–1.68 (*m*, 2H), 2.69 (*t*, 2H, J = 7.2 Hz), 6.76 (dd, 1H, J = 7.2 and 16.2 Hz), 6.87 (d, 1H, J = 16.2 Hz), 9.77 (d, 1H, J = 7.2 Hz). ^{13}C NMR δ 14.03, 22.44, 25.93, 41.15, 137.54, 145.17, 193.66, 200.36 ppm. MS: *m/z* 140 (M^+, 1), 125 (7), 111 (39), 98 (46), 97 (13), 83 (53), 70 (40), 57 (28), 56 (15), 55 (100). 4-Oxo-(*E*)-2-decenal: ^{1}H NMR δ 0.88 (*t*, 3H, J = 6.9 Hz), 1.20–1.38 (m, 6H), 1.60–1.70 (*m*, 2H), 2.69 (*t*, 2H, J = 7.2 Hz), 6.77 (dd, 1H, J = 7.2 and 16.2 Hz), 6.87 (d, 1H, J = 16.2 Hz), 9.78 (d, 1H, J = 7.2 Hz). ^{13}C NMR δ 14.23, 22.68, 23.84, 28.98, 31.75, 41.45, 137.54, 145.19, 193.66, 200.38 ppm. MS: *m/z* 168 (M^+, 1), 153 (1), 139 (41), 125 (12), 111 (9), 98 (54), 83 (56), 70 (49), 55 (100), 43 (90).

RESULTS AND DISCUSSION

4-Oxo-(*E*)-2-alkenals were readily prepared from 2-alkylfurans, using aqueous *N*-bromosuccinimide (NBS) to promote oxidative ring opening under mild conditions (Kobayashi et al., 1998). The reaction initially produces 4-oxo-(*Z*)-2-alkenals, which are completely isomerized to the (*E*)-2-isomers after several hours at room temperature in the reaction flask. 4-Oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal were obtained in one step from commercially available starting materials in 48 and 50% isolated yields, respectively. 4-Oxo-(*E*)-2-decenal was obtained in two steps, starting from furan. Thus, treatment of furan with *n*-butyl lithium, followed by addition of one equivalent of 1-iodohexane, afforded 2-*n*-hexylfuran in 89.8% yield, which was then oxidized to 4-oxo-(*E*)-2-decenal with aqueous NBS in 56% isolated yield (Scheme 1).

Several methods have been published for the syntheses of geometrical isomers of 4-oxo-2-alkenals. For example, the routes described by Ward and van Dorp (1969), Marques et al. (2000), and Zarbin et al. (2000) furnish



SCH. 1. Synthesis of (*E*)-4-oxo-2-decenal.

stereochemically pure 4-oxo-(*E*)-2-alkenals, but both routes involve multiple steps. During the course of our work, another short synthesis was reported: thus, Fernandes and Kumar (2003) found that under appropriate reaction conditions, homoallylic alcohols react with pyridinium chlorochromate to afford 4-oxo-(*E*)-2-alkenals. This reaction was proposed to proceed by the initial oxidation of the alcohol, followed by double bond migration and subsequent allylic oxidation. However, only a limited number of the homoallylic alcohol precursors are commercially available.

In summary, the short and simple syntheses of 4-oxo-(*E*)-2-alkenals described in this communication should make these compounds readily accessible to researchers. Furthermore, the route can be readily adapted to produce homologs of any desired chain length by the straightforward alkylation of furan with an alkyl halide of the appropriate chain length.

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PHENOLIC COMPOUNDS IN RED OAK AND SUGAR MAPLE LEAVES HAVE PROOXIDANT ACTIVITIES IN THE MIDGUT FLUIDS OF *Malacosoma disstria* AND *Orgyia leucostigma* CATERPILLARS

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Abstract—Phenolic compounds are generally believed to be key components of the oxidative defenses of plants against pathogens and herbivores. However, phenolic oxidation in the gut fluids of insect herbivores has rarely been demonstrated, and some phenolics could act as antioxidants rather than prooxidants. We compared the overall activities of the phenolic compounds in red oak (*Quercus rubra*) and sugar maple (*Acer saccharum*) leaves in the midgut fluids of two caterpillar species, *Malacosoma disstria* (phenolic-sensitive) and *Orgyia leucostigma* (phenolic-tolerant). Three hypotheses were examined: (1) ingested sugar maple leaves produce higher levels of semiquinone radicals (from phenolic oxidation) in caterpillar midgut fluids than do red oak leaves; (2) *O. leucostigma* maintains lower levels of phenolic oxidation in its midgut fluids than does *M. disstria*; and (3) phenolic compounds in tree leaves have overall prooxidant activities in the midgut fluids of caterpillars. Sugar maple leaves had significantly lower ascorbate: phenolic ratios than did red oak leaves, suggesting that phenolics in maple would oxidize more readily than those in oak. As expected, semiquinone radicals were at higher steady-state levels in the midgut fluids of both caterpillar species when they fed on sugar maple than on red oak, consistent with the first hypothesis. Higher semiquinone radical levels were also found in *M. disstria* than in *O. leucostigma*, consistent with the second hypothesis. Finally, semiquinone radical formation was positively associated with two markers of oxidation (protein carbonyls and total peroxides). These results

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suggest that the complex mixtures of phenolics in red oak and sugar maple leaves have overall prooxidant activities in the midgut fluids of *M. disstria* and *O. leucostigma* caterpillars. We conclude that the oxidative defenses of trees vary substantially between species, with those in sugar maple leaves being especially active, even in phenolic-tolerant herbivore species.

Key Words—Prooxidant, phenolic compound, oxidation, caterpillar, *Malacosoma disstria*, *Orgyia leucostigma*, peroxide, semiquinone radical, protein carbonyl, red oak, *Quercus rubra*, sugar maple, *Acer saccharum.*

INTRODUCTION

The phenolic compounds produced by plants represent a diverse array of over 8000 known structures (Thompson, 1964; Harborne, 1985; Bravo, 1998; Vinson et al., 2001). Along with oxidative enzymes, such as polyphenol oxidases and peroxidases, phenolic compounds are generally considered to be key components of the oxidative defenses of plants against pathogens and herbivores (Duffey and Stout, 1996). The products and byproducts of phenol oxidation include semiquinone radicals, quinones, and reactive oxygen species (e.g., hydrogen peroxide and hydroxyl radicals), each of which can damage nutrients in the gut lumens of insect herbivores or produce cytotoxic effects in their tissues (Gant et al., 1988; Felton et al., 1989, 1992; Canada et al., 1990; Zheng et al., 1997; Thiboldeaux et al., 1998; Galati et al., 2002; Hagerman et al., 2003). Thus, the oxidation of phenolics is believed to be an important step contributing to their biological activity (Canada et al., 1990; Ahmad, 1992; Appel, 1993; Summers and Felton, 1994; Pardini, 1995; Barbehenn et al., 2003b).

The physiological roles played by ingested phenolics vary, however, depending upon their chemical structures and physicochemical environments (e.g., pH, redox potential, and concentrations of oxidases, oxidants, and antioxidants) (Larson, 1995; Metadiewa et al., 1999; Sugihara et al., 1999; Galati et al., 2002; Sakihama et al., 2002; Hagerman et al., 2003). For example, if certain phenolics scavenge free radicals or otherwise function as reducing agents and they have structures that form stable semiquinone or phenoxyl radicals, they can act as antioxidants (Buettner, 1993; Hagerman et al., 1998; Halliwell and Gutteridge, 1999). Indeed, recent evidence has suggested that low molecular weight phenolics may act as antioxidants in some caterpillars (Johnson and Felton, 2001).

In this study, electron paramagnetic resonance spectrometry (EPR) was used to measure the steady-state levels of semiquinone radicals (produced by the one-electron oxidation of di- or trihydroxy phenolics) in caterpillar midgut fluids. In order to distinguish between the overall prooxidant or antioxidant

activities of foliar phenolics, semiquinone radical levels were measured in conjunction with two markers of oxidation (protein carbonyls and total peroxides). We reasoned that if ingested phenolics had an overall prooxidant activity in midgut fluids, a positive association would be observed between levels of semiquinone radicals and markers of oxidation. By contrast, if semiquinone radicals were formed during the protection of other molecules from oxidation, then an overall antioxidant activity would be indicated by a negative association. In support of this rationale, previous work has shown that tannic acid in an artificial diet acts as a prooxidant in the midgut fluids of two caterpillar species: high levels of semiquinone radicals were associated with increased levels of reactive oxygen species and markers of oxidation (Barbehenn et al., 2001, 2003b, unpublished data).

Phenolic compounds have a strong tendency to oxidize at the high pH found in many caterpillar midguts (ca. pH 10) unless there is sufficient ascorbate present to chemically reduce semiquinone radicals (Barbehenn et al., 2001, 2003b). Thus, the potential for foliar phenolics to oxidize in the midgut fluids of caterpillars was predicted by examining the ratio of ascorbate to total phenolics in red oak and sugar maple leaves (Barbehenn et al., 2003b). Based on our previous work on these tree species (Barbehenn et al., 2003a), sugar maple leaves were expected to contain low ratios of ascorbate:phenolics and to produce high levels of phenolic oxidation, while red oak leaves were expected to contain high ratios of ascorbate:phenolics and to produce low levels of phenolic oxidation following ingestion.

The two caterpillar species chosen for comparison were expected to differ in their abilities to handle ingested phenolics. *Malacosoma disstria* Hübner (Lasiocampidae), the forest tent caterpillar, is a phenolic-sensitive species that feeds on the spring foliage of a wide range of deciduous trees in North America (Stehr and Cook, 1968). *Orgyia leucostigma* Smith (Lymantriidae), the white-marked tussock moth, is a phenolic-tolerant species that also feeds on a wide range of North American trees during spring and summer broods (Baker, 1972). The degree of phenolic tolerance ascribed to the two species is based primarily on the extent to which phenolics ingested in artificial diets are oxidized in their midguts and, in the case of *M. disstria*, the formation of pupal deformities (Karowe, 1989). *O. leucostigma* maintains lower levels of phenolic oxidation in its midgut fluids than does *M. disstria* (Barbehenn and Martin 1992, 1994; Barbehenn et al., 2001, 2003b), but little work has been done on the gut biochemistries of these herbivores on their host plants.

This study examined three hypotheses: (1) ingested sugar maple leaves produce higher levels of semiquinone radicals than red oak leaves; (2) *O. leucostigma* maintains lower levels of phenolic oxidation than *M. disstria*; and (3) phenolic compounds in tree leaves have overall prooxidant activities in the midgut fluids of caterpillars.

METHODS AND MATERIALS

Trees. Red oak (*Quercus rubra* L.) (N = 14) and sugar maple (*Acer saccharum* Marshall) (N = 12) were tagged at three sites in Ann Arbor, MI, USA, the University of Michigan campus, the Matthaei Botanical Garden, and the Kuebler-Langford Park. Twigs containing terminal leaf clusters from the sunlit side of trees were cut with a pole pruner. Twigs were selected haphazardly, but damaged or diseased leaves were avoided. They were placed in a flask of water and returned to the lab for feeding experiments or were placed in labeled plastic bags and kept on ice in the dark until extracted 2–4 hr later. Leaves were sampled on the 12th of June and the 10th of July 2003. In addition, preliminary measurements were made on a group of three red oak and sugar maple trees on the 14th of May. Leaf lengths were measured repeatedly in the study trees beginning in May to determine the time when leaf expansion was complete.

A representative leaf (e.g., excluding small or young terminal leaves) was selected from within each leaf cluster. These leaves were cut in half along the length of the midrib, and the thickened portion of the midrib was removed. Leaf halves were immediately weighed (200–800 mg), ground in liquid nitrogen, and extracted in 3.0 ml of 5% (w/v) metaphosphoric acid (containing 1 mM EDTA; ambient oxygen) or nitrogen-purged ethanol (Barbehenn et al., 2003a). Samples were extracted for 30 min (ambient temperature) and then centrifuged (1000 *g*, 5 min). The extraction process was repeated, and supernatant solutions were pooled within samples. Ethanolic extracts were kept under a nitrogen atmosphere, and all samples were stored at −80°C until they were analyzed. A second leaf from each cluster was weighed, after removing the midrib, and dried at 70°C for 3 d in an open glassine envelope. The water content of these leaves was used to estimate the percent dry weight (%DW) of leaves used for chemical analysis.

Insects. The main comparisons between *M. disstria* and *O. leucostigma* were made in mid-June (on recently expanded leaves) and in mid-July (on mature leaves). *M. disstria* normally completes most of its development on expanding leaves, and EPR measurements were done to coincide with this period. *M. disstria* and *O. leucostigma* eggs were obtained from the Canadian Forrest Pest Management Institute (Sault Ste. Marie, Ontario, Canada). The diet for both species was prepared as described previously (Barbehenn et al., 2001), with the exceptions that linseed oil was substituted for wheat germ oil, methyl paraben was omitted, and sodium alginate was included (2.9% dry diet). Larvae were maintained in petri dishes in incubators (primarily at 23°C, 16-hr L, 8-hr D) until the final-instar. Colonies of both species were maintained at 18°C when it was necessary to slow their growth to synchronize their developmental stages. Final-instar larvae were assigned at random to feed on red oak or sugar maple

leaves collected from three trees of each species on the University of Michigan campus. The same trees and leaf-sampling protocol were used for all experiments. Twigs with attached leaves were placed in water in 15-ml centrifuged tubes that were placed inside ventillated plastic boxes (30 × 19 × 10 cm). Leaves in June and July were coated with 200 μl of a 70% acetone solution of sucrose (2% dry weight) to promote feeding. The percent dry weight of the added sucrose was calculated based on the dry weight of untreated leaves. A micropipet was used to dispense the solvent evenly across each leaf, placing the pipette tip parallel to the leaf surface to avoid scratching the leaf. Boxes were replaced in an incubator where larvae were fed for 2 d. Freshly treated leaves were provided on the second day, using the same trees for each caterpillar species (but different trees from the previous day). On the third day of each experiment, midgut fluids were collected from dissected larvae, as described previously (Barbehenn et al., 2001). Gut fluid samples were kept under a nitrogen atmosphere at all times to avoid artifactual oxidation (Johnson and Barbehenn, 1999).

Chemical Analyses. Ascorbic acid was measured in metaphosphoric acid extracts using high-performance liquid chromatography (HPLC) (Lykkesfeldt et al., 1995; Barbehenn, 2003). Samples were stored for 1–2 mo before analysis. Total phenolics were measured in ethanol extracts using the Prussian blue assay (Price and Butler, 1977) as modified by Graham (1992), using gum arabic to stabilize the colored product. This redox assay is based on the formation of a colored complex (ferric ferrocyanide) upon the reduction of iron (Fe III) by phenolic compounds. Samples were stored for 1–3 mo before analysis. Samples were diluted 20-fold with nitrogen-purged ethanol before analysis. Purified tannic acid, composed primarily of tetra- to octagalloylglucose (J.-P. Salminen, unpublished data), was used to construct standard curves. All assays were scaled to 200 μl to fit in a 96-well microplate, and absorbances were read at 655 nm with a Bio-Rad Benchmark microplate reader (Bio-Rad, Hercules, CA, USA). To correct total phenolics measurements for the absorbance caused by foliar ascorbate, a regression between ascorbate concentration and Prussian blue formation was determined. The Prussian blue absorbance contributed by foliar ascorbate in each sample was then subtracted from the total absorbance value for each of the samples. Ascorbate accounted for 30–40% of the "total phenolics" in oak and for 20% of the "total phenolics" in maple. The unknown phenolic compositions of the tree leaves precluded reporting phenolic concentrations on a molar basis, and therefore, all concentrations were expressed on a percent dry weight basis in purified tannic acid equivalents.

To provide a general indication of the accuracy of the Prussian blue assay, total phenolics were analyzed with HPLC or the acid–butanol method (for condensed tannins). A single sample of red oak and sugar maple leaves (including each of the trees used as food plants) was collected in mid-July 2004.

Leaf samples were lyophilized, extracted in 70% aqueous acetone, and the extracts were analyzed with HPLC using a diode array detector (Salminen et al., 1999). Hydrolyzable tannins were calculated in pentagalloyl glucose equivalents (at 280 nm), flavonoids in quercetin equivalents (at 349 nm), chlorogenic acids in chlorogenic acid equivalents (at 315 nm), coumaroylquinic acids in coumaric acid equivalents (at 315 nm), and the remaining phenolics (other than condensed tannin) as gallic acid equivalents (at 280 nm). Condensed tannins were measured with the acid–butanol method (Ossipova et al., 2001), using purified birch leaf-condensed tannins as a standard.

Protein carbonyls were measured in midgut fluids with the 2,4-dinitrophenylhydrazine (DNPH) method (Reznick and Packer, 1994; Quinlan and Gutteridge, 2000). Samples (50–110 mg) were extracted in 400 μl of pH 6.5 phosphate buffer (200 mM, nitrogen-purged) containing 20 mg of hydrated polyvinylpolypyrrolidone (Sigma). Samples were collected from five to 11 replicate larvae from each insect species on each tree species on the 13th of June, and samples from 10 to 15 larvae were collected on the 15th of July (*O. leucostigma*) and 17th of July (*M. disstria*). All samples were stored under a nitrogen atmosphere at −80°C until they were analyzed after 4–4.5 mo. Protein carbonyls were measured in thawed extracts (kept on ice) after treating 200 μl of the supernatant solutions with streptomycin sulfate (22.2 μl; 10% w/v in 50 mM, pH 7.0, HEPES buffer). After centrifugation (8000 *g*, 5 min, 4°C), proteins remaining in the supernatant solution were precipitated with trichloroacetic acid (100 μl, 28% w/v). Protein pellets were treated with 500 μl of 7 mM DNPH (Acros) prepared in 2 M hydrochloric acid (37°C, 15 min). Treated protein pellets were washed free of unbound DNPH and solubilized in a guanidine hydrochloride solution (6 M; Acros) as described by Quinlan and Gutteridge (2000). An extinction coefficient of 22,000/M for the DNPH protein carbonyl adduct at 370 nm was used to calculate protein carbonyl concentrations (Reznick and Packer, 1994; Quinlan and Gutteridge, 2000). Correction for interfering substances was made by running DNPH-free controls from each of the treatments. Protein concentrations in the sample supernatants were measured with the modified Bradford assay and quantified with a bovine serum albumin standard curve (Stoscheck, 1990). Protein carbonyl levels were expressed as nanomoles protein carbonyl per milligram protein.

Total peroxides (hydrogen peroxide and organic peroxides) were measured in methanol extracts with the FOX assay (Nourooz-Zadeh et al., 1994). *M. disstria* midgut contents (25–35 mg from the mid-midgut) were extracted in 1.0 ml of 90% methanol (nitrogen-purged), and the midgut contents of *O. leucostigma* (10–20 mg) were extracted in 0.5 ml of 90% methanol. Following centrifugation (8000 *g*, 3 min), a 35-μl aliquot of the supernatant solution was diluted with 70 μl of 90% methanol. *M. disstria* (N = 14–22/tree species) were examined on the 18th of June and the 11th of July, and *O. leucostigma*

(N = 14/tree species) were examined on the 14th of July. The FOX reagent was prepared fresh daily using ambient oxygen solvents. All assays were scaled to fit in a 96-well microplate, and absorbances were read at 540 nm with a microplate reader. Measurements were made within a period of approximately 1 hr from the time of dissection. A hydrogen peroxide standard curve was used to determine total peroxide concentrations.

Electron Paramagnetic Resonance Spectrometry. Midgut contents (20 mg) were dissected from larvae, alternating between the four caterpillar/tree species combinations. Midgut contents were extracted in 300 μl of pH 10 carbonate buffer (70 mM) containing 10% (v/v) dimethyl sulfoxide (nitrogen-purged). Samples in 2.0-ml microcentrifuge tubes were kept under a nitrogen atmosphere at all times. Samples were centrifuged for 1 min (approximately 10,000 *g*, ambient temperature). Semiquinone radicals in supernatant solutions were measured with a Bruker EMX spectrometer (Bruker Instruments, Billerica, MA, USA), using the instrumental parameters described previously (Barbehenn et al., 2003b). The time between dissecting each caterpillar and starting an EPR scan was recorded. The area of each spectrum was quantified by double integration using WinEPR software (Bruker Instruments). The sizes of semiquinone radical spectra decreased above a threshold phenolic concentration in both midgut fluid samples and in standards, apparently from semiquinone radical disproportionation (unpublished data). Therefore, the EPR spectra from a range of sample dilutions of each sample type were measured to confirm that semiquinone radical intensities increased with increasing sample mass over the full range of sample masses used. Standard curves based on tannic acid were linear over a shorter range than was necessary for quantifying the large radical spectra produced by sugar maple phenolics. Therefore, semiquinone radical intensity was expressed as the double integral area/sample mass, where the double integral area is in arbitrary units determined by instrumental parameters. Semiquinone radical intensities could be compared across all sample types in this and in previous studies because the same instrumental parameters were used in all cases. Radical intensity measured as the double integral area per milligram is directly proportional to, but approximately 10- to 17-fold larger than, measurements of spectrum height per milligram used previously (Barbehenn et al., 2003b). The identity of semiquinone or ascorbyl radicals was based upon the comparison of their spectra with standards and spectra obtained previously under identical conditions (Barbehenn et al., 2003b). As observed previously, semiquinone radical spectra from sugar maple and red oak phenolics had sigmoidal line shapes similar to those of tannic acid standards.

To correct for the decay in radical spectra through time, regressions were plotted of radical intensity vs. time. Samples of the four treatment groups were run in the 15th and 16th of May (Figure 3). These regressions included three to four samples of each type that were rescanned once or twice to extend the time

range over which data were collected. Based on these regressions, semiquinone radical intensities in May, June, and July were adjusted to the levels expected after a period of 5 min. Time periods between extracting a sample and starting an EPR scan averaged 5.0 (±0.2 min) in June and 5.2 (±0.3 min) in July, necessitating only minimal adjustments to these data. Sample sizes in each experiment ranged from eight to 15 replicate larvae for each of the four treatment groups. Midgut fluids from all four treatment groups were examined on the 10th of June, and additional *M. disstria* and *O. leucostigma* larvae were examined on the 11th and the 14th of June, respectively. Midgut fluids from *M. disstria* and *O. leucostigma* larvae were also examined on the 11th and the 14th of July, respectively.

Statistical Analyses. Concentrations of phenolic compounds and ascorbate, and ascorbate:phenolic ratios in red oak and sugar maple were compared using nested ANOVA, with site nested within tree species, and repeated measures on individual trees (PROC MIXED) (SAS, 2000). Significant effects are reported in the results. Semiquinone radical intensities, peroxides, and protein carbonyls were compared using two-way ANOVA, with insect and tree species as main effects. In May, when multiple EPR runs were made through time on the same sample, only the first EPR run on each sample was included in the ANOVA. To compare regressions of semiquinone radical intensity on time in May (Figure 3A, B), entire data sets (including multiple scans per sample) were analyzed with ANCOVA using time as the covariate (SAS, 2000). The normality of residuals was tested using PROC UNIVARIATE (SAS, 2000). If residuals could not be normalized by transformation, the significance of main effects was determined by Kruskal–Wallis tests (Wilkinson, 2000). Pairwise differences between means were examined by differences of least-squares means generated by PROC MIXED (SAS, 2000), or by Kruskal–Wallis tests. No significant differences were observed between days in experiments that were run on more than 1 d, and these data were combined for final analysis. The spatial separation of analytical equipment used in different experiments and the small sample volumes available necessitated performing separate experiments to measure each analyte in caterpillar midgut fluids. Thus, the associations between semiquinone radical formation and markers of oxidation were examined by plotting mean levels (Figure 5A, B). The associations between semiquinone radical intensities and protein carbonyl concentrations in the midgut fluids of *M. disstria* and *O. leucostigma* (Figure 5A) were examined with regressions (Wilkinson, 2000) and by ANCOVA, using semiquinone radical intensity as the covariate (SAS, 2000). Insufficient data were available to examine the association between semiquinone radical intensities and total peroxide concentrations in *O. leucostigma* statistically. However, a visual examination of this relationship in *M. disstria* and *O. leucostigma* (Figure 5B) suggested that a single linear regression for the two species was appropriate and sufficient to

examine whether semiquinone radical intensities are positively or negatively associated with the formation of peroxides.

RESULTS

Sugar maple leaves contained lower levels of ascorbate and higher levels of phenolic compounds than did red oak leaves in mid-June (Figure 1A). Similar results were obtained in mid-July (Figure 1B), with the exception that ascorbate levels were not significantly different between the species. Nevertheless, ascorbate:phenolic ratios remained significantly lower in sugar maple leaves in June (0.045 ± 0.009) and July (0.056 ± 0.007) than in oak in June (0.19 ± 0.02) and

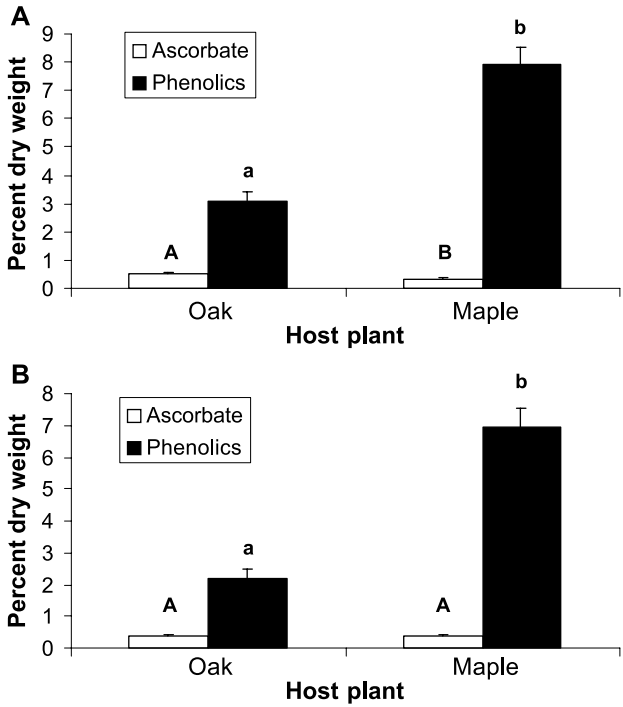

FIG. 1. Ascorbate and total phenolic levels in red oak (*Q. rubra*) and sugar maple (*A. saccharum*) leaves in mid-June (A) (soon after leaf expansion) and mid-July (B). Upper case letters that differ indicate significant differences between tree species for ascorbate ($P < 0.05$). Lower case letters that differ indicate significant differences between tree species for total phenolics.

July (0.25 ± 0.06) ($P < 0.001$). In a preliminary sample of leaves in May, ascorbate levels averaged 0.6% in both species. Total phenolics averaged 2.4 and 3.6% in red oak and sugar maple, respectively, demonstrating that levels of total phenolics doubled as sugar maple leaves matured but remained at lower levels in red oak. Therefore, the early spring foliage of sugar maple used as food in this study also had a lower ascorbate:phenolic ratio (0.17 ± 0.01) than did red oak (0.24 ± 0.02) ($P = 0.050$). Total phenolics in July 2004, expressed as the sum of the individual phenolics, measured 3.4% in red oak and 13.0% in sugar maple. To the extent that year-to-year variation in the experimental trees was negligible, the Prussian blue assay appears to have underestimated total phenolics, but provided a useful estimate of the relative amounts of phenolics in the two tree species.

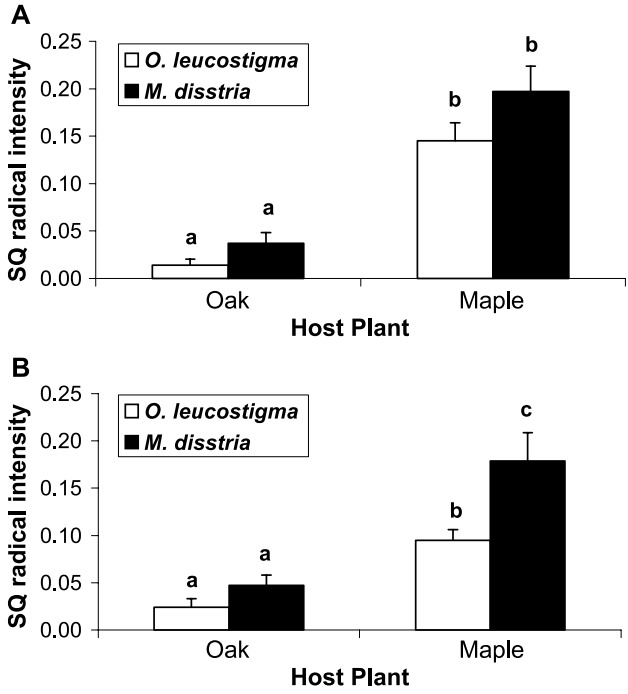

FIG. 2. Semiquinone (SQ) radical intensities in the midgut fluids of final-instar *O. leucostigma* and *M. disstria* larvae that fed on red oak or sugar maple leaves in mid-June (A) or in mid-July (B). Semiquinone radical intensity is defined as the double integral of the EPR spectrum per milligram sample, and all EPR spectra were standardized to a scan time beginning at 5 min after sample extraction. Different letters designate significant differences between the four treatments.

Semiquinone radical intensities were higher in the midgut fluids of caterpillars that were fed on maple than on oak in June and July (Figure 2A, B) ($P < 0.001$ for each month). Although semiquinone radical intensities were greater in *M. disstria* than in *O. leucostigma* in July ($P = 0.056$), they did not differ in June ($P = 0.71$). On early spring foliage, semiquinone radical intensities in *O. leucostigma* and *M. disstria* averaged 0.02 ± 0.002 SE and 0.14 ± 0.02 SE on oak, and 0.18 ± 0.01 SE and 0.30 ± 0.02 SE on maple, respectively. Thus, in May, higher semiquinone radical intensities were formed in maple-feeding caterpillars than in those feeding on oak ($P < 0.001$), and higher semiquinone radical intensities were formed in *M. disstria* than in *O. leucostigma* ($P < 0.001$). Generalizing across all 3 mo, higher semiquinone radical intensities were formed in maple-feeding caterpillars than in those

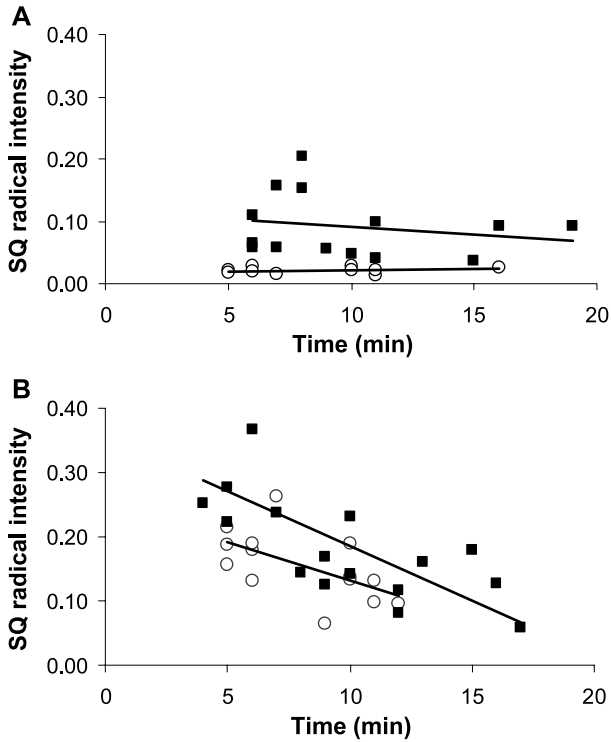

FIG. 3. Changes in semiquinone (SQ) radical intensity through time in the midgut fluids of final-instar *O. leucostigma* (open circles) and *M. disstria* (closed squares) on red oak (A) or sugar maple (B) in mid-May. Times indicate the number of minutes between the extraction of a sample and the beginning of an EPR scan of the sample.

feeding on oak ($P < 0.001$), and they were higher in *M. disstria* than in *O. leucostigma* ($P = 0.002$). Semiquinone radical intensities were higher overall in May than in June ($P = 0.002$) or July ($P = 0.001$). In comparison with semiquinone radical intensities measured previously in *M. disstria* that were fed on an artificial diet containing a mixture of 5% tannic acid and 2.3% ascorbate (Barbehenn et al., 2003b), radical intensities in *M. disstria* on sugar maple were roughly half as large.

Phenolic compounds in red oak leaves formed relatively stable semiquinone radicals in the midgut fluids of *O. leucostigma* and *M. disstria* (Figure 3A). Changes in semiquinone radical levels averaged only +0.0003 and −0.0025 intensity units/min in *O. leucostigma* and *M. disstria*, respectively. By comparison, semiquinone radicals from sugar maple phenolics had decay rates averaging −0.012 and −0.017 intensity units/min in *O. leucostigma* and *M. disstria*, respectively (Figure 3B). Thus, semiquinone radicals from sugar maple

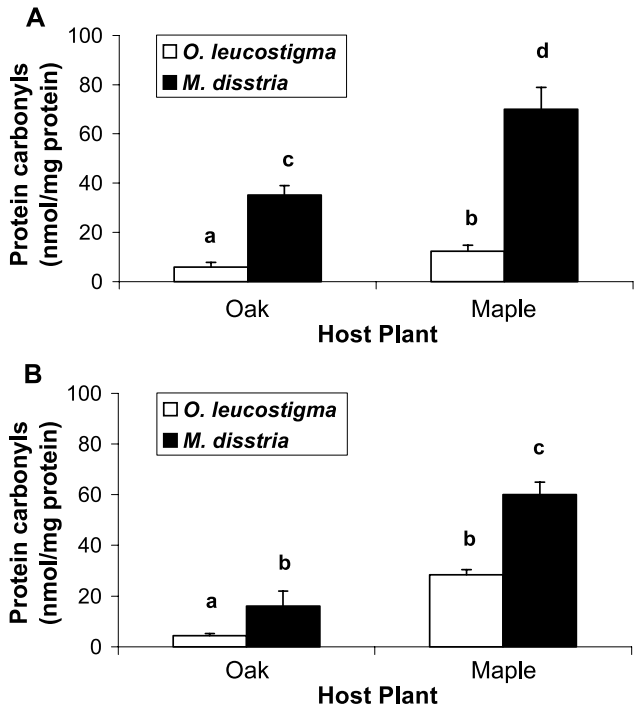

FIG. 4. Protein carbonyl levels in the midgut fluids of final-instar *O. leucostigma* and *M. disstria* larvae that fed on red oak or sugar maple leaves in mid-June (A) and in mid-July (B). Different letters designate significant differences between the four treatments.

decayed more rapidly than did those from oak in *O. leucostigma* ($P < 0.001$) and showed a similar trend in *M. disstria* ($P = 0.086$). Rates of radical decay were not significantly different between the caterpillar species when they each fed on red oak ($P = 0.508$) or sugar maple ($P = 0.792$).

The formation of brown pigments, especially in larvae that fed on sugar maple, suggested that a substantial fraction of the semiquinone radicals oxidized further to form quinones and melanin-like compounds. The gut contents of oak-feeding larvae were commonly bright green, but some browning was observed in *M. disstria* in June and July. Browning was observed in the midgut fluids of many *M. disstria* larvae when they were fed on sugar maple in June and July (and to a lesser extent in May), but the contents of *O. leucostigma* were typically dark green in the spring and summer.

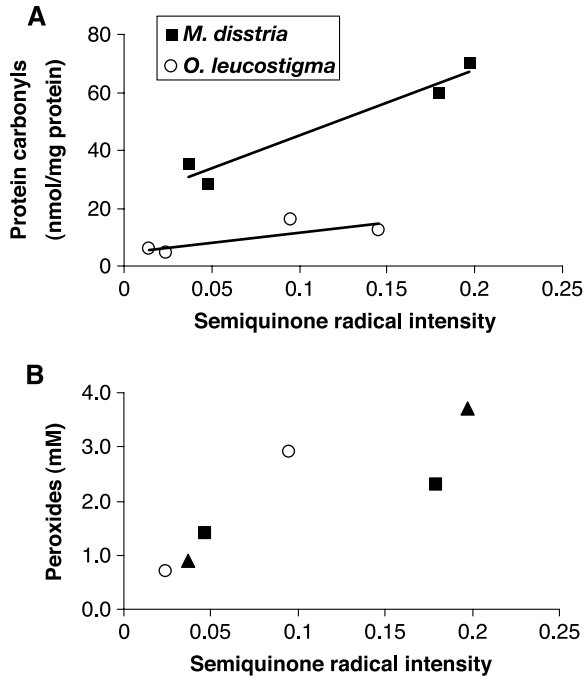

FIG. 5. Association between semiquinone radical intensities and protein carbonyl levels (A) and total peroxide levels (B) in the midgut fluids of final-instar *O. leucostigma* and *M. disstria* larvae that fed on red oak or sugar maple leaves in mid-June or in mid-July. In panel B, closed squares = *M. disstria* in July, closed triangles = *M. disstria* in June, open circles = *O. leucostigma* in July. The lower means plotted for each insect species are from larvae that fed on red oak in June or July.

TABLE 1. TOTAL PEROXIDE CONCENTRATIONS IN THE MIDGUT FLUIDS OF FINAL-INSTAR *O. leucostigma* AND *M. disstria* LARVAE THAT FED ON RED OAK OR SUGAR MAPLE LEAVES[a]

Host plant	Insect species	Date	Peroxides (mM)	*N*
Red oak	*M. disstria*	June 18	0.9 ± 0.1[a]	22
Sugar maple	*M. disstria*	June 18	3.7 ± 0.4[b]	18
Red oak	*O. leucostigma*	July 14	0.7 ± 0.1[a]	14
Red oak	*M. disstria*	July 11	1.4 ± 0.2[b]	15
Sugar maple	*O. leucostigma*	July 14	2.9 ± 0.4[d]	15
Sugar maple	*M. disstria*	July 11	2.3 ± 0.2[c]	15

[a] Data are presented as mean ± SE. Summary statistics followed by different letters (within months) are significantly different ($P < 0.05$).

Protein in midgut fluids was oxidized to a greater extent in caterpillars that were fed on sugar maple than on red oak, both in June ($P < 0.001$) and July ($P = 0.043$) (Figure 4A, B). Protein carbonyl levels were also greater in *M. disstria* than in *O. leucostigma* in June ($P < 0.001$) and July ($P < 0.001$). Protein carbonyls in *M. disstria* were positively associated with semiquinone radical intensities in their midgut fluids ($R^2 = 0.95$, $P = 0.025$) (Figure 5A). In *O. leucostigma*, there was a much weaker association between protein carbonyls and semiquinone radical intensities ($R^2 = 0.63$, $P = 0.21$), although a non-significant result in this case could be due to a small sample size ($N = 4$). ANCOVA provided confirmation that, at a given semiquinone radical intensity, protein carbonyls were formed to a greater extent in *M. disstria* than in *O. leucostigma* ($P = 0.043$). Proteins also appeared to be oxidized at a higher rate in *M. disstria* than in *O. leucostigma*, as indicated by a significant interaction between species and semiquinone radical intensity ($P = 0.046$; Figure 5A).

Total peroxide concentrations were consistently higher in larvae that were fed on sugar maple than on red oak (Table 1). However, peroxide levels in July were higher in *O. leucostigma* than in *M. disstria* on maple, the opposite of the result expected. Nevertheless, total peroxides in both caterpillar species were positively associated with semiquinone radical intensities in their midgut fluids ($R^2 = 0.72$; $P = 0.033$ for both species combined) (Figure 5B).

DISCUSSION

It has been hypothesized that phenolic compounds derive much of their anti-herbivore activities from being "oxidatively activated" (Appel, 1993). However,

few previous studies have clearly demonstrated that ingested phenolics in leaves oxidize in midgut fluids. Phenolic oxidation in the gut lumen has been suggested by studies that have measured (1) the loss of phenolics or increase in their oxidation products in frass (Felton et al., 1989; Barbehenn et al., 1996; Salminen and Lempa, 2002); (2) oxidative damage to the midgut epithelium (Summers and Felton, 1994; Bi and Felton 1995; Bi et al., 1997); or (3) the supression of ingested pathogens (Appel and Schultz, 1994; Hoover et al., 1998). However, in some of this previous work, including our own, it is likely that some fraction of phenolic oxidation occurred in frass after it left the low oxygen conditions in the gut, either from autooxidation or from polyphenol oxidase activity (Johnson and Barbehenn, 1999; Wang and Constabel, 2004). It is also possible that low molecular weight phenolics produce oxidative damage following their absorption into the midgut epithelium. The use of EPR spectrometry provides a direct measure of phenolic oxidation in the midgut fluids of caterpillars. Thus, a major finding in this study is the observation that phenolic compounds ingested in red oak and sugar maple leaves are oxidized in the midgut fluids of caterpillars. The overall prooxidant activity of these mixtures of phenolics is suggested by the positive relationships between semiquinone radical formation and two markers of oxidation in the midgut fluids from *M. disstria* and *O. leucostigma*. Although these results are correlative, it is noteworthy that previous experiments in which levels of phenolic compounds were controlled produced similar results; tannic acid ingested in an artificial diet also oxidized to produce elevated levels of semiquinone radicals, protein carbonyls, and peroxides, particularly in *M. disstria* (Barbehenn et al. 2001, 2003b, unpublished data). The results of the present study are consistent with two additional hypotheses: (1) the oxidation of phenolics and markers of oxidative damage are greater in the midgut fluids of caterpillars feeding on maple than in those feeding on oak; and (2) the levels of oxidation are greater in the phenolic-sensitive species *M. disstria* than in the phenolic-tolerant species *O. leucostigma*.

As expected, sugar maple leaves contain lower ascorbate:phenolic ratios and produce substantially higher semiquinone radical levels in caterpillar midgut fluids than red oak leaves. However, high levels of semiquinone radicals were also produced in the midgut fluids of both *M. disstria* and *O. leucostigma* when they ingested an artificial diet containing an ascorbate:gallic acid ratio of 0.21 (on a %DW basis) (Barbehenn et al., 2003b). Therefore, differences in ascorbate:phenolic ratios alone cannot explain (1) why a similar ascorbate:phenolic ratio in red oak leaves (0.25) produces a low level of semiquinone radicals in midgut fluids, (2) why high levels of semiquinone radicals are produced in larvae that feed on sugar maple in May (ascorbate:phenolics ratio of 0.17), or (3) why semiquinone radicals from sugar maple phenolics decay rapidly, while those from red oak are relatively stable in gut fluids. It appears likely that both the propensity of specific types of phenolics to oxidize and the stability of

the semiquinone radicals formed also affect the extent of oxidative damage in midgut fluids. Sugar maple leaves analyzed from July of 2004 contained high levels of ellagitannins, condensed tannins, and gallotannins, while red oak leaves were rich in condensed tannins, but contained almost no measurable ellagitannins or gallotannins (J.-P. Salminen, unpublished data). This difference in phenolic composition, along with the differences in semiquinone radical intensity and decay rates between the two tree species, leads us to suspect that sugar maple phenolics are more prone to oxidize and to damage other biomolecules than are the phenolics in red oak leaves. Other components of the oxidative defenses of maple and oak leaves, such as their oxidative enzymes, could also contribute to the observed differences in the activities of ingested oak and maple phenolics.

The relatively high levels of semiquinone radicals produced in *M. disstria* and *O. leucostigma* in May compared to June and July suggest that feeding on spring foliage does not necessarily allow these herbivores to avoid the prooxidant activities of ingested phenolic compounds. It is well known that the phenolic compositions of tree leaves change seasonally, even in cases in which there is an apparent lack of change in total phenolic levels (e.g., Salminen et al., 2004). It would be of interest to examine whether the types of phenolic compounds in immature leaves produce higher semiquinone radical intensities than those in mature leaves and to confirm whether biomarkers of oxidation are also higher in May in maple-feeding caterpillars.

As noted above, the observation that phenolic compounds are oxidized more extensively in *M. disstria* than in *O. leucostigma* is consistent with previous work on the propensity of ingested phenolics to oxidize in these species (Barbehenn and Martin, 1992, 1994; Barbehenn et al., 2001, 2003a,b). Therefore, differences in the gut biochemistries of *M. disstria* and *O. leucostigma* that have been observed on artificial diets are relevant to the more complex mixtures of phenolic compounds found in their host plants. A key conclusion of these studies has been that *O. leucostigma* maintains a more efficient ascorbate recycling system than does *M. disstria*, thereby minimizing phenolic oxidation. Higher ascorbate concentrations are found in *O. leucostigma* than in *M. disstria* on artificial diets and on tree leaves in July, but not on immature leaves in the spring (Barbehenn et al., 2001, 2003a). Thus, we are unable to explain the lower levels of semiquinone radicals formed in *O. leucostigma* on the basis of higher ascorbate levels in all cases.

Were levels of semiquinone radicals and markers of oxidation sufficiently low in *O. leucostigma* to suggest that phenolic compounds in oak can act as antioxidants in the midgut lumen? An experiment to determine this, such as one in which phenolic-poor leaves were treated with different levels of oak phenolics and levels of markers of oxidation were negatively correlated with phenolic levels, has not been done. However, the low peroxide levels in the

midgut fluids of caterpillars on artificial diets suggest that peroxide levels in the midgut fluids of *O. leucostigma* on oak were increased, rather than decreased, by oak phenolics. Peroxide levels in midgut fluids of both *O. leucostigma* and *M. disstria* on a phenolic-free diet were on the order of 50–100 μM (using the FOX and red ferrithiocyanate assays) and even peroxide levels measured on a diet containing 5% tannic acid were well below the levels of total peroxides measured in *O. leucostigma* on oak (Barbehenn et al., 2001, unpublished data). Nevertheless, red oak does appear to be a relatively benign host plant in terms of its oxidative defenses for both species of caterpillars tested.

In the case of sugar maple, the oxidative damage it generates in caterpillars could potentially explain its poor host plant quality for *M. disstria* in its northern range (Nicol et al., 1997). Increases in both protein oxidation and peroxides are potentially harmful to insect fitness (Cadenas, 1995; Felton, 1996; Halliwell and Gutteridge, 1999). However, we are unaware of studies that have clearly shown a negative relationship between the oxidation of phenolic compounds in the gut fluids of insects and their performance. Such work is needed to examine the effectiveness of plant oxidative defenses against insect herbivores more closely.

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ANTIFEEDANTS AGAINST *Hylobius abietis* PINE WEEVILS: AN ACTIVE COMPOUND IN EXTRACT OF BARK OF *Tilia cordata* LINDEN

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Abstract—Linden (*Tilia cordata*) bark was shown to contain an antifeedant effective against the large pine weevil, *Hylobius abietis*. Soxhlet extraction of inner and outer bark resulted in an extract that showed antifeedant activity in a microfeeding assay. The extract was fractionated by chromatography on silica gel using gradient elution with solvents of increasing polarity. The content of the fractions obtained was monitored by thin layer- and gas chromatography. Fractions of similar chemical composition were merged. Two of the 17 fractions showed antifeedant activity in the microfeeding assay. Nonanoic acid was identified in both of these fractions. Subsequent testing in the microfeeding assay showed that nonanoic acid possessed strong antifeedant activity against *H. abietis* adults.

Key Words—Antifeedant, Curculionidae, Coleoptera, deterrent, extract, feeding, fractionation, *Hylobius abietis*, linden, nonanoic acid, outer bark, pelargonic acid, phloem, pine weevil, soxhlet, *Tilia cordata*.

INTRODUCTION

Antifeedants are semiochemicals active in the last step of animal-host selection behavior, limiting the diet for both larvae and adults in insects (Dethier et al., 1961; Beck, 1965; Chapman, 1974) and for higher animals (Jakubas et al., 1994; Epple et al., 1996). Plant derived antifeedants are important as plant protection against predators. The neem tree produces azadirachtin, today a well

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known and commercialized compound active against a number of pest insects (Lowery and Isman, 1994; Mordue et al., 1998; Liang et al., 2003). This compound has also shown antifeedant activity against birds (Mason and Matthew, 1996). Other examples of promising plant antifeedant compounds are tryptamine, which harms reproduction in *Drosophila melanogaster* (Thomas et al., 1998), and cucurbitacins that function as feeding arrestants and toxicants against the southern corn rootworm (Barbercheck et al., 1995). Garlic oil, pulegone, and tannins reduce food consumption in birds (Avery et al., 1996; Voltz and Clausen, 2001; Hile et al., 2004) and Scots pine *Pinus sylvestris* contains pinosylvin that suppresses feeding of the snowshoe hare (Sullivan et al., 1992) and (1*S*,2*R*,4*S*,5*S*)-angelicoidenol-2-O-beta-D-glucopyranoside, a moose deterrent (Sunnerheim-Sjöberg, 1992).

This work focuses on the large pine weevil *Hylobius abietis* (L.) (Coleoptera: Curculionidae) and its feeding behavior on linden (*Tilia cordata*) bark extracts. The large pine weevil is a major pest on newly planted pine and spruce saplings in Sweden (Eidmann et al., 1996). If the young plants are left unprotected, the economic losses for the forest industry are severe (Weslien, 1998). Until recently the pyrethroid insecticide Permethrin was used to protect saplings. This insecticide has now been forbidden for use in Swedish forestry and has been replaced by Cypermethrin, which is also a pyrethroid. However, Cypermethrin has shown to be even more toxic than Permetrin (Oliveira et al., 2002). Our approach to this problem is to develop useful antifeedants that act either through smell or taste, or both, and thereby prevent the weevil from feeding. An optimal antifeedant will be an environmentally friendly compound with long-term stability to the conditions it experiences in the field. Thus, the compound should have low volatility and not decompose or be washed away under the influence of environmental factors such as oxygen, UV light, variation in temperature, and rainfall.

We have demonstrated the polyphagous habits of *H. abietis* (Månsson and Schlyter, 2004). When presented to other woody plant species and Scots pine (*P. sylvestris*) in choice tests, *H. abietis* always prefers the latter (Manlove et al., 1977; Månsson and Schlyter, 2004). However, if no choice is given, the weevils will feed on a number of plants, usually not regarded as common food sources. Thus, a broad range of possible food sources are available for the pine weevil in its natural habitat (Samuelsson, 2001). However, there are a number of woody plants on which this insect will not feed, preferring to starve. *Evonymus europaeus* and *Ilex aquifolium* were shown to belong to this group of plants (Månsson and Schlyter, 2004). However, when *Sorbus aucuparia* (L.), *Lonicera xylosteum* (L.), *Viburnum opulus* (L.), *Taxus baccata* (L.), *Syringa vulgaris* (L.), *Populus tremula* (L.), or *Tilia cordata* (Mill.) were used as food sources, another type of feeding behavior was found for the pine weevil. In this group of nonpreferred plants, weevils eat and remove only small amounts of bark and

phloem. We have also identified tree species on which the weevils feed only on the outer bark, but stop feeding once they reach the phloem (Månsson and Schlyter, 2004).

One method to investigate a plant for semiochemical antifeedant activity is to test an extract of the relevant plant tissue for activity against the relevant insect. In this way, the influence of the plant's texture is avoided. Our preliminary tests of bark extracts in microfeeding assays demonstrated that extracts of linden, *T. cordata*, bark displayed the best antifeedant qualities among eight extracted species tested. We have noted that when presented with extracts from some tree species, the weevils do not even approach controls in choice assays. Such results indicate the presence of high amounts of volatile antifeedants and interfere with statistical analysis of choice tests. Because antifeedants of high volatility are less suitable for long-term application in the field, we have focused on identifying nonvolatile antifeedants of *H. abietis* from linden bark. Antifeedant activity was assessed using our previously described microfeeding assay (Schlyter et al., 2004a).

We describe five methods for preparation of linden bark and Soxhlet extraction by different solvents. Furthermore, we describe the isolation of the fractions with the best antifeedant activity, and the identification of one active constituent from these fractions by GC-MS and NMR.

METHODS AND MATERIALS

Insect Collection and Culture. We collected adult *H. abietis* flying in May–June in Southern Sweden using established methods (Klepzig and Schlyter, 1999). Groups of 150 pine weevils were placed into ventilated plastic buckets containing moistened paper towels and *P. sylvestris* branches. To decrease the rate of the metabolism of the weevils, the buckets were stored in the dark in a cold room at 8–10°C prior to the experiments. Before initiation of an assay, the buckets were moved into a growth chamber with the parameters set to conditions similar to those found in the field at the time of weevil collection [24°C, 75% RH, and a photoperiod of 20:4 (L:D)]. Before the assays started, insects were allowed to feed on *P. sylvestris* branches for 1 wk. Thereafter, no food was provided for 6 days, and the last 24 hr before the assays they did not have access to water.

Extractions of T. cordata *Bark.* Linden branches and shoots (diam 5–30 mm) were collected during the summer in southern Sweden. Only young plant materials such as second-year shoots and branches were used. Different methods for preparation and extraction of the plant material were tested (Table 1). To determine the correct amount and concentration to use for each plate in the microfeeding bioassay, the same procedure for the initial prepa-

TABLE 1. THE FIVE EXTRACTION METHODS USED

	Bark	Method	Antifeedant Activity	Further Fractionation	Comments
A	Ground frozen	High pressure soxhlet extraction	Negative	No	Significant attraction
B	Freeze dried	Solvent soxhlet MeOH/CH_2Cl_2, 1:9, at 37°C	No	No	No activity
C	Fresh	Solvent soxhlet increasing polarity: Hexane→ CH_2Cl_2→MeOH	Positive, weak	Yes	Lost activity
D	Ground frozen	Solvent soxhlet MeOH/CH_2CL_2 1:9, at 37°C	Positive	Yes	Active in further fractionation
E	Levigated fresh in blender with MeOH	Solvent soxhlet MeOH	Positive	Yes	Reduced/lost activity

ration of the bark was used for all methods. Prior to removal of the bark from the twigs, the total area of the bark was measured. The outer and inner bark was cut or scraped off the stem and immediately extracted. The resulting extracts were concentrated *in vacuo* and solvent was added. The amount of solvent added was determined on the basis of the total bark area removed. Thus, if the bark area corresponded to, for example, 200 microfeeding assay TLC plates (each with an area of 25 mm^2) solvent was added to the extract until a volume of 200 × 10 μl was reached.

"*Frozen/Pressure*". The bark preparation was frozen in liquid nitrogen, and ground to fibers. The fiber mass was subjected to pressurized Soxhlet extraction using liquid, supercritical carbon dioxide (Rosenblum et al., 2002). In this setup, the extractor consisted of a 5-l vessel that housed a typical Soxhlet glass unit for the extraction of plant material with liquid solvents. The liquid extract was collected under pressure inside the extractor. The liquid solvent was vaporized by water heating of the bottom and sides of the extractor. On the top of the extractor, a refrigerated coil condensed the solvent vapors. The extraction cycle was controlled by monitoring temperature variation. The cycle was ended when the pressure peaked and then dropped, due to the CO_2 evaporation.

"*Freeze-Dried*". Freeze-dried bark was ground and extracted in a solvent Soxhlet apparatus (Furniss et al., 1989) for 4 hr by a mixture of dichloromethane/methanol (9:1) kept at reflux (35–45°C). In the Soxhlet apparatus (also called extractor, or chamber), the sample soaks in solvent that is periodically siphoned off, distilled, and returned to the sample. The resulting extract was concentrated *in vacuo*. To remove residual water, methanol was added to the

condensed extract followed by an additional concentration to dryness *in vacuo*. The dry weight of the extract was recorded and the extract was dissolved in dichloromethane/methanol (1:1).

"*Frozen/Solvent*". Bark was frozen in liquid N_2, then immediately pulverized in a grinder and, thereafter, treated as in the "freeze-dried" method.

"*Fresh*". Fresh bark was pulverized in a grinder, and the same batch was extracted as above with solvent Soxhlet in three steps with increasing polarity. Hexane, dichloromethane, and methanol were used as solvents and accordingly, three crude extracts were obtained.

"*Levigated*". Fresh bark was levigated to fine fiber in a blender with methanol, filtered, and then extracted with solvent soxhlet as above with the methanol from the blender.

The seven crude extracts obtained by the five extraction methods were tested for antifeedant activity in the microfeeding bioassay.

Microfeeding Bioassay. The microfeeding bioassay is a choice test consisting of a treatment and a blank on separate thin-layer chromatography plates [TLC plates (Merck #1.05552) 5 × 5 mm, aluminum sheets covered with a 0.2-mm-thick cellulose layer] (Schlyter et al., 2004b). The treatment is applied on one of the TLC plates as two drops of solution (2 × 5 μl), whereas the same amount of solvent only is added to the control plate. The dry extract was diluted so that the amount per area (10 μl) on the TLC plate (25 mm^2) corresponded to roughly the same amount per area of fresh bark. After evaporation of the solvent from the treatment and control plates, 5 μl of 1 M sucrose in water are added as a feeding stimulant. The two plates are placed together with a pine weevil in a Petri dish (diam 90 mm) and tests are run for 4 hr, $N = 12$. The cellulose layer of the control plate is often consumed within less than 4 hr.

Antifeedant Index. The results from the microfeeding assays are presented as the Antifeedant Index (AFI) (Blaney et al., 1984; Klepzig and Schlyter, 1999):

$$\mathrm{AFI} = \frac{\text{Amount fed on Control} - \text{Amount fed on Treatment}}{\text{Amount fed on Control} + \text{Amount fed on Treatment}} = \frac{\mathrm{C} - \mathrm{T}}{\mathrm{C} + \mathrm{T}}$$

A value of 1.00 corresponds to a total inhibition of feeding, a value of 0.50 a half-effect, and 0 no effect. Negative values (<0) indicate feeding stimuli. Activity was interpreted by comparing the individual 95% confidence intervals ($\alpha = 0.05$) with the theoretical values 0 (no activity) or 1 (full activity).

Fractionation. The Soxhlet extracts obtained from the "fresh," the "frozen/solvent," and the methanol extract from the "levigated" method were concentrated *in vacuo* on silica gel [5.0 g, silica gel 60 (230–400 mesh), Fluka]. The same type of silica gel (25 g) was packed into a column (diam 25 mm) and the

silica gel, impregnated with the extract, was added on top of the column. All solvents used as eluents were distilled prior to use. To elute the compounds, a gradient was used: first cyclohexane (220 ml, four column volumes), followed in order by dichloromethane/cyclohexane (220 ml, 10%, v/v), (220 ml 20%), (110 ml 30% → 90%; after each 110-ml batch, the amount dichloromethane was increased by 10%), and finally 55 ml 100% dichloromethane. The eluent was changed to methanol in dichloromethane. The methanol content was increased in steps: 10%, 15%, 30%, 50%, 80%, and 100%. The same volume of solvent (110 ml) was used in each step. Finally, the column was eluted with 110 ml of acetone. Fractions of 15 ml were collected.

All fractions were analyzed by TLC and some by GC. Those fractions that according to GC or TLC analysis contained roughly the same compounds were combined and were tested via the microfeeding assay.

Chemical Analysis. GC analyses were performed using a Varian 3400 instrument, EC-1 column (Alltech, 30 m × 0.32 mm i.d., 0.25 μm film thickness), temperature program: 40°C, 5 min, (10°C/min) to 250°C, 10 min. GC-MS analyses were carried out using a Varian 3800 GC instrument, CP-sil 5CB low bleed column (Varian, 30 m × 0.25 mm i.d., 0.25 μm film thickness), in series with a Saturn 2000 MS, ion trap detector, EI mode, temperature program as described for GC analyses. NMR analyses were carried out using a Bruker Avance DPX 250 spectrometer operating at 250.13 MHz (^{1}H) and 62.9 MHz (^{13}C), sample temperature 25°C, $CDCl_3$ as solvent, and TMS as internal standard. The reported purities of the compounds are calculated as the relative amount of integrated areas.

RESULTS

Bioactivity of Extracts. The seven extracts obtained by the different methods showed significant differences in antifeedant activity (arcsin-transformed AFI values: ANOVA, $F = 10.54$, $df = 6, 82$, $P < 0.001$). Only "frozen/solvent" fractions (AFI ± SE: Run 2: 0.93 ± 0.07, $N = 7$; Run 3: 1.00 ± 0.00, $N = 7$) showed antifeedant effects after chromatographic separation. The "fresh" method resulted in two extracts with negative AFI (Hexane: −0.64 ± 0.13, $N = 12$; CH_2CL_2: −0.29 ± 0.11, $N = 12$) showing feeding stimulation, and one inactive extract with an AFI overlapping 0 (MeOH: 0.30 ± 0.30, $N = 10$). The "freeze-dried" method yielded an extract with antifeedant activity (0.37 ± 0.14, $N = 10$) that was lost after further chromatographic separation. The "levigated" extract also showed antifeedant activity (0.64 ± 0.19, $N = 10$) that disappeared with further fractionation. The "frozen/pressure" method resulted in an extract with a negative AFI (0.47 ± 0.19, $N = 12$).

In the first set of assays of the "frozen/solvent" extract, only five plates were fed upon, giving a high variance (Figure 1a, Run 1: 0.60 ± 0.89). To allow for statistical comparisons, two additional assay runs were conducted. In both the second and the third run, the solution of extract on the treated plates was allowed to evaporate for 20 hr before testing (instead of the 20 min used before.). We found significant activity in both of these tests (Figure 1a, Run 2: 0.93 ± 0.07, $N = 7$; Run 3: 1.00 ± 0.00, $N = 7$). Out of the tested methods, this was the only one that gave extracts that furnished sufficiently active fractions after further separation by liquid chromatography. A dilution series (1:10 and 1:100) of the merged runs 2 and 3 also was performed in order to test the reliability of the method of measuring bark area as a way to obtain a similar concentration of the extract on the TLC plate as in the bark (Figure 1b). Dilution of the extract resulted in reduced activity with lower dose (Figure 1b, $r_S = 0.701$, $P < 0.001$). Only the undiluted extract had significant antifeedant activity (AFI $\gg$ 1) while the diluted extract (1:10) had an AFI overlapping 0.

Fractionation by liquid chromatography of the "frozen/solvent" extract resulted in 128 fractions. Those containing the same compounds (as judged by TLC or GC) were combined to give 17 fractions. These were tested in the microfeeding assay. Out of 17 fractions, #12 and #13 showed the strongest antifeedant activity (Figure 2, #12: 0.98 ± 0.02, $N = 9$ and #13: 0.83 ± 0.06, $N = 8$). Two fractions showed some, but not significant, antifeedant activity (#8: 0.48 ±

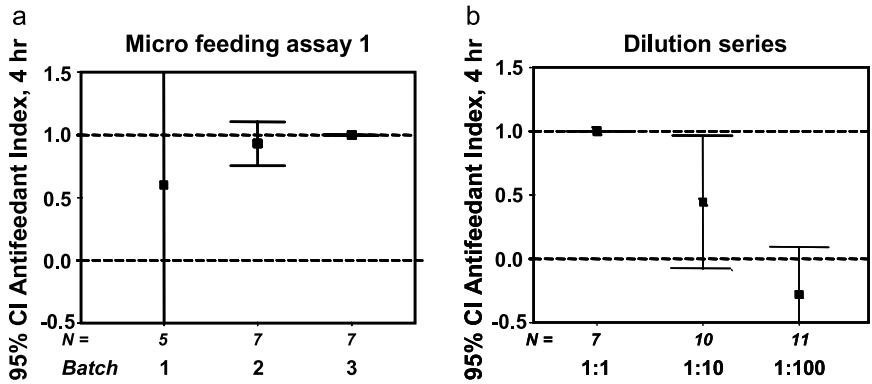

FIG. 1. a. Antifeedant activity of the crude *T. cordata* extract ($N = 12$), replicated twice. Bars show Antifeedant Index (AFI) average area consumed $(C - T)/(C + T)$, ±95% confidence interval from microfeeding assay. The number of responses in the first test was too low for statistical analysis. In the second and third tests, the solvent was allowed to evaporate during 20 hr and then complete feeding inhibition (AFI ≈ +1) was found. b. Dose response of the crude *Tilia* extract from batch 3, ($r_S = 0.701$, $P < 0.001$).

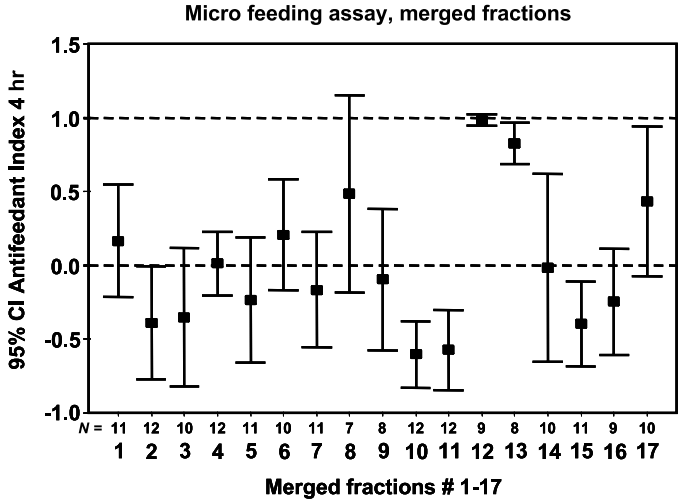

FIG. 2. Antifeedant activity of 17 fractions of *Tilia* extract in the microfeeding assay. Fraction 12 shows complete feeding inhibition (AFI ≈ +1) and 13 shows strong inhibitory tendencies (AFI ≈ 0.70–0.95) while the two fractions collected just before (10 and 11) shows significant attraction (AFI < 0).

0.27, $N = 7$ and #17: 0.43 ± 0.22, $N = 10$), while nine fractions were inactive, with an AFI overlapping 0 (#1: 0.17 ± 0.17, $N = 11$; #3: −0.35 ± 0.21, $N = 10$; #4: 0.01 ± 0.10, $N = 12$; #5: −0.23 ± 0.19, $N = 11$; #6: 0.21 ± 0.17, $N = 10$; #7: −0.16 ± 0.17, $N = 11$; #9: −0.10 ± 0.20, $N = 8$; #14: −0.02 ± 0.28, $N = 10$ and #16: −0.25 ± 0.16, $N = 9$). Four fractions showed a negative AFI not overlapping 0, i.e., feeding stimulation (#2: −0.39 ± 0.17, $N = 12$; #10: −0.60 ± 0.10, $N = 12$; #11: −0.57 ± 0.12, $N = 12$; #15: −0.40 ± 0.13, $N = 11$).

Identification of an Active Compound. The two fractions with the strongest antifeedant activity (#12 and #13, Figure 2) were analyzed by GC. Fraction #13 contained one major compound (84% purity according to GC). By comparison with the spectra of an authentic sample [Fluka], this compound was identified by GC-MS as nonanoic acid. To further verify the identity of the major compound present in fraction 13, ^{1}H and ^{13}C NMR spectra were recorded and found to be identical with those reported for nonanoic acid (Aldrich Library of ^{13}C and ^{1}H FT NMR Spectra). Fraction number 12 was analyzed by GC-MS and also found to contain nonanoic acid as the major constituent together with smaller amounts of unidentified compounds. Commercially available nonanoic acid was tested in the microfeeding assay and displayed high antifeedant activity against *H. abietis* (AFI = 0.99 ± 0.01, $N = 9$).

DISCUSSION

We have reported that *H. abietis* does not feed from the bark or the phloem of some plants including *T. cordata* (Månsson and Schlyter, 2004). We have shown that the bark of *T. cordata* contains nonanoic acid, a chemical constituent that has strong antifeedant activity against *H. abietis*. We also found a suitable method ("frozen/solvent") for extracting the bark and further fractionation of the extract without losing the antifeedant effect.

In order to isolate an active extract that did not lose its activity in the feeding assay after fractionation, we tried several different methods for the extraction of *T. cordata* bark. During the first test of the extract obtained by the "frozen/solvent" method, the number of microassay plates fed upon were only five out of 12. We know from experience that this effect was likely due to a combination of high volatility and high activity of the tested compounds. The vapor from strong repellents or arrestants, present at every position in the Petri dish, stopped the insect from accepting any of the cellulose plates, including the control. To control for this effect, we let the volatiles on the treated plates evaporate for approximately 20 hr before commencing the assays. After this new protocol, the number of plates fed upon increased to seven in both of the two tests performed, a number high enough for statistical analysis.

Nonanoic acid has been identified as minor extractive in some other species that we earlier found to have antifeedant properties (Månsson and Schlyter, 2004), although not in bark and phloem. In the leaves and husks of walnut (*Juglans regia*), for example, it is a common antifeedant against several insects (Cohen et al., 1974; Shaaya et al., 1976; Hwang et al., 1982). The acid has also been shown to occur in almost all species of animals (Fontan et al., 2002), and at low levels in many of our common foods (Jirovetz et al., 2003). Ingestion or inhalation of small amounts of nonanoic acid has no known toxic effects and has been approved as a food additive (http://www.epa.gov). In addition, the compound is degraded rapidly in the environment. Therefore, use of nonanoic acid in pesticide products appears to pose no apparent risks to humans or the environment.

In addition to nonanoic acid in the bark, blossoms from *T. cordata* contain decanoic acid (Vidal and Richard, 1986). The genus *Tilia* contains numerous other compounds, some of them with antifeedant qualities. Eighteen volatile compounds have been identified in linden honey including eugenol, carvacrol, and thymol (Muckensturm et al., 1981; Yano, 1987; Arnason et al., 1989; Blank et al., 1989; Guyot et al., 1998; Moretti et al., 1998; Vasquez et al., 1999; El-Zemity and Radwan, 2001). Essential oil from linden blossoms contain some 100 compounds (Praczko and Gora, 2001) including the antifeedants germacrene-D, limonene, linalool, and *p*-cymene (Maganga et al., 1996; Biavatti et al., 2001; Tripathi, 2002; Halarewicz-Pacan et al., 2003).

Five antifeedants known to be present in *Tilia* spp. affect *H. abietis*. Limonene inhibits the action of α-pinene, a major component responsible for the attraction of *H. abietis* to *P. sylvestris* (Nordlander, 1990). D,L-Linalool is repellent to adult pine weevils (Muller and Haufe, 1991). Apart from being an antifeedant, even low doses of carvacrol cause mortality (Schlyter et al., 2004a). Eugenol inhibits feeding at 10% doses (Schlyter, unpublished data) as does carvone (Schlyter et al., 2004b).

Thus, one single plant species like *T. cordata* provides a rich source of potent antifeedants against numerous insect species. Most likely other promising antifeedant compounds can be found among the plants on which *H. abietis* does not feed.

Nonanoic acid has strong antifeedant qualities against *H. abietis* and is a common naturally occurring plant compound with low environmental persistence and toxicity. It is commercially available and does not have to be extracted from plant material. Although nonanoic acid is commercially available and safe, there is a need to know its activity at different doses and to examine various formulations and heavier analogs. The effects of UV radiation and changes in temperature and humidity have to be evaluated for each compound. The presence of semiochemicals in linden and other woody plants gives a rich library of candidate antifeedants for future plant protection, but they have to be further examined.

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HERBIVORE-INDUCED PLANT VOLATILES TRIGGER SPORULATION IN ENTOMOPATHOGENIC FUNGI: THE CASE OF *Neozygites tanajoae* INFECTING THE CASSAVA GREEN MITE

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Abstract—A large body of evidence shows that plants release volatile chemicals upon attack by herbivores. These volatiles influence the performance of natural enemies. Nearly all the evidence on the effect of plant volatiles on natural enemies of herbivores concerns predators, parasitoids, and entomophagous nematodes. However, other entomopathogens, such as fungi, have not been studied yet for the way they exploit the chemical information that the plant conveys on the presence of herbivores. We tested the hypothesis that volatiles emanating from cassava plants infested by green mites (*Mononychellus tanajoa*) trigger sporulation in three isolates of the acaropathogenic fungus *Neozygites tanajoae*. Tests were conducted under climatic conditions optimal to fungal conidiation, such that the influence of the plant volatiles could only alter the quantity of conidia produced. For two isolates (Altal.brz and Colal.brz), it was found that, compared with clean air, the presence of volatiles from clean, excised leaf discs suppressed conidia production. This suppressive effect disappeared in the presence of herbivore-damaged leaves for the isolate Colal.brz. For the third isolate, no significant effects were observed. Another experiment differing mainly in the amount of volatiles showed that two isolates produced more conidia when exposed to herbivore-damaged leaves compared with clean air. Taken together, the results show that volatiles from clean plants suppress conidiation, whereas herbivore-induced plant volatiles promote conidiation of *N. tanajoae*. These opposing effects

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suggest that the entomopathogenic fungus tunes the release of spores to herbivore-induced plant signals indicating the presence of hosts.

Key Words—Cassava, conidiation, fungal pathogen, GC-MS, herbivore-induced plant volatiles, mite, *Mononychellus tanajoa*, *Neozygites tanajoae*.

INTRODUCTION

Plants can influence the effectiveness of the third trophic level (Price et al., 1980). For example, it is well established that natural enemies are attracted by volatile chemicals released by plants that are attacked by herbivores (Dicke and Sabelis, 1988; Dicke et al., 1990; Turlings et al., 1990). These so-called herbivore-induced plant volatiles (HIPV) help the natural enemies in locating their victims and thereby also help the plant in reducing the impact of herbivory. Whereas much work has been devoted to the effect of HIPV on predators and parasitoids, little is known about the impact on entomopathogens (Elliot et al., 2000). Recently, HIPV from the roots of *Thuja* plants were shown to attract entomopathogenic nematodes (Boff et al., 2001; van Tol et al., 2001), but other classes of entomopathogens, such as fungi, have not yet been investigated for their response to HIPV. One exception is the study by Brown et al. (1995), which suggests that tobacco plants under aphid attack produced volatiles that delay conidial germination by the entomopathogen *Pandora neoaphidis* (Remaudière and Hennebert) until after the conidia come into contact with the aphid integument. The same effect was obtained by exposing conidia to volatiles from mechanically damaged (macerated) leaves and to two alcohol-type components that are part of the blend of green leaf volatiles (GLV) (Brown et al., 1995). Hence it is not yet clear whether there is a separate role for HIPV. In this study, we addressed the question whether the fungal pathogen *Neozygites tanajoae* Delalibera, Hajek, & Humber uses HIPV to tune the production of spores to the presence of its host, the herbivorous mite *Mononychellus tanajoa* (Bondar).

The mite *M. tanajoa* is an important pest of cassava, a staple food crop widely cultivated in Africa. It lives on the underside of cassava leaves which it damages by feeding on leaf parenchyma cells, leaving discolored spots on the leaf, easily recognizable with the naked eye. To control *M. tanajoa*, biological control based on the use of phytoseiid predators and the fungal pathogen *N. tanajoae* has been developed. *N. tanajoae* is specific to *M. tanajoa* and has been found both in the Neotropics (Agudelo-Silva, 1986; Delalibera et al., 1992; Alvarez-Afanador et al., 1993) and in Africa (Bartkowski et al., 1988; Yaninek et al., 1996; Dara et al., 2001a). However, infection rates rarely exceeded 1% in Africa, whereas frequent epizootics were reported in the Neotropics (Delalibera

et al., 1992; Elliot et al., 2002). Brazilian isolates of *N. tanajoae* were, therefore, imported into Benin, West Africa, and released in experimental fields in 1999. Postrelease monitoring showed significantly higher infection rates in fields where Brazilian isolates were released (Hountondji et al., 2002). Annual epizootics have been observed in Benin since then (Hountondji et al., 2003). However, laboratory virulence tests conducted on leaf discs under optimal conditions failed to show significant differences between the indigenous and the imported isolates (Dara et al., 2001b). Given the differential epizootic potential of the indigenous and imported isolates of *N. tanajoae* in the field, we suspect genetic variation among isolates of *N. tanajoae* to play a role, e.g., with respect to climatic conditions or response to HIPV.

Previous studies demonstrated that the phytoseiid predators *Typhlodromalus aripo* DeLeon and *T. manihoti* Moraes respond to volatiles emanating from cassava leaves infested by *M. tanajoa*, as opposed to volatiles from uninfested cassava leaves, mechanically damaged leaves, and *M. tanajoa* females alone (Janssen et al., 1990; Gnanvossou et al., 2001, 2003). These results suggest the release of volatile chemicals upon herbivore attack (HIPV), but definitive proof of their presence is yet to be given. Therefore, we first carried out a GC-MS analysis to identify the compounds in the blends of infested and clean cassava plants.

To assess the sporulation response of the mite pathogenic fungus (*N. tanajoae*) to HIPV, it is important to distinguish among the various kinds of spores produced. Two types are produced during the asexual phase: spherical spores called conidia and almond-shaped spores called capilliconidia (Oduor et al., 1996a). There is also a third type, so-called resting spores, which supposedly arise in the sexual phase. Resting spores are rarely found in the field, even during epizootics (Elliot et al., 2002; Hountondji et al., 2002), but the first two types are commonly observed. Conidia are discharged from infected herbivorous mites that are mummified. Most of these immobile spores end up on the leaf in a halo around the sporulating, mummified mite. Conidia germinate to give rise to capilliconidia, which represent the infective stage of *N. tanajoae*. Production of conidia and their germination into capilliconidia require specific climatic conditions, which are only met during nighttime in certain periods of the year in the tropics: (1) cool temperature, and (2) water-saturated air (Oduor et al., 1996a,b; Elliot et al., 2002). We chose to focus on conidiation because survival—and hence timing—is more critical here than in the capilliconidial phase. Capilliconidia can survive a few days until contacting and infecting a host, but conidia loose their viability within an hour below saturation conditions (Oduor, 1995). If the climatic conditions are not met, they fail to germinate and produce capilliconidia. Therefore, we measured the relation between the production of conidia and HIPV as a potential signal of host presence.

METHODS AND MATERIALS

Fungal Isolates. We used two Brazilian isolates that were successfully introduced in Benin (Altal.brz and Colal.brz) and one isolate found in Benin (Coton.ben). The isolates were preserved as mummified, infected *M. tanajoa* referred to as mummies. Mummies of Altal.brz and Colal.brz were collected from Alto Alegre and Colas das Almas in the state of Bahia, Brazil in 1995, respectively, whereas those of Coton.ben were collected in Cotonou, Benin, in 1997. Mummies of the three isolates were conserved inside tightly sealed photographic film canisters on dry cotton wool over another layer of cotton wool soaked in glycerol (serving as humidity trap) and maintained at 4°C. The stored isolates were cultured *in vivo* at approximately 6-mo intervals to minimize loss of viability. For each experiment, a new batch of mummies was produced for each isolate to be used within a maximum of 2 wk.

Analysis of Volatile Blends. We analyzed the volatile blends from infested and clean young cassava plants (ca. 6 wk old). Plants had the same number (nine) and the same size of leaves, and they were maintained in separate cages at 25°C in climate houses. Two clean plants and four plants infested with *M. tanajoa* were tested for emission of volatiles. Infested plants carried 450–600 adult female *M. tanajoa*. The collection was done following the procedure described by Agrawal et al. (2002). Per plant, volatiles were collected from all nine detached leaves and the plant apex and were put together into a 5-l glass desiccator. Volatiles were trapped on Tenax adsorbent (90 mg) packed in a 160 × 4-mm-ID glass tube (Chrompack) by blowing purified air (ca. 100 ml/min) through the desiccator for 90 min. Incoming air was purified by drawing it through silica gel, activated charcoal, and Tenax. The Tenax tubes were stored at room temperature in the dark until before retrieving the adsorbents through thermodesorption at 250°C for 10 min in a helium flow (10 ml/min). Desorbed products were cold-trapped at −90°C (M-16200, Chrompack) and analyzed with GC-MS. Compounds were identified by comparing the mass spectra obtained with those in the Wiley-Library and our own specialized library of natural products and by comparison of retention times (see Agrawal et al., 2002).

Exposure to HIPV. Two types of experiments were conducted to assess the effect of HIPV on conidiation of *N. tanajoae*. One was conducted in closed Petri dishes to compare conidiation of the three *N. tanajoae* isolates when exposed to volatiles emanating from small, excised leaf discs that were treated in various ways. The second experiment was conducted in a system with a regulated airflow containing either clean air or HIPV emanating from cassava leaves detached from the plant.

Diffusion in Closed Dish Environment. This experiment consisted of four treatments, differing in the sources of volatiles production and clean air as control (Figure 1). Volatiles emanated from leaf discs (2 cm diam) that were

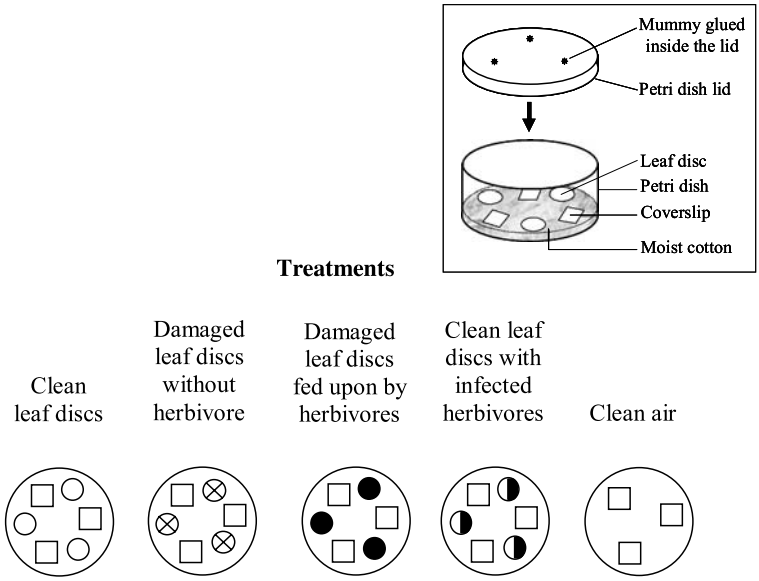

FIG. 1. Arrangement of coverslips to collect conidia (squares) and treated leaf discs (small circles) in a study of the effect of HIPV on conidiation for each isolate of the fungal pathogen *N. tanajoae* in closed dish experiment (large circle = Petri dish; see inset for a three-dimensional view of a Petri dish with mummies of the lid, illustrating the "descending conidia" setup). The volatiles to which the mummies are exposed emanate from the leaf discs, which were treated as follows: clean (open circles), damaged by the herbivore *M. tanajoa*, which were removed just before the experiment (crossed circles), infested with *M. tanajoa* females that were not infected by the fungus (black circles), and clean leaf discs with *M. tanajoa* infected and mummified by *N. tanajoae* (semi-black circles). Control dishes had no leaf discs.

excised from the first fully expanded leaves of intact cassava plants (cv Agric) and that were subjected to the following treatments prior to the experiment:

- Herbivore-damaged leaf discs from which all *M. tanajoa* and byproducts (web and feces) were removed (leaf damage scale rated 4 following the damage evaluation system proposed by Yaninek et al., 1989).
- Leaf discs on which six young adult females of *M. tanajoa*, introduced 2 hr before the experiment (leaf damage scale 2), were feeding.
- Clean leaf discs with six adult females of *M. tanajoa* mummified due to infection by *N. tanajoae* (mummies do not feed; hence no herbivore damage).
- Leaf discs excised from herbivore-free plants ("clean" leaf discs).

The method of "descending conidia" described by Papierok and Hajek (1997) was used to carry out conidiation. Three mummified mites (infected by *N. tanajoae*) were glued upside down using double-sided sticky tape, inside of the lid of a Petri dish (9 cm diam), with the distance between mummies being 4 cm. The conidia ejected from these mummies were shed over 2 × 2-cm coverslips on moist cotton at the bottom of the dish (Figure 1). Cotton inside the Petri dish was soaked with water until it reached the upper two thirds of the dish height, ensuring a maximum distance of 0.5 cm between coverslips and mummies. In between the coverslips, on the moist cotton, each dish had three leaf discs that received the same treatment (one of the four treatments mentioned earlier). Leaf discs were not provided in the control dishes. Each experiment involved five dishes per fungal isolate: four dishes for the different treatments and one for the control (Figure 1). The experiment was repeated three times. Petri dishes were closed with a lid that was fixed with two strips of tape at opposite sides of the lid to keep the mummies above the center of the coverslips. Petri dishes were covered with black cloth to avoid exposure to light (sporulation occurs at night) and incubated at 20 ± 1°C for 6 hr because previous experiments conducted with the three isolates showed that most conidia were produced within 6 hr at these conditions.

After the incubation period, dishes were left open for about 15 min to allow coverslips, covered with water droplets, to surface-dry. Conidia on the coverslips were mounted by turning the coverslips upside down on glass slides with a drop of 0.1% lactophenol blue for staining the conidia. Counts were made under a dissecting microscope (Zeiss, STEMI 2000, 45× magnifications) with inverted light. A piece of cross-ruled transparency was attached under the slide to facilitate counting.

Airflow System. Conidiation experiments were also performed in an airflow box. This was a plastic, cylinder-shaped (12 cm diam, 8 cm high) box with a lid tightly sealed with Teflon and Parafilm. There was an air inlet and an outlet to expose the mummies to an airflow permeated or not with volatile, according to treatment or control (Figure 2). Moistened cotton was placed on the bottom of this airflow box. On top of the cotton layer, there were five sporulation dishes (2.5 cm diam), with a coverslip on a thin layer of cotton on the bottom (inset in Figure 2). A single mummy was glued to the lid of the sporulation dish with double-sided sticky tape, right above the coverslip so that the conidia ejected from the mummy would end up on the coverslip. The conidia on this coverslip were counted following the same procedure as described above. To provide optimal conditions for conidiation, the experiment was carried out in the dark, in a climate room at 20°C, and at high humidity (due to moistened cotton in sporulation dish and airflow box). To facilitate air exchange with the airflow box, each sporulation dish had four equidistant holes (0.4 cm diam) along its lateral side.

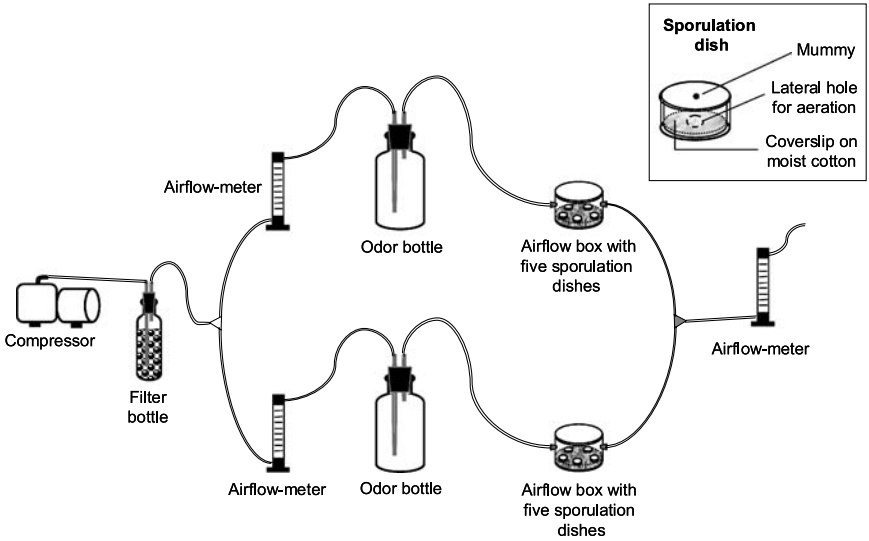

FIG. 2. Diagrammatic view of the airflow system. Air from the compressor was filtered by a bottle containing charcoal before going through airflow meters, the odor bottles (one of which contained herbivore-infested leaves; the other one was empty), the airflow boxes containing sporulation dishes (see inset), and exiting through another airflow meter. Airflow meters served to regulate the flow speed; the exit airflow meter ensures a uniform flow between the two arms of the system. Teflon tubing connected the different parts of the system.

In this experiment, HIPV (treatment) was tested against clean air (control). HIPV emanated from six young cassava leaves (third leaves from the apex) each infested for 3 hr prior to the experiment with 100 females of *M. tanajoa* (that were feeding on the leaves during the experiment) inside one of the two glass odor bottles (Figure 2). Leaves were kept fresh by inserting their petiole through a Parafilm cover into a small vial (1 cm diam, 5 cm high) with water. Airflow in the system was generated by means of a compressor (SERBATOI® AUTOCLAVI, Type ELTO, volume 50 l). The air moved through tubes from the compressor to a bottle containing a charcoal filter to make it clean, then consecutively to the airflow meters, the odor bottles, the airflow boxes, and finally to an airflow meter again (Figure 2). The airflow meters at the beginning and end of the two arms of the system are necessary to maintain a uniform airflow of 1 l/min. in both arms.

Using this setup, we assessed the effect of HIPV from *M. tanajoa*-infested cassava leaves on the conidiation of the two fungal isolates Colal.brz and Coton.ben. The experiment was replicated 10 times for each isolate. Sporulation

was allowed to take place for 24 hr. Before each experiment, airflow boxes and sporulation dishes were washed, whereas cotton wool and coverslips were replaced. Prior to putting the herbivore-infested leaves in the odor bottle and placing the mummies in the sporulation dishes, clean air was allowed to flow through both arms of the system for 2 hr. For successive experiments, allocation of clean air or HIPV was alternated between the two arms in the split tube circuit shown in Figure 2.

Data Analysis. To estimate the original number of conidia produced, it was necessary to calculate the sum of the number of nongerminated conidia, germinating conidia, and capilliconidia that had developed from conidia while ignoring the shriveled remnants of the germinated conidia.

For the closed dish experiment, data for original numbers of conidia were square-root transformed before analysis. A generalized linear model (GLM) procedure (SAS Institute Inc., 1999) was applied to test differences in conidia production between the main factors and their interactions. Isolate and treatments were treated as fixed factors, whereas experiment was treated as a random factor. Tukey's Studentized range test served to separate means at the 5% level.

With respect to the airflow experiment, data were analyzed using replicated goodness-of-fit tests against a 1:1 null hypothesis (Sokal and Rohlf, 1997, p 716). In this way, we assessed the effect of HIPV on conidiation in each replicated experiment, and across the different experiments, to account for heterogeneity among the trials. If heterogeneity was significant, groups of homogeneous replicates were identified using an unplanned comparisons procedure, and a *G*-test on pooled results within homogeneous groups of replicates was subsequently carried out to scrutinize the extent to which heterogeneity affected the validity of the total *G*-statistic based on all the experiments (Sokal and Rohlf, 1997, p 722).

RESULTS

Composition of Volatile Blends. Figure 3 shows histograms of mean chromatogram peak areas for all volatiles that contributed >5% to the total chromatogram peak area. In the blend emanating from clean leaves, five volatiles were produced in relatively large amounts, namely, 2-butanon (2), 3-pentanon (3), (*trans*)-(*E*)-2-hexanal (8), (*Z*)-3-hexen-1-ol (11), and (*E*)-2-hexen-1-ol (12). In the blend emanating from infested leaves, these volatiles were produced in higher or lower quantities, particularly the production of (*trans*)-(*E*)-2-hexanal (eight) was somewhat decreased, and that of (*Z*)-3-hexen-1-ol (11) was increased. Moreover, these blends emanating from infested leaves contained six volatiles that were only present in trace quantities in the blends from clean

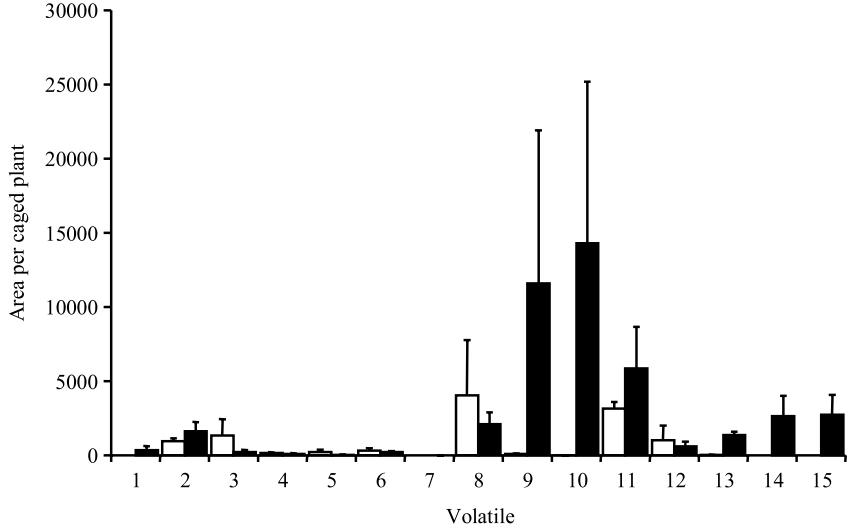

FIG. 3. Quantity of volatiles (expressed in mean peak areas ± SE) produced by leaves and apices from single clean cassava plants (open bars; $N = 2$) or from single cassava plants infested by *M. tanajoa* (closed bars; $N = 4$). The volatiles were identified by GC-MS. Only those that contributed >5% to the total peak area in the chromatograms are shown: (1) *O*-methyloxime-2-methylpropanal; (2) 2-butanon; (3) 3-pentanon; (4) 1-penten-3-on; (5) 1-butanol; (6) 1-penten-3-ol; (7) heptanal; (8) (*trans*)-(*E*)-2-hexenal; (9) (*E*)-(*trans*)-beta ocimene; (10) (3*E*)-4,8,-dimethyl-1,3,7,-nonatriene; (11) (*Z*)-3-hexen-1-ol; (12) (*E*)-2-hexen-1-ol; (13) linalool; (14) methyl salicylate; (15) 4,8,12-trimethyl-1,3(*E*),7(*E*),11-tridecatetraene.

leaves. In decreasing concentrations, these were (3*E*)-4,8-dimethyl-1,3,7-nonatriene (10), (*E*)-(*trans*)-beta ocimene (nine), 4,8,12-trimethyl-1,3(*E*),7(*E*), 11-tridecatetraene (15), methyl salicylate (14), and linalool (13) and, to a lesser extent, *O*-methyloxime-2-methylpropanal (one). Thus we found strong support for the release of volatiles by cassava plant upon herbivory by *M. tanajoa*, thereby confirming earlier inferences from behavioral experiments (Janssen et al., 1990; Gnanvossou et al., 2001). These volatiles are most likely to be herbivore-induced plant volatiles (HIPV) and not byproducts of the feces and the web (Sabelis et al., 1984; Dicke et al., 1990; Gnanvossou et al., 2001).

Closed Dish Experiment. The collective response of all three isolates of *N. tanajoa* to the treatments was close to significance (Table 1). No significant difference was observed between the conidia production of the isolates when all treatments were considered together (Table 1). However, a significant interaction was found between the treatments and the isolates (Table 1) indicating

TABLE 1. EFFECT OF VOLATILES FROM TREATED CASSAVA LEAF DISCS ON CONIDIATION OF THREE ISOLATES (ALTAL.BRZ, COLAL.BRZ, AND COTON.BEN) OF THE FUNGAL PATHOGEN *Neozygites tanajoae*, 6 HR AFTER INTRODUCING MUMMIES OF *Mononychellus tanajoa* INTO THE CLOSED DISH EXPERIMENT

Source	*df*	Sum of squares	Mean squares	*F* ratio	Significance level
Treatment	4	435.04	108.76	2.20	0.073
Isolate	2	80.06	40.03	0.81	0.447
Treatment × Isolate	8	820.16	102.52	2.08	0.044*
Experiment	2	216.47	108.23	2.19	0.117
Replicate	2	1.25	0.63	0.01	0.987
Error	112	5532.63	49.40		
Total	130	7122.97	54.79		

*Indicates a significant difference at 5% level.

differences in responses to treatments among isolates. To detect trends among the different responses of the isolates, we plotted conidia production under all five treatments for each isolate (Figure 4).

Averaged overall treatments, the isolate Coton.ben produced 292.3 conidia per mummy and showed relatively little variation in response to the different

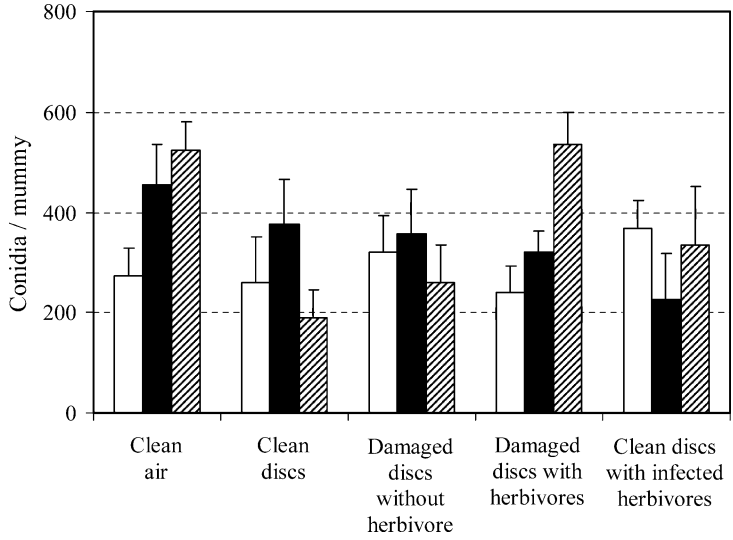

FIG. 4. Conidia production of three isolates (white bars: Coton.ben; black bars: Altal.brz; hatched bars: Colal.brz) of the fungal pathogen *N. tanajoae*, under five different treatments (see Figure 1), 6 hr after introducing mummies of the herbivorous mite *M. tanajoa* into the closed dish experiment.

treatments (coefficient of variation $V^* = 18.9\%$; Sokal and Rohlf, 1997). The isolate Altal.brz produced a mean number of 347.1 conidia per mummy and showed somewhat more variation in response to the different treatments ($V^* = 25.6\%$). Much of this variation can be attributed to the high conidia production under clean air. The isolate Colal.brz produced a mean number of 368.6 conidia per mummy, but the variation in response to the different treatments was much higher than in the other two isolates ($V^* = 44.9\%$). Much of this variation here can be attributed to the strikingly high conidia production under clean air and when exposed to volatiles from leaf discs infested by *M. tanajoa*.

The variation expressed by the isolate Colal.brz in the bar plot points to a trend in responses to clean air as well as to volatiles from leaf discs fed upon by *M. tanajoa*. This trend was also indicated by the nearly significant difference between the treatments (Table 1). To identify potential causes for this trend, a Tukey test was applied following the GLM analysis to separate means between the treatments. This showed a significant difference ($P < 0.05$) between clean air and volatiles from clean leaf discs: 428.8 ± 41.46 conidia per mummy vs. 275.1 ± 47.48 conidia per mummy (mean ± SE), respectively. Furthermore, given the high variability and large differences in the responses to the treatments by the isolate Colal.brz, an ANOVA was conducted for this isolate alone, and significant differences in conidia production were found between the treatments ($P = 0.003$, $F = 4.01$, $df = 4$, $N = 45$). Subsequent Tukey tests to compare the means showed a significant difference between conidia production ($P < 0.05$) under volatiles from leaf discs fed upon by *M. tanajoa* and volatiles from clean leaf discs, but no significant difference between leaf discs fed upon by *M. tanajoa* and clean air (Figure 4).

Airflow System. Given that Colal.brz gave the most striking differences between clean air and volatiles from clean leaf discs and virtually no difference between clean air and volatiles from leaves fed upon by *M. tanajoa*, we decided to challenge the latter result by repeating the experiment with the following modifications: (1) using intact leaves as volatiles sources (instead of leaf discs), (2) starting with a higher infestation of herbivorous mites on cassava leaves (30× more mites than in closed dish experiment), and (3) allowing the herbivorous mites to feed longer on cassava leaves (3 d instead of 2 hr). Using the replicated *G*-test for total *G*-statistics, we found that conidia production was significantly higher in an air stream with volatiles from cassava leaves infested by *M. tanajoa* than under clean air conditions (404.7 conidia per mummy vs. 354.0 conidia per mummy; hence 14% more conidia; total $G = 967.52$, $df = 10$, $P < 0.001$). In total, there were five significant replicates in favor of the control with a difference in conidia production of only 46% (±20.6 SE) of the treatment (Figure 5). There were four significant replicates in favor of the treatment (volatiles from infested leaves), and here the difference was 187% (±71.9 SE) of the control (Figure 5). Indeed, heterogeneity among replicates

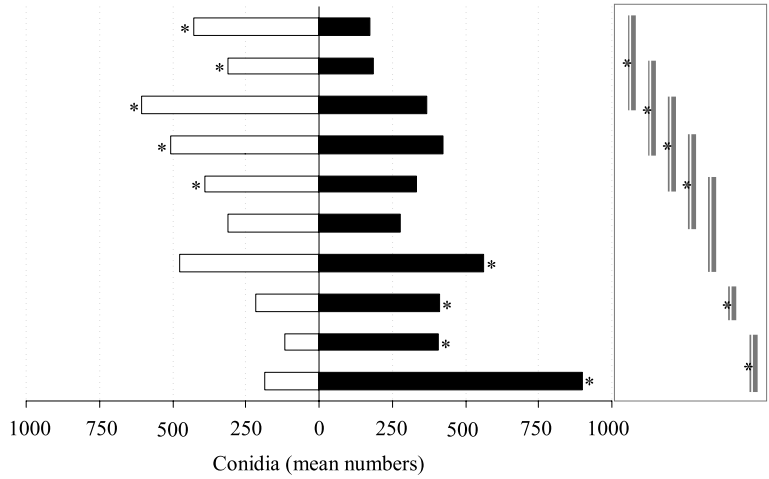

FIG. 5. Conidia production of a Brazilian isolate of *N. tanajoae* (Colal.brz) in the presence (black bars) and absence (open bars) of volatiles from intact cassava leaves infested by the herbivorous mite *M. tanajoa* in the airflow system experiment. Replicates of each treatment were carried out simultaneously with a replicate of the other treatment, and the results are therefore shown pairwise for 10 replicates. Significant differences among simultaneous replicates of the two treatments are indicated by an asterisk. Since replicated *G*-test indicated significant heterogeneity among the replicates, unplanned comparisons were carried out to test for homogeneity among replicates. The right-hand panel shows the groups of homogeneous replicates; significant differences between the two treatments using the pooled *G*-statistic within homogenous replicates are indicated by an asterisk.

was significant (heterogeneity $G = 933.52$, $df = 9$, $P < 0.001$). Unplanned comparisons showed groups of one, two, and more often three replicates that were homogeneous (Figure 5, right-hand panel). Taken together, the comparisons indicated a large variability among replicates (i.e., small homogeneous groups). It should be noted that whenever homogeneous groups showed a significantly higher effect of HIPV on conidia production in the treatment, the numerical difference was more pronounced than when the higher effect was found in the control.

To test whether the absence of a response in the closed dish environment implies the same response under conditions where volatiles come from intact leaves infested by a larger population of herbivorous mites feeding for a longer period, we also assessed conidiation of another isolate, i.e., Coton.ben. We found that conidia production of this isolate was significantly higher in an air stream with volatiles from cassava leaves infested by *M. tanajoa* than under

clean air (251.0 conidia per mummy vs. 183.8 conidia per mummy; hence 37% more conidia). This can be inferred from the replicated *G*-test for total *G*-statistic (total $G = 693.11$, $df = 10$, $P < 0.001$). In total, there were two replicates with significantly more conidia under clean air stream, whereas there were eight replicates with significantly more conidia under an air stream with volatiles from infested leaves (Figure 6). Significant heterogeneity was observed among replicates (heterogeneity $G = 588.93$, $df = 9$, $P < 0.001$). Unplanned comparisons showed that apart from one group of two replicates there were four groups of four replicates that were homogeneous (Figure 6, right-hand panel). Replicated *G*-tests within the latter four homogeneous groups were all significant and all in favor of a higher production of conidia when exposed to HIPV. Note that the homogeneous group size for Coton.ben is somewhat larger than for the other isolate (Colal.brz), indicating a relatively lower level of heterogeneity.

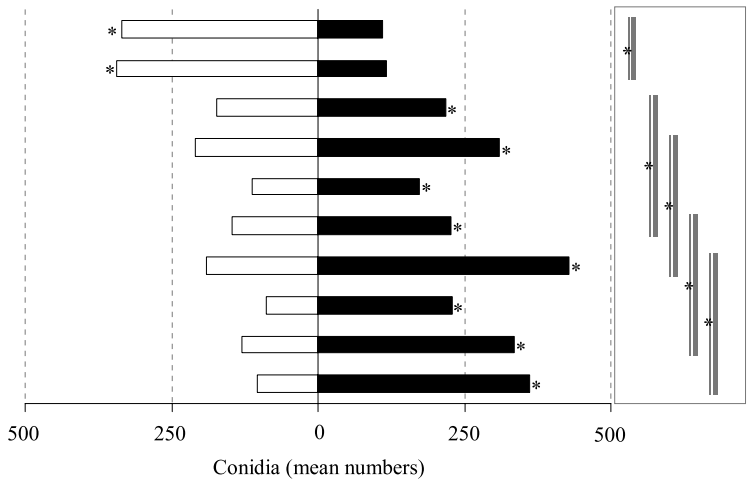

FIG. 6. Conidia production of a Benin isolate of *N. tanajoae* (Coton.ben) in the presence (black bars) and absence (open bars) of volatiles from intact cassava leaves infested by the herbivorous mite *M. tanajoa* in the airflow system experiment. Replicates of each treatment were carried out simultaneously with a replicate of the other treatment and the results are, therefore, shown pairwise for 10 replicates. Significant differences among simultaneous replicates of the two treatments are indicated by an asterisk. Since replicated *G*-test indicated significant heterogeneity among replicates, unplanned comparisons were carried out to test for homogeneity among replicates. The right-hand panel shows the groups of homogeneous replicates; significant differences between the two treatments using the pooled *G*-statistic within homogenous replicates are indicated by an asterisk.

DISCUSSION

Based on the results of our closed dish experiments, we conclude that volatiles from clean leaves (green leaf volatiles; GLV) suppressed conidiation in the two Brazilian isolates (Colal.brz and Altal.brz), whereas volatiles from leaves infested by *M. tanajoa* stimulated conidiation in the Brazilian isolate Colal.brz (Figure 4). Evidence for inhibition by GLV comes from the significantly lower number of conidia produced in the presence of volatiles emanating from excised clean leaf discs than produced with clean air. Evidence for stimulation by HIPV was only found for the Brazilian isolate Colal.brz. It is based on the higher number of conidia produced in the presence of volatiles from *M. tanajoa*-infested leaf discs than produced under exposure to volatiles from clean leaf discs.

It is puzzling that conidia production did not differ between the treatment with clean air and that with volatiles from leaf discs fed upon by *M. tanajoa*. To further analyze this, we carried out a similar experiment, but now following another setup (i.e., the airflow system) in which the amount of volatiles from infested leaves was increased (six intact leaves instead of three excised leaf discs; 30 times more *M. tanajoa* feeding for 3 d instead of 2 hr). This experiment showed that for the Benin isolate (Coton.ben), conidia production is significantly higher when exposed to volatiles from infested leaves than when exposed to clean air (Figure 6). For the Brazilian isolate (Colal.brz), we found much heterogeneity among replicates and apart from a bias in favor of higher conidia production under exposure to HIPV we did not obtain statistical evidence for an effect of HIPV (Figure 5). Possibly, each isolate has a unique optimum in its response to different concentrations of HIPV. The significant effects for the Brazilian isolate (Colal.brz) using the closed dish experiment and that for the Benin isolate (Coton.ben) are consistent with the hypothesis that HIPV stimulates conidiation.

Inhibition of conidiation in response to volatiles from herbivore-free plants has been reported earlier for the aphid pathogen *P. neoaphidis* when exposed to volatiles from macerated tobacco leaves (Brown et al., 1995). Inhibition of growth was also reported in response to isothiocyanate in other insect fungi (e.g., Klingen et al., 2002) and in response to phytoalexins in plant pathogens (Baily, 1970; Fraser, 1970). Some fungi were also found to produce volatiles with a self-inhibiting effect (Chitarra et al., 2004). Whereas evidence for inhibition by volatile chemicals exists, stimulation of conidiation by herbivore-induced plant volatiles is a novel finding. It should be noted that our experiments were done under climatic conditions that are optimal for sporulation, even in the absence of chemical signals (Oduor et al., 1996a). Thus the effects of HIPV and GLV were inevitably of a relative nature. Therefore, it would be worthwhile to repeat them under conditions that are marginal for sporulation.

From a functional point of view, it is in the interest of the entomopathogenic fungus to delay conidiation until HIPV signals that herbivores are nearby. However, this leaves unexplained why conidiation readily takes place in clean air, even more so because inside a mummy the fungus would be able to survive under dry conditions for more than 8 mo (Oduor et al., 1995). We hypothesize that the fungus does not gain by delaying sporulation in an environment without cues from plants, and it may only successfully infect in the event that there is an unlucky herbivore passing by.

Whereas predators and parasitoids are well known to exploit HIPV as a source of information on the location of their victims, entomopathogens have received little attention in this respect. Infochemicals in general have been shown to play a role in this class of natural enemies (baculovirus: Felton and Duffey, 1990; Hoover et al., 1998; entomopathogenic fungi: Brown et al., 1995; entomopathogenic nematodes: Choo et al., 1989; Lei et al., 1992; Lewis et al., 1992, 1993, 1996; Grewal et al., 1993a,b, 1994; Kanagy and Kaya, 1996; Wang and Gaugler, 1998; Boff et al., 2001; Boff and Smits, 2001; van Tol et al., 2001; Cutler and Webster, 2003), but most of these studies concern entomopathogenic nematodes. Among them, recent work by van Tol et al. (2001) and Boff et al. (2001) showed that HIPV from roots of weevil-infested *Thuja* plants attracts the entomopathogenic nematode *Heterorhabditis megidis*. However, fungi are essentially motionless, and attraction cannot, therefore, play a role. Here we showed that HIPV may help to tune conidia production to the presence of herbivores on plants. This paves the way for further tests on the benefits to the plant of releasing volatiles upon herbivore attack.

Another major question for future research is "which components of the volatile blends emanating from herbivore-free and herbivore-infested cassava plants are responsible for inhibition or stimulation?''. It is interesting to note that (*trans*)-(*E*)-2-hexanal, a major component of the blend of volatiles from herbivore-free cassava plants (Figure 3), has been shown to have an inhibiting effect on conidiation of the aphid pathogen *P. neoaphidis* (Brown et al., 1995). As shown by our GC-MS analysis (Figure 3), there are other components of GLV [e.g., (*Z*)-3-hexen-1-ol; (*E*)-2-hexen-1-ol; 3-pentanon; 2-butanon], and HIPV [e.g., (3*E*)-4,8,-dimethyl-1,3,7,-nonatriene; (*E*)-(*trans*)-beta ocimene; 4,8,12-trimethyl-1,3(*E*),7(*E*),11-tridecatetraene; methyl salicylate; linalool], and these are yet to be tested for inhibition or stimulation of conidiation in *N. tanajoae*.

The conidiation responses of the entomopathogenic fungus to HIPV showed much more heterogeneity than the behavioral responses of predatory mites to HIPV (Janssen et al., 1990; Margolies et al., 1997; Gnanvossou et al., 2001; Aratchige et al., 2004). For example, our experiments resulted in mummies yielding 1000 conidia and mummies yielding none, whereas the average number of conidia was about 250. If our hypothesis on the function of HIPV for the

plant and the entomopathogen holds, then why is there so much variability? One explanation may be that we did not sufficiently standardize the conditions of the host and/or the entomopathogen. Whereas we chose adult females as the only hosts under test, we did not select a uniform female size because this is a hard task to carry out with arthropods as small as mites. Variation in host size may cause variation in conidia yield and thereby create noise in our experimental data. Pathogen quality may also have varied in our experiments, for example, because within-host density of hyphal bodies (preceding spore formation) may differ between hosts due to variation in the number of capilliconidia infecting the host or due to variation in host quality. Our current method to propagate the entomopathogen *in vivo* is such that these variations inevitably arise. Development of *in vitro* culturing methods would be a breakthrough in helping to standardize pathogen quality. If further standardization would not substantially reduce variability in the response to HIPV, then there may be a functional reason for its existence. Entomopathogens have a risky life because they are immobile and the infective stages are especially short-lived and sensitive to abiotic conditions. This is why entomopathogens may not use HIPV as the only signal for sporulation, but possibly several other cues related to the presence of potential hosts (e.g., feces, silk). If there are many cues affecting sporulation, and the presence of those cues varies in time and space, then one may either expect entomopathogens to show the full repertoire of responses or a subset if the ability to perceive the cues entails significant costs. In the latter case, variability in responsiveness to the different cues is expected, even among the descendants of a single spore if there is a selective advantage to spreading the risk.

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ANTENNAL ELECTROPHYSIOLOGICAL RESPONSES OF THREE PARASITIC WASPS TO CATERPILLAR-INDUCED VOLATILES FROM MAIZE (*Zea mays mays*), COTTON (*Gossypium herbaceum*), AND COWPEA (*Vigna unguiculata*)

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Abstract—Many parasitic wasps are attracted to volatiles that are released by plants when attacked by potential hosts. The attractiveness of these semiochemicals from damaged plants has been demonstrated in many tritrophic systems, but the physiological mechanisms underlying the insect responses are poorly understood. We recorded the antennal perception by three parasitoids (*Cotesia marginiventris*, *Microplitis rufiventris*, and *Campoletis sonorensis*) to volatiles emitted by maize, cowpea, and cotton plants after attack by the common caterpillar pest *Spodoptera littoralis*. Gas chromatography-electroantennography (GC-EAG) recordings showed that wasps responded to many, but not all, of the compounds present at the physiologically relevant levels tested. Interestingly, some minor compounds, still unidentified, elicited strong responses from the wasps. These results indicate that wasps are able to detect many odorant compounds released by the plants. It remains to be determined how this information is processed and leads to the specific behavior of the parasitoids.

Key Words—GC-EAG, electrophysiology, *Cotesia marginiventris*, *Microplitis rufiventris*, *Campoletis sonorensis*, induced plant odor.

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INTRODUCTION

Many plants attacked by herbivorous insects indirectly defend themselves by emitting semiochemicals that act as attractants for natural enemies of the attackers (Dicke and Sabelis, 1988; Turlings et al., 1990; Steinberg et al., 1993; Agelopoulos and Keller, 1994; Geervliet et al., 1994; Röse et al., 1997; Du et al., 1998; Shiojiri et al., 2000; Neveu et al., 2002). At least 12 families of plants produce volatiles in response to herbivory (Dicke, 1999). The compositions of the volatiles produced are variable, but isoprenoids, lipoxygenase-derived volatiles, and aromatics (derived from either the phenylalanine–ammonia–lyase pathway or from other amino acids) commonly dominate the blends released by insect-damaged plants (Whitman and Eller, 1990; Tumlinson et al., 1992; Rutledge, 1996). The emission of volatiles after attack by Lepidoptera is induced by elicitors in the oral secretions of herbivores (Dicke et al., 1993; Turlings et al., 1993; Mattiacci et al., 1995). Two different types of elicitors have been identified, a β-glucosidase from *Pieris rapae* (Lepidoptera: Pieridae) (Mattiacci et al., 1995), and fatty acid derivatives, such as volicitin isolated from *Spodoptera exigua* (Lepidoptera: Noctuidae) (Alborn et al., 1997; Turlings et al., 2000).

Spodoptera spp. (Lepidoptera: Noctuidae) larvae are important pests of various cultivated plants, including maize (*Zea mays mays*), cotton (*Gossypium herbaceum*), and cowpea (*Vigna unguiculata*). In response to larval feeding, these plants emit a particular odor (Turlings, 1990; McCall et al., 1993; Röse et al., 1996) that is perceptible even by humans (Gouinguené, personal observation). These pests can be parasitized by various wasps, including *Cotesia marginiventris* (Hymenoptera: Braconidae), *Microplitis rufiventris* (Hymenoptera: Braconidae), and *Campoletis sonorensis* (Hymenoptera: Ichneumonidae). The induced odors emitted by maize, cotton, and cowpea plants during feeding by *Spodoptera* spp. are attractive to each of these parasitoids (Turlings et al., 1995; Röse et al., 1998; C. Tamó and T. Degen, personal communication). According to the species of plant, the induced odor can vary, although some common substances are present in the different blends (Turlings et al., 1991; Röse et al., 1998; Hoballah et al., 2002). *C. marginiventris* and *C. sonorensis* originate from the new world, while *M. rufiventris* occurs in the old world, in particular in north Africa. These are generalists, although *M. rufiventris* is found more often parasitizing *S. littoralis* feeding on cotton plants (C. Tamó, personal communication). The different hosts of these parasitoids are often generalists, and feed on various plant families, including maize, cotton, and cowpea.

Although the responses of parasitoids to induced plant odors have been intensively studied in terms of behavior, the physiological mechanisms underlying the behavior are largely unknown. Coupled high-resolution gas chromatog-

raphy (GC) and electrophysiology techniques can be used to identify physiologically active compounds from complex natural product extracts (Wadhams, 1990). This approach has been used to determine active components in the odor blend of maize for *C. flavipes*, a parasitoid of the stemborer spp (Ngi-Song and Overholt, 1997). Female *C. flavipes* respond to six compounds (an unidentified C_5 compound, heptanal, (*Z*)-3-hexenyl acetate, (*E*)-ocimene, linalool, and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT)) in the odor of maize infested with *Chilo partellus* larvae (Lepidoptera: Pyralidae). Using simple EAG recordings, *C. sonorensis* has been shown to respond to green leaf volatiles, monoterpenes (α-pinene, myrcene, limonene), and with lower sensitivity to sesquiterpenoids (gossonorol, β-caryophyllene oxide, β-caryophyllene, α-humulene) (Baehrecke et al., 1989). So far, no data are available on the perception of induced odor by *C. marginiventris* and *M. rufiventris*. EAG recordings have also been used to test the sensitivity of antennal perception in some other parasitoids (Lecomte and Pouzat, 1985; Salom et al., 1991; Jyothi et al., 2002). Only a few studies on parasitoids have been done using coupled GC and EAG systems. Pettersson et al. (2000) found that the bark beetle parasitoid, *Rotrocerus xylophagum* (Hymenoptera: Pteromilidae), did not respond to the major compounds present in the bark oil. More recently, Smid et al. (2002) compared the responses of a specialist and generalist parasitoid of *Pieris* caterpillar to attacked Brussels sprouts, and showed that the two parasitoid species responded similarly to a large number of compounds present in the odor of damaged plants. Interestingly, two compounds were only perceived by the specialist parasitoid (*Cotesia rubecula*) (Smid et al., 2002).

The aim of this study was to use the GC-EAG approach to determine and to compare the antennal perception of compounds in the induced odor blends of maize, cotton, and cowpea for the three generalist parasitoids *C. marginiventris*, *M. rufiventris*, and *C. sonorensis*, which show strong similarities in host range and life cycle.

METHODS AND MATERIALS

Insects. *C. marginiventris*, *M. rufiventris*, and *C. sonorensis* were provided by the Laboratory of Animal Ecology and Entomology, University of Neuchâtel, Switzerland. The rearing of the three parasitoids follows the same procedure. Second instar larvae of *S. littoralis* were exposed to female parasitic wasps for at least 2 hr, then wasps were removed and the larvae reared on artificial diet (Syngenta, Basel, Switzerland). When the parasitic larvae are ready to pupate, they exit the host and form cocoons that can be easily collected. Cocoons were placed in plastic boxes (13.5 × 7.5 × 6 cm) with wet cotton to

maintain humidity and drops of honey for food. Newly-emerged adult parasitoids were sexed, and placed in plastic cups (6.5 × 5.5 cm diam.), and were provided with honey and wet cotton. They were kept in a climate chamber (20°C, 40% RH) and 2- to 3-d-old females were used in experiments.

Collection of Induced Odors. Air entrainment samples of induced odor of cotton, maize (variety Delprim), and cowpea were used, and were prepared at the University of Neuchâtel. Three seeds of cotton, maize, and cowpea were sown in plastic pots, filled with fertilized potting soil (Coop, Switzerland). Plants were grown in a climate chamber at 23°C, 60% RH, and under 50,000 lm/m^2 (16L:8D). Fifteen-d-old plants were used. To induce the plants to emit volatiles, 30 *S. littoralis* larvae (second instar) were allowed to feed on the three plant species for 2 d prior to the first odor collection. The volatile collection system has been described in detail (Turlings et al., 1998) and was modified as follows. It consisted of five vertically placed glass cylinders (7 cm i.d., 43 cm high). The pot with the three plants was placed in a glass pot (7 cm diam., 8.5 cm high), which fits in the vertical cylindrical glass. Purified and humidified air was pushed into each cylinder at a rate of 1 l/min and flowed around the plant. Around the base of each cylinder, an opening served as a port that could hold the collection traps. For collections, air was drawn (0.8 l/min) through a Super-Q adsorbent trap (Heath and Manukian, 1994), while the rest of the air vented through the hole in the top, thus preventing external, impure air from entering. The automated part of the collection system (Analytical Research System, Gainesville, FL, USA) controlled flow through the trap. The climate chamber (CMP4030, CONVIRON, Winnipeg, Canada) in which the collection cylinders were housed was kept at 17.5°C. Due to the heat irradiation, the temperature inside the cylinders was 23 ± 3°C. During the photophase (16L:8D) light intensity was approx. 20,000 lm/m^2.

Two collections were done during the day, starting 2 and 9 hr after the beginning of the photophase. Each collection lasted 6 hr. After each collection, traps were extracted with 250 μl of methylene chloride (Lichrosolv, Merck, Switzerland). The same plants were used for volatile collection the next day. At the end of the 2 d, the samples from the four collections of the same plant species were pooled. To obtain control samples, the same conditions were used to collect volatiles from undamaged plants, pot and soil only, and empty glasses.

Electrophysiology. Electroantennogram (EAG) recordings from 2- to 3-d-old female wasps were made using Ag–AgCl glass electrodes filled with saline solution (composition as Maddrell (1969), without glucose). The insect was anaesthetized by chilling, and the head was excised and inserted in the tip of the indifferent electrode. The tips of the two antennae were inserted into the recording electrode. Signals were passed through a high impedance amplifier

(UN-06, Syntech, The Netherlands) and analyzed by using a customized software package (Syntech, The Netherlands).

Coupled Gas Chromatography-Electroantennography (GC-EAG). The coupled GC-EAG system, in which the effluent of the GC column is simultaneously directed to the antennal preparation and the GC detector, has been described (Wadhams, 1990). Separation of the volatiles was achieved on an AI 93 GC equipped with a cold on-column injector and an FID. The column used was a 50 m × 0.32 mm i.d. HP-1 column (non polar). Oven temperature was maintained at 40°C for 2 min and then programmed at 5°C/min to 100°C and then at 10°C/min to 250°C. The carrier gas was hydrogen (15 psi). The outputs from the EAG amplifier and the FID were monitored simultaneously and analyzed with the software package (Syntech, the Netherlands). A standard stimulation was done at the beginning and at the end of each recording to correct for the loss of sensitivity of the preparation. The stimulus (100 μg of (*Z*)-3-hexenol) was applied on a filter paper strip, inserted in a Pasteur pipette, and a puff of odor was flushed on the EAG preparation. For the correction, we assumed that the decrease in sensitivity is linear with the time. Data were then normalized to the standard as follows:

$$\frac{A}{\mathrm{EAG(std1)} + (\mathrm{EAG(std2)} - \mathrm{EAG(std1)})\dfrac{\mathrm{RT(A)} - \mathrm{RT(std1)}}{\mathrm{RT(std2)} - \mathrm{RT(std1)}}}$$

where A is the amplitude (mV) of the EAG response to compound A; EAG(std1) is the EAG response to standard at the beginning of the recording; EAG(std2) is the EAG response to standard at the end of the recording; RT(A) is the retention time of compound A; RT(std1) is the time when the stimulation at the beginning of the recording was done; RT(std2) denotes the time when the stimulation at the end of the recording was done. For each induced odor tested, responses from 10 females were recorded.

Coupled Gas Chromatography-Mass Spectrometry (GC-MS). GC-MS was used to tentatively identify compounds giving rise to the peaks associated with EAG activity from the induced odor blend of maize, cowpea, and cotton. A capillary GC column (50 m × 0.32 mm i.d. HP-1) fitted with a cold on-column injector was directly coupled to a mass spectrometer (VG Autospec, Fisons Instruments). Ionization was by electron impact at 70 eV, 250°C. The oven temperature was maintained at 30°C for 5 min and then programmed at 5°C/min to 250°C. Peak identities were determined by comparison of the spectra with those of authentic compounds from synthesis or other botanical sources and confirmed by comparison of Kovat's indices.

Statistical Analysis. Normalized EAG responses of the three parasitoids to the different plants odor were compared using nonparametric tests. On each compound that activated a response from the parasitic wasps, a Kruskall–Wallis

comparison was done, when it was shown that the mean responses were different, Mann–Whitney pairs comparisons were realized to determine which parasitoid was different from which. For such comparison, the level of significativity was corrected using the Bonferroni correction, thus $\alpha = 0.017$.

RESULTS

Figures 1, 2, and 3 present representative GC-EAG recordings from the 10 replicates from each wasp species and each induced odor (60 recordings in total). Most of the major compounds elicited EAG responses from the three parasitoids. Some minor compounds also evoked strong responses (Figures 1, 2, and 3). The greatest responses were obtained from the green leaf volatiles (peaks a, b, n), whereas lower responses were recorded later in the GC-EAG analytical traces. This difference may reflect a differences in sharpness of the GC peaks and the number of molecules that hit the antennae at the same time and should not be interpreted as difference in strength of perception. Table 1

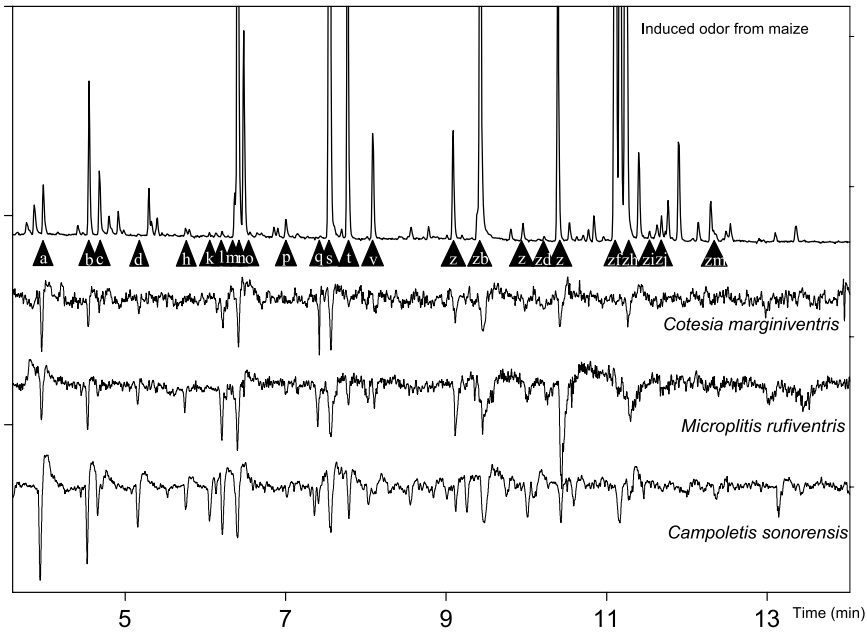

FIG. 1. GC-EAG traces of *Cotesia marginiventris*, *Microplitis rufiventris* and *Campoletis sonorensis* in response to the caterpillar-induced odor from maize.

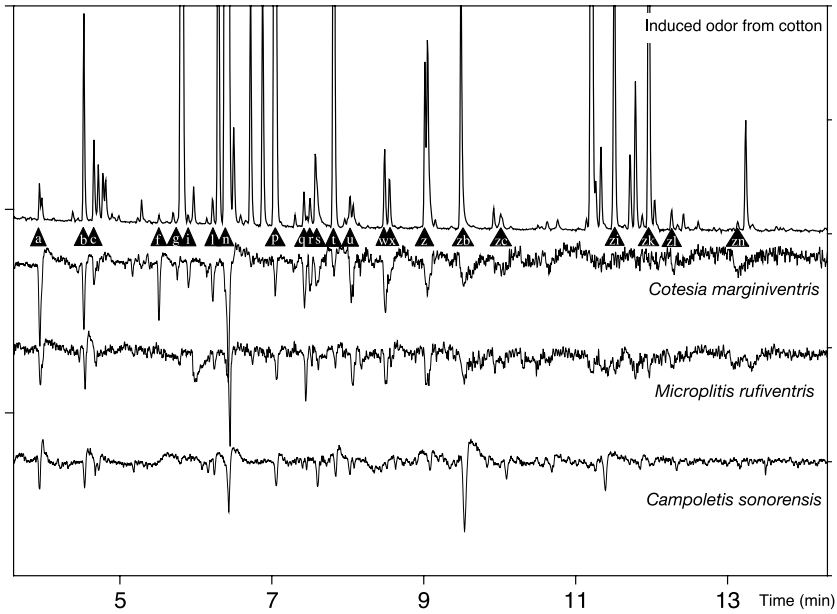

FIG. 2. GC-EAG traces of *Cotesia marginiventris*, *Microplitis rufiventris* and *Campoletis sonorensis* in response to the caterpillar-induced odor from cotton.

gives the identity and normalized EAG value of all the electrophysiologically active compounds in the three induced odor samples tested. The letter in the first column refers to the letters in Figures 1, 2, and 3. Only a few small responses were recorded from the volatile samples from undamaged control plants (Figure 4). For example, in odor from undamaged maize, these responses were associated with the constitutive compounds, like linalool (s).

For maize, a total of 25 compounds in the induced blend gave detectable responses from the three parasitoids combined (Figure 1 and Table 1). EAG responses from *C. marginiventris* were elicited by 24 compounds. *C. sonorensis* females responded to 20 compounds (Figure 1). *C. marginiventris* showed high responses to (*E*)-2-hexenal (b), (*Z*)-3-hexenyl acetate (n), linalool (s), neryl or geranyl acetate (ze), and unknown 5 (l) (Table 1). Normalized responses to (*E*)-2-hexenal (b) were higher than the response of the two other parasitic wasps (comparison with *M. rufiventris*, $P = 0.004$; with *C. sonorensis*, $P = 0.005$), while responses to DMNT were lower ($P = 0.005$ in both comparisons). Neryl or geranyl acetate (ze) gave the highest response (20.6%) from *M. rufiventris* (Table 1), and that response was significantly higher than the one from *C. sonorensis* ($P = 0.007$). *M. rufiventris* responded weakly to unknown 5 (l) as

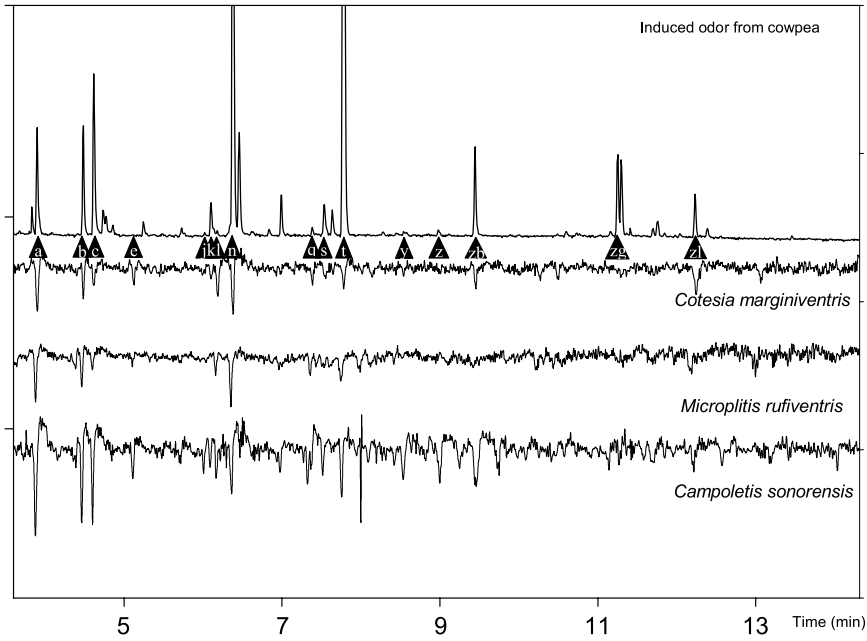

FIG. 3. GC-EAG traces of *Cotesia marginiventris*, *Microplitis rufiventris* and *Campoletis sonorensis* in response to caterpillar-induced odor from cowpea.

compared to *C. marginiventris* ($P < 0.001$) and *C. sonorensis* ($P < 0.001$). *C. sonorensis* showed higher responses to linalool (s), (*Z*)-3-hexenyl acetate (n), (*Z*)-3-hexenol (f), (*Z*)-3-hexenal (a), and DMNT (t). *C. sonorensis* had lower responses than *C. marginiventris* to (*E*)-2-hexenal (b) ($P = 0.005$). Although not statistically different, responses to methyl anthranilate (zc) by *C. sonorensis* tended to be low compared to the ones of the two other parasitoids. Benzyl acetate (v) evoked no responses in *C. sonorensis*. Interestingly, all three parasitoids responded strongly to geranyl acetate (ze).

A total of 23 compounds were perceived in the induced odor of cotton (Figure 2). As in maize plants, some of the minor compounds elicited relatively strong EAG responses in *C. marginiventris* (i and l, Figure 2). *C. sonorensis* and *C. marginiventris* appeared to have similar sensitivity to most of the compounds from induced odor of cotton. These wasps showed a greater sensitivity to green leaf volatiles (compounds a, b, and c) than *M. rufiventris* (Figure 2 and Table 1), which had significantly lower response to (*Z*)-3-hexen-1-ol (c) ($P = 0.015$ and $P = 0.01$, respectively). Compared to the other two wasps, *C. marginiventris* showed particularly strong responses to methyl-(*Z*)-3-hexa-

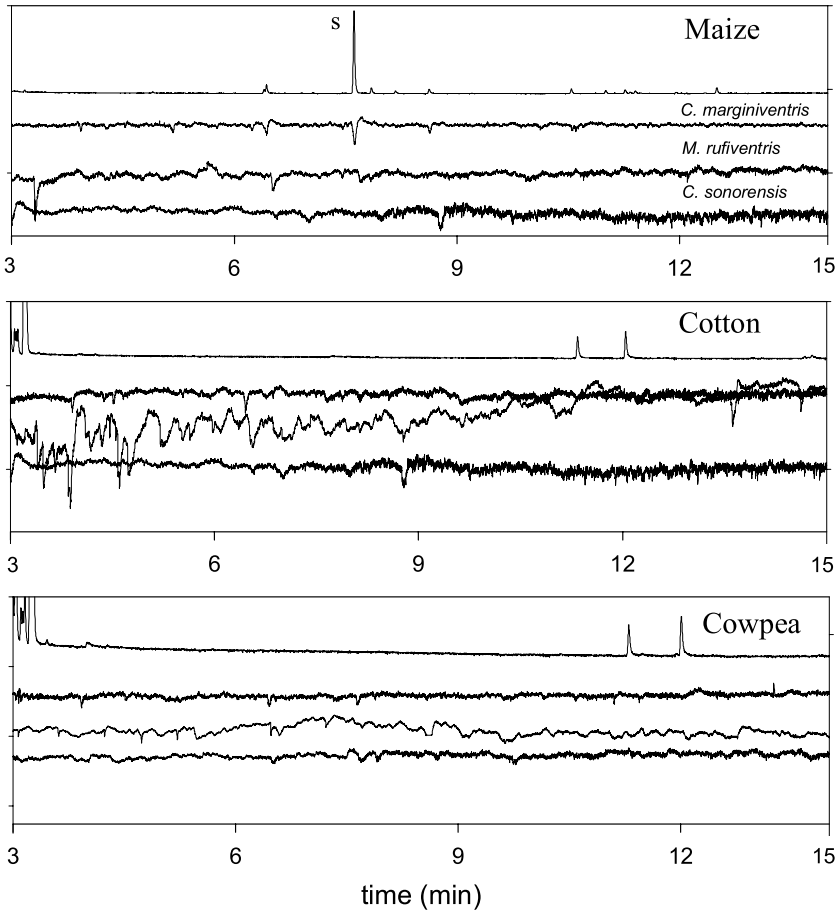

FIG. 4. GC-EAD traces of *Cotesia marginiventris*, *Microplitis rufiventris* and *Campoletis sonorensis* in response to odor from undamaged cotton, maize and cowpea.

noate (f) ($P < 0.001$ both with *M. rufiventris* and *C. sonorensis*), nerolidol (zl), and unknown 8 (zn) (although not significantly different). *M. rufiventris* was less responsive to (*Z*)-3-hexen-1-ol (c), methyl benzoate (q), and DMNT (t) compared to *C. marginiventris* ($P = 0.015$, $P = 0.001$, $P = 0.011$, respectively) and *C. sonorensis* except to DMNT ($P = 0.010$, $P < 0.001$, $P = 0.043$). *M. rufiventris* did not respond to unknown 3 (i), unknown 5 (l), or humulene (zi).

The induced blend of cowpea was less complex than those from maize and cotton (Figure 3). Only 16 compounds elicited EAG responses from the parasitoids (Figure 3). For all parasitoids, responses to green leaf volatiles were

TABLE 1. MEAN NORMALIZED EAG VALUES (STANDARD ERROR) OF THE COMPOUNDS IN THE INDUCED MAIZE, COTTON AND COWPEA ODOR, WHICH ARE PERCEIVED BY AT LEAST ONE SPECIES OF PARASITOID TESTED

Type	Peak No. (see Figures 1–3)	Compound	*C. marginiventris*			*M. rufiventris*			*C. sonorensis*		
			Maize	Cotton	Cowpea	Maize	Cotton	Cowpea	Maize	Cotton	Cowpea
Fatty acid derivative	a	(*Z*)-3-Hexenal	11.9 (2.5)	11.2 (2.5)	13.8 (1.1)	9.2 (1.1)	9.3 (1.3)	9.0 (1.3)	11.4 (1.3)	9.5 (1.8)	5.5 (1.2)
	b	(*E*)-2-Hexenal	6.5 (1)	11.9 (2.2)	9.9 (1.3)	2.3 (0.8)	7.8 (2)	6.8 (1.2)	1.3 (0.4)	15 (2.2)	4.9 (1.1)
	c	(*Z*)-3-Hexen-1-ol	7.1 (1.7)	5.7 (1.8)	6.4 (1.2)	10.9 (1.3)	1.9 (1)	4.6 (1.7)	12.4 (1.1)	6.3 (1.2)	4.4 (1.2)
	f	Methyl (*Z*)-3-hexenoate		10.4 (2)			0.8 (0.3)			1.2 (0.5)	
	h	(*Z*)-2-Heptenal									
	k	1-Octen-3-ol[a]	4.7 (3.3)		4.6 (0.9)	7.6 (1.8)		0.8 (0.6)	2.8 (0.5)		1.5 (0.7)
	n	(*Z*)-3-Hexenyl acetate	17.4 (4)	19.5 (4.1)	16.8 (2)	12.8 (2.4)	13.8 (1.9)	12.2 (1.7)	14.2 (1.6)	12.7 (2)	5 (1)
	o	(*E*)-3-Hexenyl acetate	3.3 (1)			3.8 (1.1)			2.9 (0.7)		
	r	(*Z*)-2-Hexenyl propionate		7.7 (1.8)			2.9 (0.9)			4.4 (1.5)	
	u	(*Z*)-3-Hexenyl butanoate		6.2 (2.1)			5.2 (1.8)			3.9 (0.9)	
	w	(*E*)-3-Hexenyl butanoate		11.3 (2.9)			5.7 (1.5)			3.6 (0.5)	
	x	(*E*)-2-Hexenyl butanoate		0.5 (0.5)							
	z	(*Z*)-3-Hexenyl 3-methyl butanoate		8.1 (1.5)	1.9 (0.8)		6.7 (1.7)			2.6 (0.6)	1.5 (0.9)
	zj	Pentadecane	1.9 (1.1)			3.5 (2.1)			2 (0.7)		
Amino acid derivative	q	Methyl benzoate	3.7 (1.5)	7.6 (1.8)	8.1 (1.2)	4.4 (1.3)	1.6 (0.7)	1.3 (0.6)	3.3 (0.9)	8.5 (0.9)	1.4 (0.6)
	v	Benzyl acetate	2.3 (0.8)			1.9 (0.7)					
	y	Methyl salicylate			4.7 (1.1)			0.4 (0.3)			0.8 (0.5)
	za	Phenylethyl acetate	5.5 (1.4)			12.3 (1.9)			8 (2.5)		
	zb	Indole	9.7 (1.7)	7.7 (2.6)	7.3 (1.4)	7.5 (2.1)	4.9 (1.2)	2.3 (1)	7.8 (1.2)	5.9 (1.6)	2.4 (0.5)
	zc	Methyl anthranilate	6.5 (2.5)	5.2 (2.2)		6.1 (2.3)	2.3 (1)		0.9 (0.4)		
Isoprenoid or	g	α-Phellandrene		1.3 (0.7)			0.9 (0.4)			3.5 (0.6)	

derivative	m	β-Myrcene	1.8 (0.5)			1.3 (0.5)			1.6 (0.8)		
	p	(*E*)-Ocimene	1.1 (0.4)	7.7 (1.4)		2.8 (0.6)	5.6 (1.8)		1.6 (0.7)	5.6 (1)	
	s	Linalool[a]	10.7 (1.8)	2.1 (1.9)	2.9 (0.9)	11.9 (2.1)	4.6 (0.8)	1.3 (0.5)	14.6 (2.5)	4.1 (0.4)	1.2 (0.6)
	t	4,8-Dimethyl-1, 3,7-nonatriene (DMNT)	2.2 (0.8)	6.1 (1)	8.6 (0.9)	7.3 (1.43)	2 (0.9)	7.9 (1)	7.5 (0.8)	5.3 (1)	0.8 (0.5)
	ze	Neryl or Geranyl acetate[a]	13.8 (3.2)			20.6 (1.8)			10.7 (2)		
	zf	β-Caryophyllene	4 (1.6)			3.2 (1.5)			1.9 (0.5)		
	zg	Bergamotene			3.9 (1.6)			5.3 (1.7)			2.3 (0.6)
	zh	(*E*)-β-farnesene	8.5 (1.8)			9.6 (2.3)			6 (1)		
	zi	Humulene	5.6 (2.6)			0.4 (0.4)			0.8 (0.4)	1 (0.7)	
	zl	Nerolidol[a]		9.8 (3.2)	10.4 (1.5)		2.4 (1.4)	5 (1.1)		1.2 (0.6)	8.2 (4.4)
	zm	4,8,12-Tetramethyl-1,3, 7,11-tridecatetraene (TMTT)	8.4 (2.4)			3.7 (2.2)			2.1 (0.9)		
	d	Unknown 1	5.6 (1.4)			3.10 (1.13)			2.8 (0.9)		
	e	Unknown 2			3.7 (1.1)			0.7 (0.4)			1.4 (0.4)
	i	Unknown 3		6.8 (1.6)						3 (0.6)	
	j	Unknown 4			3.3 (0.8)			1.7 (1.1)			1.7 (0.9)
	l	Unknown 5	9.24 (2.30)	6.3 (1.3)	6 (1.4)	0.46 (0.33)		3.6 (0.9)	5.1 (0.7)	3.5 (0.5)	2 (0.5)
	zd	Unknown 6	2.34 (1.49)			2.7 (1.53)			3.5 (1.7)		
	zk	Unknown 7					2 (1.1)			2.4 (0.8)	
	zn	Unknown 8		7.8 (3.1)			0.9 (0.9)			3.1 (0.9)	

[a] Stereochemistry undefined.

dominant, but *C. marginiventris* also responded relatively well to nerolidol (zl) and methyl benzoate (q) ($P < 0.001$ for both *M. rufiventris* and *C. sonorensis*) (Figure 3). *C. marginiventris* showed a tendency to higher responses compared to the two other parasitoids (Table 1), while *M. rufiventris* and *C. sonorensis* responded similarly to the compounds in cowpea induced odor (Table 1). *C. sonorensis* responded significantly with lower intensity to DMNT (t) ($P < 0.001$), while responses from *C. marginiventris* and *M. rufiventris* were not different ($P = 0.72$). Some compounds did not activate the olfactory receptors, even when released in large amounts. The most striking example was α-pinene (peak between g and i) in the induced cotton odor (Figure 2). Again, all three species were sensitive to some minor compounds (f, g, i, and l), which may be of importance in the recognition of specific host plants. In intact plants, the emission of volatiles was extremely low, which led to no activation of the olfactory receptors on wasps' antennae (Figure 4).

DISCUSSION

The release of volatile compounds when plants are attacked by insect herbivores, and the attractiveness of such induced compounds to natural enemies, have been shown in various studies (Turlings et al., 1990; Steinberg et al., 1993; Geervliet et al., 1994; Dicke and Vet, 1998). In some systems, there are key compounds for the attraction of parasitoids, such as 6-methyl-5-hepten-2-one, which is an indicator of the presence of specific host aphids for the parasitoid, *Aphidius ervi* (Du et al., 1998). In other systems, the role of the various compounds in the induced odor blends appears to be more complex (Dicke et al., 1990; Vet et al., 1990; Turlings et al., 1995; Röse et al., 1998). In this study, we compared the olfactory perception of three female parasitoids to the induced odor of maize, cotton, and cowpea to determine if there are key compounds mediating host location. The results showed that many, but not all, of the compounds in the induced odors were perceived by the parasitoids. There were some differences in the intensity of response to particular compounds, indicating potential differential sensitivity of the parasitoids to the various volatiles. For example, *C. sonorensis* and *M. rufiventris* both responded to linalool, but when the amount of this compound was reduced, as it is in the induced odor of cowpea, no EAG activity was detected, whereas *C. marginiventris* still responded at these concentrations. For an accurate determination of the response threshold of the different parasitoids, dose-response measurements should be made for each compound perceived and for each wasp. The absence of responses from odor blends of control plants indicates that the undetectable amount of odor released in the absence of attack by herbivorous insects may not

be useful at long distance. It cannot be excluded, however, that undetectable amounts of specific plant odors may be detected at short distance. Although it is difficult to distinguish any specific pattern of response from the parasitoids due to the high number of EAG active compounds in the different blends tested, minor compounds may play a more important role than previously thought. The strong EAG responses elicited by these semiochemicals suggests this. While the major compounds are ubiquitous plant secondary metabolites, and may provide general information indicating herbivore-damaged plants, information on the identity of the herbivore doing the damage may be indicated by differences in ratios between the different compounds (De Moraes et al., 1998), and minor compounds may also be key in specific host recognition. Some of these minor compounds were not present in the odor of all three plants, being more plant- than host-specific.

No specific relation between the origin of female parasitoids and their preferred plant/host complex could be detected. For example, *M. rufiventris*, which occurs in Egypt, is more prevalent on cotton plants than the two other parasitoids (C. Tamò, personal communication), but this was not reflected in the antennal responses. It is also curious that, in general, the wasps showed relatively poor responses to DMNT, TMNT, and several sesquiterpenes, which are typical for caterpillar-induced odor blends and have been assumed to be key attractants. This poor perception of these compounds, however, corresponds well with results from behavioral assays designed to pinpoint key parasitoid attractants (Hoballah et al., 2002: M D'Alessandro, M. Held, T. Turlings, personal communication).

A recent study measured the olfactory responses of *C. glomerata*, a generalist parasitoid, and *C. rubecula*, a specialist parasitoid of *Pieris rapae*. No obvious differences were found in the detected range of compounds released by Brussels sprouts when attacked by caterpillars (Smid et al., 2002). This confirms the difficulty of detecting any differences in peripheral perception among parasitoids. As observed for the parasitoids in the present study, *C. glomerata* and *C. rubecula* show a broad olfactory capability responding to 20 compounds in the cabbage volatiles (Smid et al., 2002). The wasps' ability to detect a wide range of compounds in the induced odors of maize, cotton, and cowpea plants suggests that they use a wide range of compounds to identify plants that carry hosts. However, not all detected compounds are expected to have an effect on behavior. The attraction of female parasitoids could be due to a few specific compounds in the blend, or to a specific mixture of compounds. This is testable, as is the relative importance of the minor compounds on parasitoid behavior. Identification of the minor EAG active compounds in the different blends may reveal interesting prospects for application and should improve our understanding of the recognition by female parasitoids of plants attacked by potential hosts. More detailed study should be done at the single sensillum level in order

to determine the specificity of olfactory cells to the different compounds present in the induced blends.

The results show that these parasitic wasps have a wide olfactory capability and that there is little difference among species of parasitoids. Further studies to understand the mechanisms mediating host location by parasitic wasps may help in developing methods to optimize the efficiency of natural enemies as biological control agents.

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GRAPEFRUIT OIL ENHANCES ATTRACTION OF MEXICAN FRUIT FLIES TO A SYNTHETIC FOOD-ODOR LURE

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Abstract—We investigated the attractiveness of grapefruit oil to the Mexican fruit fly. Only high concentrations were attractive in laboratory wind-tunnel bioassays. Attraction of flies to grapefruit oil was not enhanced if they had previous experience with grapefruit. In citrus orchard experiments, undiluted grapefruit oil attracted Mexican fruit flies and enhanced attraction to traps baited with a synthetic food-odor lure emitting ammonia and other nitrogenous chemicals. This is the first demonstration of host fruit odor increasing attraction to another type of attractive blend in Mexican fruit fly. These results indicate differences in the way the flies respond to undiluted grapefruit oil compared with previously tested fruit odors.

Key Words—Diptera, Tephritidae, *Anastrepha ludens*, attractant, grapefruit oil.

INTRODUCTION

It is well known that host plant odors are generally attractive to phytophagous insects. Further, host plant odors often enhance attraction to pheromone in Lepidoptera, Coleoptera, and Diptera (Landolt and Phillips, 1997). In Tephritidae, host fruit odors were commonly demonstrated to be attractive for oviposition, feeding, and mating (Fletcher and Prokopy, 1991; Landolt et al., 1992; Jang and Light, 1996). Additive or synergistic effects from combining host or plant odors with pheromones or bacteria odors were reported for several

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tephritids including Mediterranean fruit fly (*Ceratitis capitata*) (Dickens et al., 1990), papaya fruit fly (*Toxotrypana curvicauda*) (Landolt et al., 1992), and apple maggot (*Rhagoletis pomonella*) (MacCollom et al., 1994). Combinations of host or plant odors with other types of attractive blends sometimes were less attractive than expected in Tephritidae. For example, traps containing both host fruit and Nulure baits were less attractive than traps with only host fruit bait to protein-fed female Oriental fruit flies (*Bactrocera dorsalis*) (Cornelius et al., 2000).

Combinations of fruit odors with other types of attractants have always been less attractive than the more attractive of the two lure types in the Mexican fruit fly (*Anastrepha ludens* Loew) (Diptera: Tephritidae). Robacker and Garcia (1990) reported that fermented-fruit odor decreased attraction of sexually active females to pheromone, and that pheromone decreased attraction of hungry females to the fruit odor. Robacker (1991) showed that combinations of fermented-fruit chemicals with bacterial odor were less attractive to sugar-hungry flies than fruit chemicals alone. Finally, Robacker and Heath (1997) found that fermented-fruit chemicals decreased attraction to a mixture of ammonia, methylamine, and putrescine (AMPu) tested in a citrus orchard.

In this work, we tested grapefruit oil in laboratory wind-tunnel bioassays to determine the most attractive concentration and to investigate whether attraction of flies to the oil would increase after flies were exposed to grapefruit. Two experiments were conducted in citrus orchards to measure the attractiveness of grapefruit oil by itself and in combination with a synthetic food-odor lure containing ammonia, methylamine, and putrescine.

METHODS AND MATERIALS

Insects and Test Conditions. Mexican fruit flies were obtained from a culture that originated from yellow chapote fruit (*Casimiroa greggii*), a native citrus host of the fly, collected in Nuevo Leon, Mexico, in 2000. Laboratory conditions for holding and testing flies were 22 ± 2°C, 50 ± 20% relative humidity, and photophase from 06:30 to 19:30 hr. Laboratory tests were conducted between 09:00 and 16:00 hr using 3- to 13-d-old flies.

Experiments 1A and 1B: Wind-Tunnel Bioassay of Grapefruit Oil Dose–Response Relationship. The purpose of these experiments was to relate test quantity of grapefruit oil to attractiveness. Grapefruit oil (product code 7553, no lot numbers given by the manufacturer) was obtained from Now Foods (Bloomingdale, IL, USA). Grapefruit oil was tested undiluted, and as serial 1 to 10 dilutions down to 0.01% oil in hexane.

Bioassays were conducted in a Plexiglas wind tunnel with the dimensions of 0.3 × 0.3 × 1.2 m. Each end of the wind tunnel was screened to allow

airflow. The downwind end contained a baffle system to create a uniform airflow through the chamber. Air was pulled through the chamber at 0.4 m/sec by an exhaust fan connected to the downwind end. Air exiting the chamber was directed into an exhaust hose and removed to the outdoors. In addition to the direct exhaust from the wind tunnel, this room contained inlet and outlet vents to bring new air into the room from outdoors and remove air from the room to the outdoors. Air entered and exited the room at the rate of eight complete air replacements per hour. The top of the chamber had two circular openings (12.8 cm diam) with Plexiglas covers, located at each end of the chamber, to allow easy access to the chamber's interior. A 75-W "soft white" light bulb (General Electric Co., Cleveland, OH, USA) in a reflecting lamp was positioned 17 cm above the downwind end of the chamber. The purpose of this light was to minimize random flying into the upwind end of the chamber by using the flies' positive phototactic reaction. Overhead lighting was provided by fluorescent "cool white" lights (F40CW, General Electric).

Bioassays were conducted by using flies of two feeding regimes: (1) sugar-fed, protein-starved (from eclosion) (Experiment 1A) and (2) sugar-starved (for 2 d), protein-starved (Experiment 1B). Mixed-sex groups of 25–40 flies (overall sex ratio was 1:1 but individual cartons varied) were kept in 473-ml cardboard cartons with screen tops until used in tests. To conduct a trial, 100 μl of sample (containing various amounts of grapefruit oil) were put onto a 3 × 3 cm piece of filter paper that was attached to the upwind side (not visible to approaching flies) of a yellow plastic card (10.5 × 15.5 cm) suspended in the upwind end of the wind tunnel. One carton of flies was placed under the downwind opening. Flies were given 5 min to leave the carton, fly or walk upwind, and contact the yellow card. Upwind movement was scored if flies passed a point two-thirds of the distance from the release carton to the card. Seven replications of each experiment were conducted.

Experiment 2: Wind-Tunnel Bioassay of Effect of Experience with Grapefruit on Attraction to Grapefruit Oil. The purpose of this experiment was to determine if attraction of flies to grapefruit oil would increase if the flies had previous experience with grapefruit. The grapefruit oil lure used in this experiment was a 4-ml glass vial containing 1 ml of undiluted grapefruit oil with a cotton wick. The lure was fastened to the upwind side of a yellow plastic card. A plastic card without a lure was used as control for testing both naïve and grapefruit-experienced flies. Bioassays were conducted as in Experiments 1A and 1B.

Test flies were from two groups: naïve flies and grapefruit-experienced flies. Both groups were held in Plexiglas cages (20.5 × 20.5 × 20.5 cm) (200 flies per cage) with screen tops containing a diet mixture of sugar and yeast hydrolysate (U. S. Biochemical, Cleveland, OH, USA) and water supplied separately. Half of the cages were provisioned with a grapefruit (*Citrus paradisi*,

variety Rio Red) from a local orchard beginning when flies eclosed. Grapefruits had a 2-cm-diam piece of peel removed to enhance the release of peel volatiles. All cages were kept in the same laboratory where bioassays were performed, but those with grapefruits were kept near an exhaust fan to vent grapefruit odor to outdoors. Test flies were older than 4 d to allow at least several days for flies to learn the odor of grapefruit. The four treatments (2 fly types × 2 lure treatments) were tested in random order in each replication. Thirteen replications of the experiment were conducted.

Experiments 3A and 3B. Citrus Orchard Evaluation of Grapefruit Oil, Anastrepha Lure, and their Combination. The purpose of these experiments was to determine the attractiveness of grapefruit oil and its combination with nitrogenous food odors in the field. The grapefruit oil lure used in these experiments was a 4-ml glass vial containing 1 ml of undiluted grapefruit oil with a cotton wick. *Anastrepha* fruit fly lures obtained from IPM Tech (Portland, OR, USA) were used as synthetic food odor lure. These lures contain ammonia, methylamine, and putrescine, and are based on an attractant for the Mexican fruit fly reported by Robacker and Warfield (1993).

The experiments were conducted in two citrus orchards located near the laboratory in Weslaco, TX, USA. The first orchard contained several varieties of oranges, lemons, and tangerines. One row of Valencia sweet oranges (*Citrus sinensis*) and one row of Dancy tangerines (*C. reticulata*) were used for tests. The other orchard contained only Rio Red grapefruit from which two rows were used for experiments. Within each row, four linear blocks of four trees each were chosen with one buffer tree between each block. Testing was done during autumn of 2003 in the first orchard and winter–spring of 2004 in the second. All trees were laden with nearly ripe or ripe fruit initially, but fruits were stripped from the grapefruit orchard with three to four replications remaining during spring testing.

Four lure treatments were tested in each experiment: grapefruit oil, *Anastrepha* lure, their combination, and no lure. *Anastrepha* lures were used for the duration of the autumn replications, then replaced with new lures that were used for the duration of the winter–spring replications. Grapefruit oil lures were replaced weekly. One each of the four treatments was tested in each block. Positions of treatments within each block were randomized for the first test of each series. A trial lasted 1 wk, after which flies were counted and traps were serviced as necessary. Positions of treatments in consecutive trials were not randomized but were moved sequentially within each block. Traps were hung one to a tree, north of center, at 1–2 m height. Each week, approximately 2000 flies were distributed uniformly onto rows of trees adjacent to the test rows. Mixed-sex groups of 180–200 flies were kept in 473-ml cardboard cartons under laboratory conditions (described above) until released in the test orchard. Flies were irradiated, due to quarantine laws, with 70–92 Gy (Cobalt 60) 1–2 d

before adult eclosion. Flies were fed sugar and water until they were released in test plots 3–12 d after eclosion.

Experiment 3A was a test of the lures on Intercept AM traps (IPM Tech). Intercept traps are yellow cardboard rectangles (14 × 23 cm) coated with Stickem Special (Seabright Laboratories, Emeryville, CA, USA) on both sides. Lures were fastened onto trap hangers above traps such that the bottom of the lure contacted the top of the trap. Traps were replaced each week. Seventeen replications (weeks) were conducted; eight during autumn in the tangerines section of the first orchard, and nine during winter–spring in grapefruits of the second orchard.

Experiment 3B was a test of the lures in yellow-bottom Multilure traps (Florence Agri Investment, Inc., Miami, FL, USA). Multilure traps are plastic McPhail-like traps with a clear, colorless top and an opaque, colored bottom that serves as a liquid reservoir for drowning captured flies. Grapefruit oil lures were taped inside the tops. *Anastrepha* lures were modified by removing the two plastic bags containing the attractants from the factory supplied mesh bag. The two plastic bags were inserted into the lure basket of the multilure traps. Traps contained water with 0.01% Triton®. (Rohm and Haas Co., Philadelphia, PA, USA) as a wetting agent. The no-lure trap contained only water and Triton. Water was replaced each week in all traps. Fourteen replications (weeks) were conducted; six during autumn in the oranges section of the first orchard, and eight during winter–spring in grapefruits of the second orchard.

Statistical Analyses. All experiments were conducted as randomized complete blocks. For wind-tunnel data, the numbers of males and females that moved upwind or landed on the odor source were divided by the total numbers of males or females in the carton used in the trial. These proportions were transformed by arcsin of the square root for data analyses (Snedecor and Cochran, 1967). Proportions of 0 were replaced with $1/4N$ before transformation (N is the number of males or females in the carton). For field tests, replications over time were treated like replications over space (blocks of trees) for the purpose of statistical analyses. Transformed percentages from the wind tunnel bioassays, and the numbers of flies captured on traps in field tests, were subjected to analysis of variance using SuperANOVA (Abacus Concepts, 1989). Means separations were done by Fisher's protected least significant difference method (LSD) (Snedecor and Cochran, 1967).

RESULTS

Experiments 1A and 1B: Wind-Tunnel Bioassay of Grapefruit Oil Dose–Response Relationship. Sugar-fed flies were not attracted to grapefruit

oil at any test quantity (Figure 1A). Sugar-starved males were attracted to the two highest test quantities of grapefruit oil but not to any lower quantities (upwind movement: $F = 6.4$; $df = 5, 44$; $P < 0.001$; landings: $F = 4.3$; $df = 5, 44$; $P < 0.01$) (Figure 1B). Sugar-starved females showed a similar trend but no individual test quantities were significantly more attractive than blanks or other quantities (Figure 1B). However, single-*df* contrasts of the means for the two

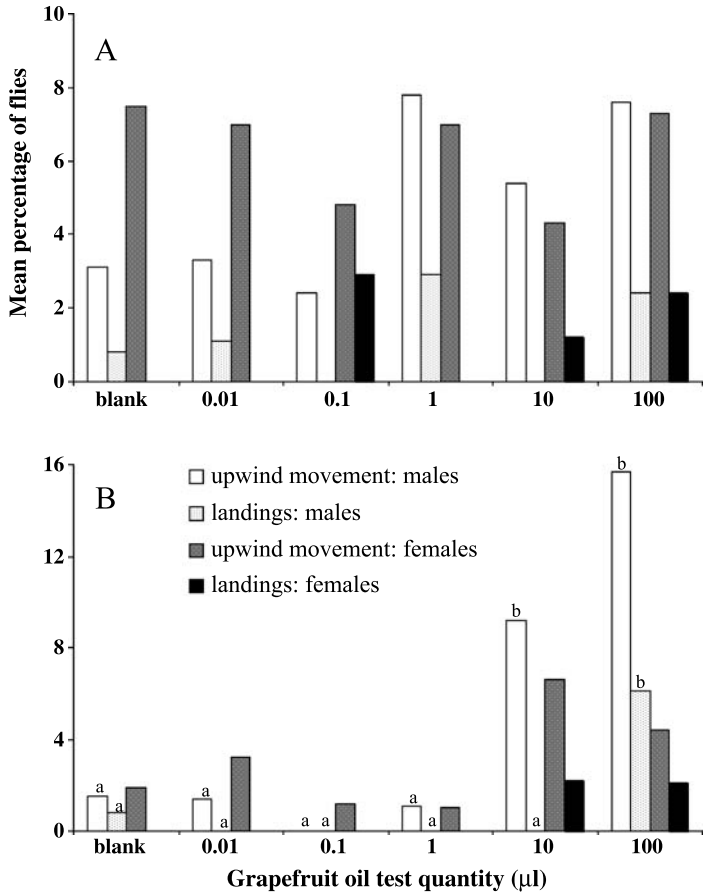

FIG. 1. Attraction of sugar-fed (A) and sugar-starved (B) Mexican fruit flies to various test quantities of grapefruit oil in wind-tunnel bioassays. For sugar-starved males, means in the same data series (upwind movements or landings) with different letters above the bars were significantly different ($P < 0.05$) by Fisher's protected LSD. No individual means were significantly different for sugar-fed males and females, and sugar-starved females.

highest test quantities vs. the blank and lower quantities were significant (upwind movement: $F = 5.6$; $df = 1, 44$; $P < 0.05$; landings: $F = 7.8$; $df = 1, 44$; $P < 0.01$).

Experiment 2: Wind-Tunnel Bioassay of Effect of Experience with Grapefruit on Attraction to Grapefruit Oil. Experience with grapefruit did not enhance attraction of either male or female flies to grapefruit oil. The treatment effect (for the four test sample × experience treatments) was significant only for upwind movements by males ($F = 2.9$; $df = 3, 36$; $P < 0.05$). However, in this instance, the percentage of naïve males to move upwind toward the blank yellow card (12.3%) was significantly greater than response to either of the grapefruit oil treatments (grapefruit oil/naïve males, 2.7%; grapefruit oil/ experienced males, 4.5%) but not significantly greater than response by experienced males to the blank yellow card (7.1%).

Experiment 3A. Citrus Orchard Evaluation of Grapefruit Oil, Anastrepha Lure, and their Combination on Interceptor Traps. Results are shown in Table 1. Analysis of the complete dataset indicated that traps with grapefruit oil were not significantly more attractive than unbaited traps. However, when traps with *Anastrepha* lures and those with the combinations were not included, traps with grapefruit oil were significantly more attractive than unbaited traps to both males ($F = 9.5$; $df = 1, 114$; $P < 0.01$) and females ($F = 7.0$; $df = 1, 114$; $P < 0.01$). Traps with both lures were significantly more attractive than those with only *Anastrepha* lures to males but not to females (complete dataset). Traps with either *Anastrepha* lures or the lure combinations were more attractive than unbaited traps and traps with only grapefruit oil to both males and females. Results from the autumn replications did not differ from those from the winter–spring replications. Also, results from winter–spring replications in which trees had ripe fruit did not differ from those in which trees had no fruit.

Experiment 3B. Citrus Orchard Evaluation of Grapefruit Oil, Anastrepha Lure, and their Combination in Multilure Traps. Results are shown in Table 2. Traps with grapefruit oil were more attractive than unbaited traps to females.

TABLE 1. ATTRACTION OF MEXICAN FRUIT FLIES TO INTERCEPTOR AM TRAPS BAITED WITH GRAPEFRUIT OIL, IPM TECH *Anastrepha* FRUIT FLY LURES, OR THEIR COMBINATION IN A CITRUS ORCHARD

	Males	Females	Total
Blank	0.2 ± 0.05 a	0.2 ± 0.05 a	0.4 ± 0.08 a
Grapefruit oil	0.5 ± 0.08 a	0.6 ± 0.12 a	1.1 ± 0.17 a
Anastrepha lure	1.8 ± 0.22 b	2.0 ± 0.30 b	3.8 ± 0.47 b
Combination	2.7 ± 0.41 c	2.5 ± 0.33 b	5.2 ± 0.65 c

Values are mean flies captured per week (±SE). Means in the same column followed by different letters are significantly different at the 5% level by Fisher's protected LSD (males: $F = 29.4$; $df = 3, 24$; $P < 0.001$; females: $F = 29.3$; $df = 3, 34$; $P < 0.001$).

TABLE 2. ATTRACTION OF MEXICAN FRUIT FLIES TO MULTILURE TRAPS BAITED WITH GRAPEFRUIT OIL, IPM TECH *Anastrepha* FRUIT FLY LURES, OR THEIR COMBINATION IN A CITRUS ORCHARD

	Males	Females	Total
Blank	0.2 ± 0.08 a	0.2 ± 0.08 a	0.5 ± 0.14 a
Grapefruit oil	0.9 ± 0.23 a	1.2 ± 0.21 b	2.1 ± 0.36 b
Anastrepha lure	2.9 ± 0.38 b	3.4 ± 0.40 c	6.3 ± 0.70 c
Combination	3.1 ± 0.41 b	4.3 ± 0.47 d	7.4 ± 0.79 c

Values are mean flies captured per week (±SE). Means in the same column followed by different letters are significantly different at the 5% level by Fisher's protected LSD (males: $F = 29.3$; $df = 3, 21$; $P < 0.001$; females: $F = 42.6$; $df = 3, 21$; $P < 0.001$).

When traps with *Anastrepha* lures and the lure combinations were not included in the analysis, traps with grapefruit oil were also significantly more attractive than unbaited traps to males ($F = 9.4$; $df = 1, 97$; $P < 0.01$). Traps with both lures were significantly more attractive than those with only *Anastrepha* lures to females but not to males (complete dataset). Traps with either *Anastrepha* lures or the lure combinations were more attractive than unbaited traps and traps with only grapefruit oil to both males and females. As in Experiment 5, time of year and presence of fruit did not affect results.

DISCUSSION

Grapefruit oil was only weakly attractive to male and female Mexican fruit flies in wind-tunnel bioassays and citrus orchard experiments. In contrast, fermented chapote fruit and low concentrations of chemicals from the fermented chapote fruit previously proved highly attractive to Mexican fruit flies in laboratory experiments (Robacker et al., 1990a,b; Robacker, 1991) and in one field test (Robacker and Heath, 1996), although chapote chemicals were not very attractive in another field test (Robacker and Heath, 1997). Considering similarities in experimental protocols in at least some of the work with chapote odor and grapefruit oil, the most prudent conclusion is that grapefruit oil is not very attractive to Mexican fruit flies.

Despite its weak attractiveness, grapefruit oil enhanced attraction of both males and females to *Anastrepha* lures in citrus orchard experiments. All previous experiments in which fruit odors were added to either bacterial odors or to a chemical blend similar to that emitted by the *Anastrepha* lures resulted in negative interactions of the odor types (see Introduction).

Grapefruit oil was not more attractive to grapefruit-experienced than to naïve flies. Previous experiments demonstrated that Mexican fruit flies caged

with peel-damaged grapefruits learned the odor of grapefruits and subsequently increased their attraction to low concentrations of grapefruit peel extracts (Robacker and Fraser, 2005). In the current work, the results suggest that flies that learned grapefruit odor did not recognize grapefruit oil as the same odor. The apparent lack of recognition could be due to quantitative differences (low vs. high concentrations) or qualitative differences. The grapefruit oil used in this work probably was qualitatively different from the odor of Rio Red grapefruit to which flies were exposed. The oil was purchased from Now Foods and most likely was not derived from Rio Red grapefruit. However, exposure to grapefruits increased attraction to oranges in a previous work (Robacker and Fraser, 2003) even though the odor of oranges is noticeably different from that of grapefruit.

The nature of the attraction response to grapefruit oil is not known at this time but it appears to be different from the nature of responses of Mexican fruit flies to fruit odors tested in earlier work. To summarize the evidence for this assertion: (1) attraction to grapefruit oil was weak compared with attraction to fermented chapote odor; (2) grapefruit oil enhanced attraction to a blend of attractive nitrogenous chemicals but a combination of chapote-odor chemicals inhibited attraction to the same blend of nitrogenous chemicals; and (3) attraction to grapefruit oil did not increase after exposure to grapefruit whereas attraction to a low concentration of grapefruit-peel extract did increase after exposure to grapefruit.

Although the nature of the attraction is not known, it is interesting that the origin of the attraction is a plant essential oil. The "parapheromone" methyl eugenol, a powerful attractant to males of numerous *Bactrocera* species, was discovered as a minor constituent of citronella oil, another plant essential oil (Howlett, 1915). As recently as 15 years ago, parapheromones such as methyl eugenol were considered by some as "one of the great mysteries of tephritid biology" (Cunningham, 1989). The physiological basis of the attraction to methyl eugenol was not unraveled until the last decade led in part by the efforts of Shelly, Tan, and Nishida (Shelly and Dewire, 1994; Tan and Nishida, 1996). What seemed mysterious attraction just 15 years ago now has been explained as attraction of males to a chemical that serves both as a precursor to a pheromone that attracts females and as an allomone that deters predation (Tan and Nishida, 1998). While the nature of the attraction of Mexican fruit flies to grapefruit oil is very different from the attraction of *Bactrocera* to parapheromones, the possibility exists that the ultimate source of the attractiveness may be a minor component of the oil that may be highly attractive at greater concentrations.

Also relevant to our work is the finding that female papaya fruit flies respond to papaya odor differently depending upon the odor concentration and their mating status (Landolt et al., 1992). Papaya odor, mixed with a constant amount

of pheromone, was most attractive to unmated females at a relatively low concentration but most attractive to mated females at the highest concentration tested. These results suggest the physiological basis of attraction of unmated and mated females to papaya may differ. A similar phenomenon could be occurring in Mexican fruit fly in that attraction to high concentrations of grapefruit odor may have a different physiological basis than attraction to low ones.

We propose three tentative explanations for the results reported here. First, attraction to undiluted grapefruit oil may be an aberrant behavior caused by overloading receptors that normally function to receive lower concentrations of odorants. Second, a minor component of grapefruit oil that has an unknown role in the ecology of Mexican fruit flies may be responsible for the unusual attraction properties. Finally, it is possible that an as yet undiscovered physiological/motivational state may exist in Mexican fruit fly (and other Tephritidae) that causes attraction to fruit odors at high concentrations.

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Vitex agnus-castus IS A PREFERRED HOST PLANT FOR *Hyalesthes obsoletus*

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Abstract—*Hyalesthes obsoletus* Signoret (Homoptera: Cixiidae) is a polyphagous planthopper that transmits stolbur phytoplasma (a causative agent of "yellows" disease) to various weeds, members of the Solanaceae, and wine grapes (*Vitis vinifera* L.) in Europe and the Middle East. Planthoppers were collected by hand vacuuming eight native plant species. *Vitex agnus-castus* L., a shrub in the Verbenaceae, hosted the largest number of *H. obsoletus*, although *Olea europaea* L. also served as a host for adults. Using a Y-olfactometer, we compared the planthoppers relative preference for *V. agnus-castus*, *Convolvulus arvensis*, and *V. vinifera*. *V. agnus-castus* was more attractive to both male and female *H. obsoletus* than the other plants. *H. obsoletus* antennal response was stronger to volatiles collected from *V. agnus-castus* than from Cabernet Sauvignon variety of *V. vinifera*. To determine if *V. agnus-castus* would serve as a reservoir for the pathogen, *H. obsoletus* were collected from leaf and stem samples of native *V. agnus-castus*, and were tested by polymerase chain reaction (PCR) for the presence of phytoplasma DNA. While 14% and 25% (2003 and 2004, respectively) of the insects tested positive for phytoplasma DNA, none of the plant samples tested positive. To determine if *V. agnus-castus* could serve as a host plant for the development of the planthopper, we placed emergence cages beneath field shrubs and enclosed wild-caught *H. obsoletus* in a cage with a potted young shrub. We found adult *H. obsoletus* in the emergence cases and planthopper nymphs in the soil of the potted plant. We concluded that *V. agnus-castus* is attractive to

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H. obsoletus, which seems to be refractory to phytoplasma infections and warrants further testing as a trap plant near vineyards.

Key Words—EAG, "yellows" disease, spread control, management.

INTRODUCTION

The level of infection of a crop by phytoplasma is dependent on the number and abundance of insect vectors and of alternative/reservoir plants harboring the pathogen, provided that the planting material is not the source of infection. In some cases, such as the aster yellows of carrots, lettuce, and celery in the midwestern U.S., the relationship between the vector and native phytoplasma-infected host plants has been elucidated (Hoy et al., 1992). In other cases, such as the stolbur phytoplasma infection in various crop plants in Europe, the role of alternative host plants in disease epidemiology is not completely understood.

Hyalesthes obsoletus Signoret (Homoptera: Cixiidae), is a polyphagous vector of stolbur phytoplasma to some weeds, plants in the Solanaceae (Brcak, 1979), and wine grapes (*Vitis vinifera* L.) (Maixner, 1994; Sforza et al., 1998) in Europe, and is a suspected vector to wine grapes in Israel (Klein et al., 2001). Grape vines are probably not among the preferred plants of *H. obsoletus*, as it survives poorly on them (Maixner, 1994; Sforza et al., 1998). Brcak (1979) reported that *H. obsoletus* does not leave its primary central and east European host, *Convolvulus arvensis* L., as long as the plant remains suitable for feeding. Furthermore, he reported that as long as the planthopper remained on *C. arvensi*s, a natural source of the phytoplasma, there were no observed incidents of stolbur infection in crops. *H. obsoletus* has been found on other weeds in addition to *C. arvensis*, some of which are also potential phytoplasma reservoirs. In vineyards in Germany, Maixner et al. (1995) demonstrated that *C. arvensis* and *Solanum nigrum* L. were infected with stolbur phytoplasma and could serve as an inoculum reservoir. In contrast, Weber et al. (1997) showed that in vineyards where *Ranunculus bulbosus* L. was the dominant weed, there were fewer infected *H. obsoletus*, and *R. bulbosus* was never found to harbor the phytoplasma. In France, *H. obsoletus* was found to complete its life cycle on *Lavendula hybrida* Reverchon (Moreau and Leclant, 1973), *C. arvensis*, *Lavendula angustifolia* Mill., and *Lepidium draba* L. (Sforza et al., 1999), and was shown to be infected by stolbur phytoplasma (Cousin and Moreau, 1991; Sforza et al., 1998).

Yellows disease was first observed in wine grapes in Israel in the 1980s, and has had a devastating effect. To date, over 50 ha of vineyards have been uprooted due to this disease. Vineyards in affected areas have been surveyed for the presence of phloem feeding insects, such as leafhoppers and planthoppers

(Klein et al., 2001; Orenstein et al., 2003), and several candidate species, including *H. obsoletus*, have been identified.

Alternative plant hosts for the phytoplasmas have not been identified in Israel. During the middle of the growing season (June–August), there are few living plants, and virtually no leafhoppers or planthoppers are captured in vineyards (Klein et al., 2001; Orenstein et al., 2003). Because the last precipitation in wine-grape production areas is in mid-April (Orenstein et al., 2003), by May all nonirrigated areas are dry and only deep-rooted plants remain green throughout summer. Two generations of *H. obsoletus* are found annually in Israeli vineyards; there is a small peak population of adults in May and early June, and another larger peak in September and October (Klein et al., 2001; Orenstein et al., 2003). The goal of this research was to determine the native host plant(s) of *H. obsoletus* and their ability to serve as a reservoir for phytoplasmas.

METHODS AND MATERIALS

Native Plant Screening. This study took place in the Golan Heights at an elevation of 350 m in the south to 800 m in the north. We collected insects from native plants that remained green throughout the summer; *Amaranthus retroflexus* L., *C. arvensis*, *Inula viscose* L., *Myrtus communis* L., *Salix alba* L., *Tamarix* sp., and *Vitex agnus-castus* L. We used a modified leaf blower (Echo model No. PB 1000), in which air intake and exhaust ports were switched, and intake was fitted with a fine mesh nylon bag. Vacuum sampling collects all insects indiscriminately and does not rely on their attractiveness to a trap. In the first year (2003), a minimum of three plants of each species were sampled two to four times in May–June and once in September in the central-southern Golan. During the second year (2004), a minimum of three plants of each species were sampled two to four times in May–June and eight times from September to November; *V. agnus-castus* was sampled in central and northern Golan. *H. obsoletus* were found during spring in an olive (*Olea europaea* L.) orchard, and therefore sampled twice in spring and eight times in autumn.

Captured *H. obsoletus* were counted and recorded by sex when collected in large numbers. Live specimens were maintained at room temperature in screen-covered (50 mesh) boxes containing putative feeding plants until they were used in further trials.

Plant Choice Assay. The host plant preference of *H. obsoletus* was determined by olfactometry. The olfactometer consisted of a Y-shaped glass tube, 2 cm diam. The base was 9 cm long, and the two arms of the olfactometer were each 16 cm in length. Each arm was attached to a flow meter and an odor source container (glass sphere, 12 cm diam) into which test plant branches were

placed. In each case, charcoal-filtered air (1 l min^{-1}) was forced through each arm of the apparatus. There were six replicates of each plant pairing: *V. agnus-castus* vs. *C. arvensis* and *V. agnus-castus* vs. *V. vinifera*. *V. agnus-castus* was used in each pair comparison because of the larger number of planthoppers captured in this species. In Israel, the majority of *V. vinifera* are grafted on Richter 110, but three other rootstocks are also used. We tested Richter 110 and Castel 216. In each replicate (six replicates with females and six with males), a group of five to seven planthoppers was placed in the base tube of the Y-olfactometer and allowed to move upwind towards either of the two arms of the olfactometer. Trial periods were 5 min in length, and the location of each test planthopper was recorded at the conclusion of each trial. To compensate for any positional bias, the olfactometer was rotated 180° after every three replicates. In order to avoid contamination by remaining plant volatiles, the olfactometer was washed with acetone and dried after each experiment.

Volatile Collection. Volatiles were collected from blooming *V. agnus-castus* and Cabernet Sauvignon variety of *V. vinifera*. As noted above, samples of *V. vinifera* from both rootstocks were used. Charcoal purified air was forced through a 500-ml glass sampling jar containing a plant branch, then through a 200-mg SuperQ trap at a rate of 0.2 l min^{-1} to collect organic chemicals released from test plants. Traps were washed with pentane to elute the captured volatiles. The resulting extract was concentrated to a volume of 10 μl and stored at −20°C.

Electroantennograms. In order to examine antennal sensitivity to volatiles of various host plants, electroantennograms (EAG) were recorded from detached antennae of male and female *H. obsoletus* exposed to test volatiles. The base of each antenna was inserted into a glass capillary tube, with a silver electrode lining serving as a grounding probe, while the tip of the antennae was inserted into a second capillary tube, with a silver electrode lining serving as a recording probe. Both glass capillaries were filled with 0.1 N KCl. Antennae were positioned by using a micromanipulator (INR-05, Syntech, Hilversum, The Netherlands). Two microliters of concentrated plant volatiles were applied to an approximately 1 cm^2 piece of filter paper (Whatman No. 1). One microliter of pentane was applied to filter paper to serve as a blank. The filter paper was allowed to air dry for 20 sec to allow the solvent to evaporate, and was then placed inside a glass Pasteur pipette (15 cm long). The pipette tip was inserted through a side hole in a glass tube (0.6 cm diam and 15 cm long) through which charcoal-filtered, humidified air flowed at 0.5 ml min^{-1}. Within each stimulus pipette that contained the filter paper with plant volatiles, 3 ml of air were forced into the constant air stream by a mechanical puffing that delivered puffs of 0.4-sec duration. At least 2-min intervals were maintained between each release of plant volatiles. The presentation order of stimuli for one antenna was randomized within the set (two extracts and one blank). EAG recordings were

made by using a serial data acquisition controller (Syntech IDAC-232, The Netherlands).

H. obsoletus Development on V. agnus-castus. To determine if *V. agnus-castus* serves as a host plant upon which *H. obsoletus* can complete its development, two evaluation methods were used: evaluation of *H. obsoletus* emerging from wild *V. agnus-castus* and development of caged *H. obsoletus* on potted *V. agnus-castu*s. Emergence boxes were made of a 50 × 50 × 40 cm wooden frame covered on five sides with 50 mesh screening. One box was placed, open side down, under the canopy of each of three *V. agnus-castus* plants in the center-south of the Golan at the beginning of May 2003 and again at the beginning of September. One yellow sticky trap was placed in each box to catch any emerging adult insects, and traps were replaced once every 2 wk. In May 2004, one box was placed under the canopy of each of 15 *V. agnus-castus* plants. One yellow sticky trap was placed in each box to catch any emerging adult insects, and traps were replaced once every 2 wk until emergence ceased; this was repeated in autumn starting at the beginning of September and continuing until emergence ceased. Additionally, in spring, 50 mesh screening was draped over the bottom branches of two different shrubs and secured to the ground, encompassing a semicircular area 1.5 m from the trunk. One yellow sticky trap was suspended on a branch from each of these shrubs and changed as described.

In September 2003, 40 adult *H. obsoletus* were vacuum-collected and placed in a 1 × 1 × 1 m cage covered with 50 mesh screening and containing one *V. agnus-castus* plant. The shrub was planted in a 10-l pail: the bottom 2/3 of the potting mix was a mixture of 70:30 peat moss:volcanic gravel, and the top 1/3 was a mixture of 1:1 coconut fiber:perlite. The plant was watered with an automatic irrigation system. Since adults are difficult to discern on the plant through the net, in early May of the next year, when the first adults were observed in the field, the pot was removed from the cage and the soil around the roots was carefully examined for presence of *H. obsoletus* nymphs.

Since *C. arvensis* is a known host in Europe, in May 2003 and 2004, we dug up >50 plants in areas where *V. agnus-castus* were also found, and searched the roots for *H. obsoletus* nymphs.

Phytoplasma Infection in V. agnus-castus. Samples of *H. obsoletus* adult males and females were analyzed individually by polymerase chain reaction (PCR) to determine if they were positive for phytoplasma. Plants on which were they were captured were observed for typical signs and symptoms of phytoplasma infection (proliferation of small leaves, "witches' broom," or yellow leaves) through the duration of the study, and they were analyzed by PCR for presence of phytoplasma at the end of the season (October) in 2003 and 2004.

DNA Extraction and Polymerase Chain Reaction. DNA extracted from phytoplasma-infected *Vinca minor* L. (periwinkle) was used as a positive

control, and DNA extracted from asymptomatic *V. minor* served as a negative control. Insects were homogenized individually in 150 μl extraction buffer, and 1 g of plant tissue was homogenized in a total of 3 ml of extraction buffer (2% cetyl trimethyl ammonium bromide (CTAB), 100 mM Tris–HCl pH 8.0, 1.4 M NaCl, 40 mM EDTA, 0.2% β-mercaptoethanol), then put in a 65°C water bath for 30 min with periodic vortexing. Both insect and plant samples were centrifuged (1500 × *g* for 5 min), the supernatant was mixed with an equal volume of chloroform:isoamyl alcohol (24:1 v/v), and recentrifuged (20,000 × *g* for 5 min). The supernatant was mixed with 0.6 volume of isopropanol for 15 min then centrifuged (20,000 × *g* for 20 min) to pellet the DNA. The extracted DNA was washed twice with 70% ethanol, then resuspended in 60 μl distilled H_2O, and stored at −20°C.

PCR amplification of DNA was performed by using a Tgradient thermocycler (Tamar Laboratory Supplies, Israel). The purified DNA from plant and insect samples was passed through one 30-cycle PCR reaction in the presence of universal primers defined as rU3 and fU5 (Lorenz et al., 1995). Amplification was carried out in a total of 20 μl; 0.1 μl (2.5 units μl^{-1}) *Taq* polymerase (Promega), 16.5 μl amplification buffer (supplied with *Taq* polymerase, and dNTPS), and 5–10 ng test DNA. The PCR reaction began a 5-min 94°C heat shock step followed by a 2-min step at 50°C, a 2-min step at 72°C, 35 cycles of 92°C (30 sec), 55°C (30 sec), 72°C (45 sec). In the last cycle, the 72°C step was extended for 7 min as an elongation step. The amplified PCR products were analyzed by electrophoresis of 12 μl of reaction mixture in 1.5% agarose gel (40 mM Tris–HCl, pH 7.5; 20 mM acetic acid; 1 mM EDTA), stained with ethidium bromide, and visualized with a UV transilluminator. The size standard 100-bp DNA ladder used in gels was obtained from MBI Fermentas.

Statistical Analysis. Comparisons between the numbers of planthoppers caught from the different plant species were analyzed by one-way analysis of variance (ANOVA), and means were separated by the Tukey HSD multiple range test using JMP 5.1 software (SAS software). Results of the olfactometer tests were analyzed by the log-likelihood ratio (*G*-statistic). Kruskal–Wallis test was used to compare the EAG responses to the headspace preparations and the control. Medians were separated by the Median-Notch method. All tests were conducted at $\alpha = 0.05$ level.

RESULTS

Native Host Plants. In the first year, more specimens of *H. obsoletus* were present on *V. agnus-castus* than or any other plant species (df 7, 118, $F = 153.86$, $P < 0.001$) (Table 1). *H. obsoletus* were found on *M. communis* only in autumn.

TABLE 1. SURVEY OF THE NUMBER OF *Hyalesthes obsoletus* FOUND ON NATIVE PLANTS IN 2003

Species	Samplings	Total *H. obsoletus*
Amaranthus retroflexus	13	4 b*
Convolvulus arvensis	18	2 b
Inula viscose	12	0 b
Myrtus communis	15	15 b
Salix alba	12	0 b
Tamarix sp.	12	0 b
Vitex agnus-castus	18	549 a
Vitis vinifera	26	13 b

*Data followed by different letters are significantly different at $\alpha = 0.05$.

Repeated sampling of four wild *V. agnus-castus* shrubs in June yielded 218 *H. obsoletus* individuals with a male:female ratio of 58:42. Vacuuming the same four shrubs in October yielded 564 specimens, with a male:female ratio of 46:54. In spring of the second year, more specimens of *H. obsoletus* were present on *V. agnus-castus*, followed by olives, than for any other plant species ($df = 9, 51$, $F = 87.47$, $P < 0.001$) (Table 2). In autumn, significantly more specimens of *H. obsoletus* were present on *V. agnus-castus* than on all other plants ($df = 10, 210$, $F = 34.04$, $P < 0.001$) (Table 2). *H. obsoletus* were found on olive trees in spring, and only until mid-October in autumn.

TABLE 2. SURVEY OF THE NUMBER OF *Hyalesthes obsoletus* FOUND ON NATIVE PLANTS IN 2004

	Spring		Autumn	
Plant species	Samplings	Total *H. obsoletus**	Samplings	Total *H. obsoletus*
Amaranthus retroflexus	6	2 c	24	1 C
Convolvulus arvensis	6	1 c	18	5 C
Inula viscose	6	0 c	24	0 C
Myrtus communis	6	0 c	24	13 C
Olea europaea 1 yr	4	39 b	3	2 C
Olea europaea 3 yr	3	29 b	15	56 C
Salix alba	6	0 c	24	0 C
Tamarix sp.	6	0 c	24	0 C
Vitex agnus-castus South	9	294 a	22	728 A
Vitex agnus-castus North			21	156 B
Vitis vinifera	9	5 c	18	4 C

*Data followed by different letters are significantly different at $\alpha = 0.05$.

TABLE 3. OLFACTOMETER RESULTS FOR MALE AND FEMALE *Hyalesthes obsoletus* GIVEN A CHOICE BETWEEN *Vitex agnus-castus* (*V. A.-C.*) AND *Convolvulus arvensis* (*C. A.*) OR *Vitex agnus-castus* AND *Vitis vinifera* (*V. V.*)

Sex	Choice	*df*	G-statistic	*P*
Male	*V. a.-c.* × *C. a.*	2	10.66	<0.005
Female	*V. a.-c.* × *C. a.*	2	8.46	<0.025
Male	*V. a.-c.* × *V. v.*	2	22.32	<0.001
Female	*V. a.-c.* × *V. v.*	2	61.76	<0.001

Since *H. obsoletus* was present in large numbers on *V. agnus-castus*, further analysis was possible. Significantly fewer *H. obsoletus* were found on *V. agnus-castus* in the northern Golan (El Rom), and only until the end of October, whereas in the south-center of the Golan, they were abundant until the end of November. In October 2004, the monthly average low temperature in the north was 14.0°C, whereas in the center-south it was 16.5°C. By November, the average low temperature was 7.5°C in the north and 11.3°C in the center-south.

Olfactometer and Electroantennogram Trials. In an olfactometer assay, both sexes of *H. obsoletus* significantly preferred *V. agnus-castus* over grape vines (*V. vinifera*) and *C. arvensis* (Table 3), although the number of females choosing *V. agnus-castus* over grape vines was higher than that of males. Conversely, more males chose *V. agnus-castus* over *C. arvensis*. There was no rootstock effect.

Antennal response of adults (Table 4) was significantly higher to *V. agnus-castus* volatiles than to grape vines (Cabernet Sauvignon) or the blank control ($df = 3, 20$, $F = 9.58$, $P < 0.001$). The antennal response to the grape vine was not different from the response to the blank control. There was no rootstock effect.

TABLE 4. RESULTS OF DETACHED *Hyalesthes obsoletus* ANTENNAE STIMULATED BY VOLATILES ISOLATED FROM *Vitex agnus-castus* AND CABERNET SAUVIGNON VARIETY OF *Vitis vinifera* ON TWO DIFFERENT ROOT STOCKS (CASTEL 216 AND RICHTER 110)

Sample	Number of replicates	Mean (mV) ± standard deviation
Blank	6	390 ± 122 b*
Vitex agnus-castus	6	695 ± 125 a
Vitis vinifera (Richter)	6	455 ± 71 b
Vitis vinifera (Castel)	6	498 ± 89 b

*Data followed by different letters are significantly different at $\alpha = 0.05$.

TABLE 5. STICKY TRAP CATCHES OF *Hyalesthes obsoletus* IN EMERGENCE CAGES, 2004

No. *Vitex agnus-castus*	Capture technique	No. *Hyalesthes obsoletus*												
		May 14	May 31	June 11	June 20	September 1	September 8	September 23	October 1	October 12	October 24	November 5	November 8	November 15
15	Cage	14	13	2	0	4	3	7	3	0	16	8	0	1
2	Drape	37	0	0	0									

H. obsoletus Development on V. agnus-castus. H. obsoletus were caught on sticky traps in emergence boxes placed under *V. agnus-castus* in the field. In the first year (2003) a total of eight adults were caught in May; in autumn, 10 adults were caught from the beginning of September until the middle of October. In the second year (2004), more cages were set up and emergence was monitored in spring and autumn (Table 5). In spring, adults emerged for a maximum of 6 wk, whereas in autumn they emerged over a period of almost 3 mo. In the spring, approximately half the area under each of two shrubs was covered with net; because the entire emergence area was not covered, few planthoppers were caught. Assuming a nonclustered distribution for this species, an estimated mean number of 37 (2 × number captured from 1/2 of each shrub) adult *H. obsoletus* emerged from each shrub. In May, we found 25 third to fifth instar *H. obsoletus* nymphs on the caged *V. agnus-castus* that were exposed to mated females in September. No *H. obsoletus* nymphs were found on any of the 50 + *C. arvensis* plants that were uprooted in spring of 2003 and 2004.

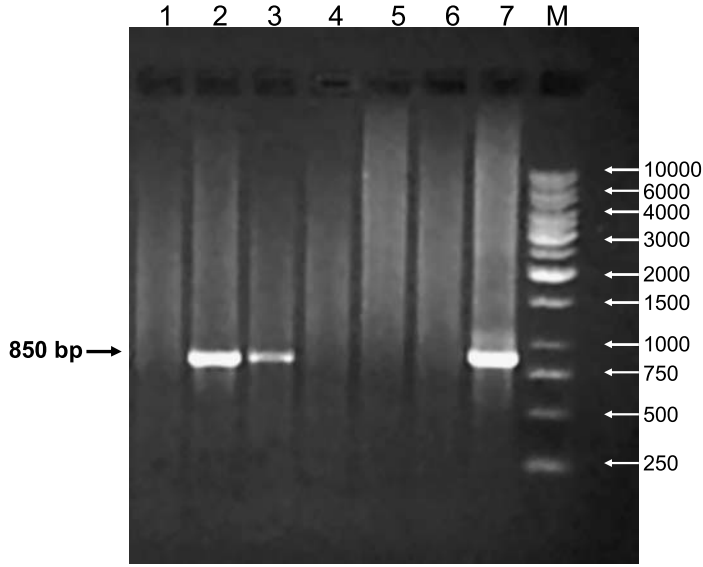

FIG. 1. Photograph of 1.5% agarose gel stained with ethidium bromide, showing amplified products from universal primers rU3 and fU5 for carrots and leafhoppers. Lane: M. molecular markers (in addition to those indicated, 8000, 5000, 4000, 3500, 2500 bp are also present), 1–3 *Hyalesthes obsoletus*, 4–5 *Vitex agnus-castus*, 6 healthy *Vinca minor*, 7 Stolbur-infected *Vinca minor*.

V. agnus-castus Infection with Phytoplasma. V. agnus-castus plants in the field were examined for signs and symptoms of phytoplasma infection. Although no symptoms were observed throughout the entire season, leaf and stem samples were collected from each plant in October and were analyzed by PCR for presence of phytoplasma DNA. Results of these analyses were negative for all 40 *V. agnus-castus* samples and healthy periwinkle, and were positive for phytoplasma-infected periwinkle (Figure 1). Of the *H. obsoletus* collected from these plants, 12.24% of the females (6 of 49 individuals) and 1.92% of the males (2 of 52 individuals) tested positive in 2003, and 17.78% of the females (8 of 45 individuals) and 7.5% of the males (3 of 40 individuals) tested positive in 2004.

DISCUSSION

This is the first study identifying *V. agnus-castus* as the preferred developmental host plant of adult *H. obsoletus* as observed, by vacuum sampling, on *A. retroflexus*, *C. arvensis*, *M. communis*, *O. europaea*, and *V. agnus-castus*. Other than *C. arvensis*, these are native plants that remain green throughout the year. This is consistent with findings of other researchers in Europe (Hoch and Remane, 1986; Maixner et al., 1995; Sforza et al., 1998). In central and eastern Europe, *C. arvensis* is the primary host for the planthopper (Brcak, 1979); however, in Israel aerial portions of this plant are completely dried by May when the first generation of *H. obsoletus* appears, except in irrigated areas where it is usually controlled along with other weeds.

C. arvensis does not appear in the field until after the first rains, usually in November–December, when *H. obsoletus* are usually no longer found (Orenstein et al., 2003). Since the weed and the planthopper are temporally different, it was not surprising that examination of the roots of more than 100 *C. arvensis* yielded no *H. obsoletus* nymphs. Furthermore, based on olfactometer and electroantennagram bioassays with *C. arvensis* and *V. agnus-castus*, the planthopper responded differently to host plant volatiles; response was higher to *V. agnus-castus*. Our results indicate that *V. agnus-castus* is a viable developmental host for the planthopper. When adult *H. obsoletus* were contained in a cage with young potted *V. agnus-castus*, nymphs developed in the same length of time as planthopper development in the field. Differences in the numbers of *H. obsoletus* captured on the shrub in the northern (fewer individuals) vs. central-southern Golan are consistent with previous findings on the distribution of the planthopper in vineyards (Orenstein et al., 2003) and with the distribution of phytoplasma (less incidence in the north) (Orenstein et al., 2001).

Having determined that *V. agnus-castus* is a preferred host plant and supports the complete development of *H. obsoletus*, it was necessary to determine

if the shrub would serve as a reservoir for the pathogen. To date, no signs or symptoms of yellows disease have been observed in *V. agnus-castus* or other wild plants examined in this study. Phytoplasmas can be detected in plants that do not exhibit symptoms of infection, and the part of the plant assayed can be critical to detection (Constable et al., 2003). Asymptomatic plants may be resistant or tolerant to phytoplasma infection. Fourteen percent to 25% of the *H. obsoletus* adults tested by PCR were positive for phytoplasma. Sforza et al. (1998) found between ~28% and 39% of *H. obsoletus* in France were infected with phytoplasma. Since there is a latent period between insect feeding on an infected plant and its capability to transmit the phytoplasma, a positive PCR result does not necessarily mean that the specific insect is capable of transmitting the phytoplasma. However, since these insects were collected in October, at the end of the second peak of *H. obsoletus* activity (Klein et al., 2001; Orenstein et al., 2003), it is reasonable to assume that some were capable of transmitting phytoplasma.

The attraction of *H. obsoletus* to *V. agnus-castus*, as well as the apparent lack of phytoplasma infection in this plant species, suggests that there is potential for using *V. agnus-castus* as a trap plant near vineyards to direct them away from grapevines. Identifying the volatile plant attractants and incorporating them in traps may be effective in protecting crops from inoculation by *H. obsoletus*. We are currently working on identifying the dominant components of the attractant compounds.

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SUGARS IN MEDITERRANEAN FLORAL NECTARS: AN ECOLOGICAL AND EVOLUTIONARY APPROACH

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Abstract—High-pressure liquid chromatography analyses of 73 plant species showed that the nectars of phrygana (East Mediterranean garrigue) mainly contain sucrose, glucose, and fructose, and traces of 10 minor sugars. Although the sucrose/hexose ratio was not related to plant life habit, ecological constraints had a detectable effect in shaping sugar composition. This was detected by distinguishing the phryganic plant species into "spring–summer" and "winter" flowering, with the distinction made on the basis of the water deficit in the study area. Plants flowering in spring–summer had a higher rate of "high sucrose" (i.e., sucrose/hexose ratio $\geq$0.5; 60.8% of the plant species) vs. "low hexose" nectars (i.e., ratio <0.5; 39.2%). The ratio was reversed in winter flowering species (36.4% vs. 63.6% with "high sucrose" and "high hexose," respectively). Sucrose/hexose ratios were associated with plant family. The highest values were those of Lamiaceae, which differed significantly from the "low sucrose" Liliaceae and Apiaceae. Based on recorded plant–pollinator interactions in the community, the present data provide evidence of a partitioning of nectar resources by the existing pollinator guilds within the community, based on the sugar profiles of nectar (all sucrose/hexose ratios for all interactions). Among all major groups, bees and wasps (aculeates) preferred "high sucrose" nectars, which differed significantly from syrphids, anthomyid a.o. flies, and beetles that visited "low sucrose" nectars. Similarly, butterflies visited "lower sucrose" nectars compared to bees. Within families, only Megachilidae could be clearly characterized as "high sucrose" consumers, differing in this respect from all the remaining insect groups including most other bee families. This confirms previous findings that Megachilidae have a key position in Mediterranean

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communities where they probably constitute a selective factor for "high sucrose" nectars.

Key Words—Nectar sugars, sugar ratio, sucrose, glucose, fructose, hexoses, sugar preference, evolutionary constraints, bees, Megachilidae, phrygana, Mediterranean ecosystems, pollination ecology.

INTRODUCTION

Among the major rewards to pollinators, floral nectar is unique, and likely subject to selection pressures that result in nectar differences among closely related plants pollinated by different animals (Pyke and Waser, 1981; Baker and Baker, 1982). The literature on floral nectars is considerable. Amount and sugar concentration of nectars are related to pollinator type (Percival, 1961, 1965; Baker and Baker, 1975), especially the hexose/sucrose ratio (Wykes, 1952; Percival, 1961; Baker and Baker, 1979, 1982, 1983, 1990; Southwick, 1982; Stiles and Freeman, 1993; Petanidou et al., 1996). Amino acids in nectars have also received attention, especially their significance to pollinators (Gottsberger et al., 1984; Baker and Baker, 1986; Petanidou et al., 1996; Petanidou, unpublished data).

Several large-scale studies have focused on the relative concentration of sugar components of nectar. In her pioneering, semiquantitative study on nectar of 889 species, Percival (1961) distinguished sucrose-dominant, balanced sugar, and hexose dominant nectars, which she found related to plant family affinity as well as to pollinator type (Percival, 1965). Baker and Baker (1982, 1990) confirmed Percival's results corroborating the correlation between tubular flowers and sucrose richness, and the tendency of open flowers to be hexose-rich. They further placed the sugar profile into a coevolutionary context, by also considering the plant's pollination mode, referred to as the "pollination syndrome." Based on the nectar analysis of 765 species of different origin, they found that the sugar profile [measured in weight as sucrose/hexose ratio: S/(G + F)] could be associated with the plant's pollination syndrome: plants with sucrose-dominant (>0.999) and sucrose-rich (0.999–0.500) nectars are pollinated by hummingbirds, butterflies, and long-tongued bees, whereas plants with hexose-rich (0.499–0.100) and hexose-dominant (<0.100) nectars are pollinated by short-tongued bees and flies. In another study focusing on a wide range of hummingbird-visited plants in Costa Rica, Stiles and Freeman (1993) found that the sucrose concentration of floral nectars decreased with elevation while fructose concentration increased. All the above results were based on the assumption that a plant is pollinated by a certain insect group, and have not been tested by considering the actual response of the pollinators, i.e., all plant–pollinator interactions observed in the community. However, it is commonly

known that actual pollinators may differ from those suggested by the pollination syndrome, and most plants receive visits of a large array of pollinators.

Little is known of the nectar composition of Mediterranean plants, and certainly not at the community level (Dafni et al., 1988; Petanidou et al., 1996, 2000). Due to water shortage in Mediterranean communities, several aspects of pollination ecology are influenced by climate, such as nectar quantity and concentration (Herrera, 1985; Petanidou and Vokou, 1990; Petanidou and Smets, 1995), flower and nectary structure (Petanidou et al., 2000), pollen calorific value (Petanidou and Vokou, 1990), flowering time (Petanidou and Vokou, 1993; Petanidou et al., 1995), and pollinator assemblages (Petanidou and Ellis, 1993, 1996).

In this work, I investigated the role of nectar sugars in phrygana (East Mediterranean garrigue community). In these communities, pollinator assemblages are not as variable as in other arid, tropical, and temperate systems (no birds, no bats, few bumblebees), with the majority of plants being pollinated by wild bees (Petanidou and Ellis, 1993, 1996; Petanidou and Potts, 2005). As this is the first community study from the area, my specific questions were: (1) What sugars dominate in the Mediterranean nectars? (2) What are the major constraints shaping nectar sugar ratios (phylogenetic, ecological, coevolutionary): Are nectar sugar ratios related to plant family (phylogenetic constraints, as found by Percival, 1961) and pollinator assemblages (coevolutionary constraint, as previously concluded by Percival (1965), Baker and Baker (1983), and Stiles and Freeman (1993)), or do plant flowering period and/or life habit also matter (ecological constraints), as it is normally the case with other pollination characteristics in the Mediterranean?

I carried out the investigation in a typical phrygana, and considered all nectariferous plants occurring in the phryganic community (30 ha) for which nectar collection was practical, with flowering times dispersed throughout the year. As all species were native and typical for the phrygana, this allowed me to explore the effect of ecological constraints. In addition, the large number of plants studied (73) allowed investigation within plant families (phylogenetic constraints). Previous studies within the same community allowed me to use a detailed plant–pollinator database to explore any coevolutionary effects. The latter approach, i.e., matching the nectar sugar composition and the response of pollinator assemblages based on plant–pollinator interactions at a community level, is novel.

METHODS AND MATERIALS

Study Site, Plant Species, and Field Measurements. I studied the nectar of 73 phryganic species occurring in the nature reserve of the “I. and A. Diomedes

Botanical Garden of Athens University" located 10 km west of the center of Athens, Greece. The site and the phryganic community have been described in earlier studies conducted by Petanidou and Ellis (1993, 1996) and Petanidou et al. (1995).

Nectar volume, flower depth, and other measurements used in this study were taken from Petanidou and Smets (1995). Nectar for laboratory analyses was collected from flowers selected at random using the same plants from which nectar volume was measured. All flowers used were at their first day of anthesis, covered in bud stage with bridal veil on the eve of the collection day to prevent nectar removal by insects. Nectar was collected the following day, always towards noon to early afternoon (1100–1400 hr) except for *Capparis spinosa* sampled between 0930 and 1000 hr. The nectar of each flower was picked up directly on a Whatman No. 1 small paper wick and fixed on a stainless steel pin that had been cleaned with acetone. The paper wicks, placed on styrofoam blocks, were left to air-dry. They were stored in airtight containers over silica gel until analysis. Touching with the fingers or other possible contaminating means was carefully avoided (Petanidou et al., 1996).

Nectar Analysis. Sugar analysis was carried out with high-pressure liquid chromatography (HPLC) (Dionex, Sunnyvale CA, USA). Before analysis, the nectar content of each wick was dissolved in 1 ml of distilled water in a microcentrifuge tube by intermittent vortexing at room temperature for at least 1 hr. Tubes were centrifuged to remove paper particles (Petanidou et al., 1996). Analysis was made directly on a CarboPac PA1 anion-exchange column, and quantified by a pulsed amperometric detector. Flow rate was 1 ml min^{-1}. The elution conditions were 100 mM NaOH for 4 min, a linear gradient from 0 to 30 mM Na acetate in 100 mM NaOH over 16 min, a linear gradient of 30–100 mM Na acetate in 100 mM NaOH over 30 min, and finally 300 mM NaOH for 10 min. The column was regenerated with 1 M NaOH for 10 min and equilibrated for 20 min with starting buffer after every run. I investigated the presence of 15 sugars, viz. glucose, fructose, sucrose, sorbitol, mannitol, ribose, melibiose, maltose, stachyose, arabinose, mannose, rhamnose, lactose, trehalose, and gentiobiose, all of which have been reported to occur in floral nectars (cf. review by Baker and Baker, 1983). I did not consider xylose, which is present in specific nectars of the South African species of *Protea* and *Faurea* (Nicolson and Van Wyk, 1998). Sugar quantification was performed on the peak areas by comparison with external standards.

Flower Visitors. In order to detect whether pollinator species had a differential response to the different sugar contents of flowers, I used the community data matrix from Petanidou (1991), which was also used as a basis for Petanidou and Vokou (1993), Petanidou and Ellis (1996), and Petanidou and Potts (2005). This matrix contained plant–pollinator interactions for 70 of the 73 plant species analyzed. All flower visitors were considered pollinators if they

visited the flowers repeatedly irrespective of their "quality" (pollinator efficiency). This is normally done in such community studies (Waser and Ollerton, 2005). Excluded from the analyses were *Echinops sphaerocephalus* subsp. *albidus* and *Teucrium chamaedrys* for which there were no pollinator data, as well as *Romulea linaresii*, which was not visited by insects. The 70 plant species were visited by 576 insect species, resulting in 1930 plant–pollinator interactions.

The families Andrenidae, Halictidae, and Colletidae were considered collectively to comprise of short-tongue bee species, whereas the families Anthophoridae, Apidae, and Megachilidae were composed of long-tongue ones (Petanidou and Ellis, 1996; Michener, 2000).

Data Analysis. Values from all laboratory analyses were calculated per flower. This was the average of several separate runs (3–11, except in a few cases), each one on the basis of one flower where nectar secretion was sufficient (e.g., Lamiaceae). In cases where nectar secretion per flower was low, samples were pooled for HPLC analysis.

I used the same sucrose/hexose ratio as Baker and Baker in all their papers: sucrose/(glucose + fructose), all sugar amounts calculated in weights (Baker and Baker, 1983). Differences among plant families in sucrose/hexose ratios were tested using one-way ANOVAs. Seven plant families were considered in the analysis aiming to explore any phylogenetic affinity of nectar composition. Apart from being the most species-rich in the phryganic community (with ≥ 3 plant species), the plant families considered in the data analysis should not be closely related in terms of their phylogenetic history, the independence control based on the plant evolution tree appearing in Dodd et al. (1999), which is enriched with pollination mode data. The families tested were Boraginaceae, Asteraceae, Lamiaceae, Fabaceae, Liliaceae, Apiaceae, and Ranunculaceae, whereas the remaining families were treated as a separate eighth group. The family Dipsacaceae, represented by three species in the study phrygana, does not appear in the tree by Dodd et al. (1999); thus, it was treated together with the remaining species of the eighth group. Similarly, differences among insect groups in their response to flowers was explored by comparing the nectar sugar ratios of the plant species visited by all species of the insect group, considering all ratio values of all plant–pollinator interactions.

In order to investigate the importance of flowering time in shaping nectar composition, I considered the differential water regime (i.e., the relative evapotranspiration vs. rainfall) throughout the year. According to the data taken from ombrothermic curves of the area (cf. Petanidou and Ellis, 1993), two periods were distinguished: (1) April 1–September 15, when evapotranspiration exceeds rainfall, and (2) September 16–March 31, when rainfall exceeds evapotranspiration. Plant species having their midpoint of flowering in either period were assigned as "spring–summer" or "winter" flowering species.

TABLE 1. PLANT SPECIES STUDIED FOR THEIR NECTAR SUGARS BY HPLC[a]

Date of nectar collection	Plant species	Abbreviation	Life form	Nectar volume (μl flower^{-1})	Flower depth (mm)	Midpoint of flowering (calendar day)
Amaryllidaceae						
21.10.92	*Sternbergia lutea* Orph. ex Nym. subsp. *sicula* (Tin. ex Guss.) D.A. Webb	Sg	geo	1.33	10.4	297
Apiaceae						
16.7.92	*Eryngium campestre* L.	Ey	herb	0.00	1.9	193
7.4.92.	*Scandix australis* L. subsp. *australis*	Sc	ther	0.03	0.0	73
4.6.92	*Thapsia garganica* L.	Tg	herb	0.02	0.0	136
9.4.92	*Tordylium apulum* L.	Ta	ther	0.01	0.0	105
Asteraceae						
13.4.93	*Calendula arvensis* L.	Ca	ther	0.01	3.6	80
14.6.93	*Centaurea orphanidea* Heldr. & Sart. ex Boiss. subsp. *orphanidea*	Co	ther	0.01	11.2	165
25.4.92	*Centaurea raphanina* Sibth. & Sm. subsp. *mixta* (DC.) Runemark	Cr	herb	0.21	21.6	117
3.5.92	*Chrysanthemum coronarium* L.	Cc	ther	0.01	4.3	128
13.7.92	*Echinops microcephalus* Sibth. & Sm.	Ec	herb	0.13	7.4	190
9.8.92	*Echinops sphaerocephalus* L. subsp. *albidus* (Boiss. & Spruner) Kozuharov	Es	herb	0.16	7.4	220
2.6.92	*Helichrysum stoechas* DC. subsp. *barrelieri* (Ten.) (Nyman)	Hs	frut	0.00	4.4	136
26.4.92	*Hypochaeris achyrophorus* L.	Ha	ther	0.01	3.5	114

4.6.92	*Pallenis spinosa* (L.) Cass.		Ps	ther	0.00	2.2	144
1.5.92	*Phagnalon graecum* Boiss. & Heldr.		Pg	frut	0.15	4.7	125
28.4.92	*Reichardia picroides* (L.) Roth		Rp	herb	0.03	8.3	107
1.5.92	*Tragopogon porrifolius* L. subsp. *porrifolius*		Tp	ther	0.01	7.7	110
Boraginaceae							
7.4.92	*Alkanna tinctoria* (L.) Tausch		At	herb	0.34	4.9	95
13.4.93	*Anchusa variegata* (L.) Lehm.		Av	ther	0.45	6.2	62
3.6.92	*Echium creticum* L.		Ea	herb	2.89	6.9	178
12.7.92	*Heliotropium europaeum* L.		He	ther	0.05	2.0	276
13.7.92	*Heliotropium hirsutissimum* Grauer		Hh	ther	0.06	4.6	277
Brassicaceae							
19.4.93	*Eruca vesicaria* Cav. subsp. *sativa* (Mill.) Thell.		Ev	ther	0.13	9.2	113
2.5.92	*Sisymbrium orientale* L.		So	ther	0.01	3.2	119
Campanulaceae							
27.4.92	*Campanula drabifolia* Sibth. & Sm. subsp. *drabifolia*		Cf	ther	0.03	6.0	118
Capparidaceae							
13.6.94	*Capparis spinosa* L. var. *inermis* Turra	Cs		frut	42.05	6.2	188
Caryophylaceae							
25.4.92	*Petrorhagia velutina* (Guss.) P.W. Ball & Heywood	Pv		ther	0.04	16.0	106
9.4.92	*Silene colorata* Poir.	Si		ther	0.06	5.9	95

TABLE 1. CONTINUED

Date of nectar collection	Plant species	Abbreviation	Life form	Nectar volume (μl flower^{-1})	Flower depth (mm)	Midpoint of flowering (calendar day)
Cistaceae						
30.4.92	*Cistus parviflorus* Lam.	Cp	frut	0.05	0.0	125
2.5.92	*Cistus salvifolius* L.	Ci	frut	0.02	0.0	104
Convolvulaceae						
13.7.92	*Convolvulus arvensis* L.	Cv	herb	0.05	2.5	170
8.7.92	*Convolvulus cantabrica* L.	Cn	herb	0.06	2.5	169
Cucurbitaceae						
15.7.92	*Ecballium elaterium* (L.) A. Rich.	Ee	herb	0.03	0.7	214
Dipsacaceae						
5.6.92	*Pterocephalus papposus* (L.) Coult.	Pp	ther	0.03	5.3	123
12.5.93	*Scabiosa atropurpurea* L.	Sa	ther	0.01	4.5	125
27.4.92	*Tremastelma palaestinum* (L.) Janch.	Tm	ther	0.05	5.7	116
Ericaceae						
1.11.93	*Erica verticillata* Forssk.	En	frut	0.00	3.6	363
Euphorbiaceae						
11.4.93	*Euphorbia acanthothamnos* Heldr. & Sart. ex Boiss.	Eu	frut	0.25	0.0	79
Fabaceae						
16.5.93	*Anthyllis hermanniae* L.	Ah	frut	0.01	3.1	132
8.4.92	*Astragalus monspessulanus* L.	Ao	herb	0.28	10.4	102
17.4.93	*Hymenocarpos circinnatus* (L.) Savi	Hc	ther	0.01	1.9	96
29.4.92	*Psoralea bituminosa* L.	Pso	herb	0.24	7.8	135
8.4.92	*Trifolium stellatum* L.	Ts	ther	0.04	8.3	98

Globulariaceae						
11.4.93	*Globularia alypum* L.	Ga	frut	0.01	4.5	77
Iridaceae						
17.11.92	*Crocus cancellatus* Herb.	Ce	geo	0.19	92.0	298
25.2.94	*Romulea linaresii* Parl. subsp. *graeca* Bég.	Ro	geo	0.07	5.7	33
Lamiaceae						
11.6.93	*Ballota acetabulosa* (L.) Benth.	Ba	herb	0.14	8.6	160
26.2.94	*Lamium amplexicaule* L. subsp. *amplexicaule*	La	ther	0.20	14.6	74
15.5.93	*Phlomis fruticosa* L.	Pf	frut	2.52	16.1	108
28.4.92	*Prasium majus* L.	Pm	frut	7.48	9.8	114
14.4.93	*Salvia triloba* L.f.	St	frut	7.74	11.9	94
9.4.92	*Salvia verbenaca* L.	Sb	ther	0.33	7.1	85
16.5.93	*Satureja thymbra* L.	Sj	frut	0.05	7.6	137
2.6.92	*Stachys cretica* L. subsp. *cretica*	Sy	herb	0.59	7.4	137
5.6.92	*Teucrium chamaedrys* L.	Td	frut	0.50	7.9	135
4.6.92	*Teucrium polium* L. subsp. *capitatum* (L.) Arcang.	Te	frut	0.06	4.2	156
8.7.92	*Thymus capitatus* (L.) Hoffmanns. & Link	Tc	frut	0.10	5.4	171
Liliaceae						
30.4.92	*Allium subhirsutum* L.	Ab	geo	0.03	0.0	106
20.10.92	*Asparagus acutifolius* L.	Af	geo	0.02	0.0	268
6.4.92	*Asphodelus aestivus* Brot.	Am	geo	2.44	3.8	85
25.2.94	*Fritillaria graeca* Boiss. & Spruner subsp. *graeca*	Fg	geo	0.06	24.2	85
24.2.94	*Muscari commutatum* Guss.	Mu	geo	0.01	5.5	54
24.2.94	*Muscari neglectum* Guss. ex Ten.	Mn	geo	0.01	4.8	39

TABLE 1. CONTINUED

Date of nectar collection	Plant species	Abbreviation	Life form	Nectar volume (μl flower^{-1})	Flower depth (mm)	Midpoint of flowering (calendar day)
14.4.93	*Ornithogalum exscapum* Ten. *O. graecum* C. Zahariadi	Oc	geo	0.05	0.0	70
21.10.92	*Scilla autumnalis* L.	Su	geo	0.01	0.0	290
6.9.92	*Urginea maritima* (L.) Baker	Um	geo	0.64	0.0	261
Malvaceae						
5.6.92	*Alcea pallida* (Willd.) Waldst. & Kit.	Ap	herb	2.54	0.0	160
Ranunculaceae						
10.7.92	*Delphinium peregrinum* L.	Dp	ther	0.52	16.0	185
5.6.92	*Nigella arvensis* L.	Ng	ther	0.30	3.3	169
13.4.92	*Ranunculus sprunerianus* Boiss.	Ra	geo	0.08	1.4	98
Resedaceae						
27.4.92	*Reseda alba* L.	Re	herb	0.10	0.0	104
Rutaceae						
5.6.92	*Ruta graveolens* L.	Rg	herb	0.32	0.0	145
Thymelaeaceae						
27.2.93	*Thymelaea hirsuta* (L.) Endl.	Th	frut	0.00	2.9	25

[a] Life forms are: geophytes (geo), therophytes or annuals (ther), herbaceous perennials (herb), frutescent or woody perennials (frut). Life form, flower depth, nectar volume, and midpoint of flowering are after Petanidou et al. (1995) and Petanidou and Smets (1995). Nomenclature used is according to The International Plant Names Index (2004).

Before any statistical application, the data were tested for normality, and if not normally distributed, nonparametric tests were applied (Kruskal–Wallis ANOVA, Spearman *R* correlation). When necessary, Kruskall–Wallis ANOVAs were followed by posthoc Mann–Whitney *U* tests and application of the ultra conservative Bonferroni correction (Pagano and Gauvreau, 1993). Whenever used, mean values are followed by their SEs.

RESULTS

Table 1 contains the list of all 73 plant species, with some floral attributes possibly related to sugars contained in the nectars. All 13 sugars used as references in the HPLC analyses were found in the nectars of phrygana flowers, and some additional unknown peaks were also found (Table 2). As expected, the most common sugars were the "big three": glucose, fructose, and sucrose (Baker and Baker, 1983). The remaining 10 contributed little to the sugar profile of phryganic nectars. Among these, sorbitol had the most significant contribution in a few cases with >1% in total nectar sugars, followed by mannose and melibiose.

Based on the % of sucrose contained in the total nectar sugars (in nmol) and the sucrose/hexose ratios (in weight), the phryganic plant families can be distinguished into three groups: the first with "high sucrose" nectars (Ranunculaceae, Lamiaceae, Fabaceae), a second with "low sucrose" nectars (Apiaceae, Liliaceae), and a third, mixed group, encompassing Asteraceae, Boraginaceae, and the remaining families (Table 3). No plant family has a close phylogenetic relationship to any other family within the same or different group (Dodd et al., 1999).

Plant families differed in their sucrose/hexose ratios (K–W $H_{(7,73)} = 35.6$; $P < 0.001$; Table 3). *A posteriori* comparisons showed that these differences were due to the higher ratios of Lamiaceae (M–W *U* tests after applying Bonferroni correction, $P = 0.006$ and $P = < 0.001$, for the pairs Lamiaceae–Liliaceae and Lamiaceae–other families, respectively). The results are the same if the outlier *Anthyllis hermanniae* is not considered.

The values of the sucrose/hexose ratios were related to flower depth (Spearman rank $R = 0.394$, $P < 0.001$; Figure 1) and nectar volume ($R = 0.383$, $P < 0.001$), especially when the outlier *A. hermanniae* was not considered ($R = 0.441$, $P < 0.001$; $R = 0.426$, $P < 0.001$, respectively). (Being an outlier, this species is considered separately or not at all in the statistical analyses.)

The sucrose/hexose ratio was not time-dependent (Spearman *R* correlation against midpoint of flowering, $P = 0.749$). However, when considering "spring–summer" and "winter" species flowering within different water regimes (see "Data Analyses" in METHODS AND MATERIALS), I found

TABLE 2. SUGAR COMPOSITION DATA OF THE NECTARS OF THE PHRYGANIC PLANTS[a]

Plant species	N	Glucose	Fructose	Sucrose	Sorbitol	Mannitol	Ribose	Melibiose	Maltose	Stachyose	Arabinose	Mannose	Lactose	Trehalose	Unknown sugars	% Contribution to the total sugars		S: (G + F) (in weight)
		(nmol flower^{-1})														Minor sugars	Sucrose	
Sg	8 (3)	313 ± 91.4	319 ± 73.7	289 ± 73.3	11 ± 2.4										28 ± 12.3	4.0	30.1	0.87
Ey	12 (1)	113	142	12											8	2.9	4.2	0.09
Sc	9 (4)	148 ± 42.0	121 ± 31.9	52 ± 14.1	1 ± 0.3											0.3	16.1	0.37
Tg	5 (3)	513 ± 72.3	689 ± 84.3	20 ± 6.3												0.0	1.6	0.03
Ta	15 (4)	50 ± 1.5	58 ± 2.8	17 ± 2.6	0.4 ± 0.2							0.3 ± 0.1				0.6	13.3	0.29
Ca	12 (3)	50 ± 21.6	52 ± 19.3	3 ± 0.9												0.0	3.2	0.06
Co	13 (4)	12 ± 2.6	8.1 ± 2.2	110 ± 57.6												0.0	84.7	10.51
Cr	11 (3)	62 ± 5.1	15 ± 3.3	491 ± 100.2												0.0	86.5	12.20
Cc	24 (5)	58 ± 24.4	50 ± 16.8	4 ± 0.9	6 ± 1.2											5.4	3.0	0.06
Ec	8 (5)	443 ± 71.8	1201 ± 100.4	10 ± 2.9				11 ± 3.0								0.7	0.6	0.01
Es	4 (4)	285 ± 50.9	281 ± 44.3	188 ± 59.0											3 ± 0.9	0.3	25.0	0.64
Hs	40 (4)	31 ± 3.8	38 ± 5.9	33 ± 12.5				0.2 ± 0.1								0.2	31.9	0.89
Ha	51 (3)	104 ± 66.4	111 ± 73.5	4 ± 2.5				1 ± 0.6								0.5	1.8	0.03
Ps	18 (2)	4 ± 1.2	2 ± 0.8	1 ± 0.3												0.0	15.0	0.34
Pg	12 (1)	58	63	3												0.0	2.4	0.05
Rp	20 (3)	78 ± 44.4	78 ± 32.4	46 ± 18.9												0.0	22.8	0.56
Tp	14 (3)	168 ± 54.1	185 ± 54.4	40 ± 15.9				1 ± 0.4								0.3	10.2	0.22
At	6 (4)	421 ± 94.8	148 ± 50.3	567 ± 119												0.0	49.9	1.89
Av	13 (5)	216 ± 59.7	130 ± 44.0	385 ± 81.3					0.5 ± 0.1	0.4 ± 0.1	1 ± 0.0					0.3	52.5	2.11
Ea	5 (5)	652 ± 80.3	157 ± 44.8	3684 ± 540.7												0.0	82.0	8.65
He	8 (3)	134 ± 51.8	137 ± 62.8	15 ± 4.3												0.0	5.1	0.10
Hh	8 (3)	146 ± 65.3	153 ± 59.5	188 ± 67.9												0.0	38.6	1.19
Ev	5 (3)	469 ± 82.8	483 ± 88.9	4 ± 1.6												0.0	0.4	0.01
So	5 (3)	283 ± 56.4	315 ± 77.8	5 ± 0.9											8 ± 3.1	1.3	0.8	0.02
Cf	6 (3)	308 ± 73.8	357 ± 35.8	529 ± 84.4												0.0	44.3	1.51
Cs	5 (5)	27972 ± 8486.2	27242 ± 9269.9	15098 ± 4295.8												0.0	21.5	0.52
Pv	8 (2)	376 ± 77.4	435 ± 109.4	4 ± 2.0	2 ± 0.9			5 ± 2.0								0.8	0.5	0.01
Si	4 (4)	91 ± 22.3	85 ± 22.3	3 ± 1.1												0.0	1.4	0.03
Cp	10 (3)	763 ± 50.8	806 ± 22.8	1820 ± 149.9	4 ± 1.0											0.1	53.7	2.20
Cl	10 (3)	1442 ± 100.9	1628 ± 201.8	578 ± 72.3	31 ± 7.8							22 ± 5.3				1.4	15.6	0.36
Cv	6 (3)	325 ± 72.7	368 ± 79.7	204 ± 62.9	22 ± 4.6											2.4	22.2	0.56

Cn	10 (4)	293 ± 63.9	336 ± 50.1	10 ± 3.8	11 ± 3.2									1.7	1.5	0.03
Ee	14 (3)	47 ± 14.6	47 ± 15.2	69 ± 9.3										0.0	42.3	1.39
Pp	14 (2)	151 ± 69.1	155 ± 73.0	128 ± 53.7										0.0	29.5	0.79
Sa	8 (3)	155 ± 50.2	157 ± 49.9	48 ± 13.2										0.0	13.3	0.29
Tm	24 (3)	147 ± 14.3	166 ± 10.6	69 ± 7.0										0.0	18.1	0.42
En	6 (5)	5 ± 0.9	5 ± 0.9	0										0.0	0.0	0.00
Eu	14 (2)	84 ± 7.9	111 ± 16.3	21 ± 4.6		0.3 ± 0.1	4 ± 1.6						6 ± 1.7	4.4	9.2	0.20
Ah	11 (4)	0.3 ± 0.1	0.3 ± 0.1	41 ± 15.0										0.0	98.5	121.75
Ao	13 (6)	265 ± 36.7	25 ± 5.5	898 ± 174.8			2 ± 0.1		1 ± 0.0	0.1 ± 0.0				0.2	75.4	5.88
Hc	13 (2)	30 ± 7.2	34 ± 8.3	35 ± 12.6	0.4 ± 0.2	0.4 ± 0.2								0.7	34.9	1.03
Pso	15 (3)	40 ± 6.8	49 ± 7.3	618 ± 192.8										0.0	87.4	13.21
Ts	3 (3)	78 ± 37.0	89 ± 42.0	78 ± 56.4										0.0	31.8	0.89
Ga	6 (1)	51	35	17										0.0	16.4	0.37
Ce	13 (3)	218 ± 23.2	210 ± 11.1	109 ± 12.4								0.3 ± 0.0		0.1	20.3	0.48
Ro	4 (4)	155 ± 47.1	180 ± 57.4	41 ± 13.1										0.0	11.0	0.23
Ba	10 (10)	47 ± 18.0	11.9 ± 4.7	279 ± 61.8										0.0	82.7	9.05
La	4 (4)	68 ± 11.6	14 ± 2.7	120 ± 23.6										0.0	59.3	2.77
Pf	10 (10)	266 ± 48.6	1258 ± 232.9	2559 ± 518.4										0.0	62.7	3.19
Pm	9 (9)	980 ± 190.9	267 ± 68.3	3653 ± 852.5										0.0	74.6	5.57
St	5 (5)	974 ± 281.2	1407 ± 430.9	4233 ± 1289.6										0.0	64.0	3.38
Sb	7 (7)	244 ± 69.1	109 ± 32.5	399 ± 93.0										0.0	53.1	2.15
Sj	6 (6)	62 ± 27.5	97 ± 35.6	232 ± 72.4										0.0	59.3	2.77
Sy	10 (10)	155 ± 93.5	384 ± 153.4	1509 ± 283.7										0.0	73.7	5.32
Td	3 (3)	559 ± 84.7	692 ± 88.4	2011 ± 101.0										0.0	61.6	3.05
Te	10 (10)	116 ± 42.4	349 ± 76.9	775 ± 86.5										0.0	62.4	3.16
Tc	8 (8)	51 ± 9.0	176 ± 17.7	121 ± 13.7										0.0	34.8	1.01
Ab	10 (3)	122 ± 64.9	125 ± 66.3	53 ± 7.5										0.0	17.6	0.41
Af	55 (6)	93 ± 34.8	127 ± 47.4	1 ± 0.6				0.3 ± 0.1	0.1 ± 0.0					0.2	0.5	0.01
Am	3 (3)	7844 ± 601.3	8035 ± 702.8	5446 ± 507.7										0.0	25.5	0.65
Fg	1	18	175	112										0.0	36.7	1.10
Mu	11 (11)	68 ± 12.7	51 ± 14.9	2 ± 0.8										0.0	1.5	0.03
Mn	11 (11)	116 ± 21.8	134 ± 26.7	2 ± 1.4										0.0	0.8	0.02
Oc	3 (3)	262 ± 36.6	123 ± 10.2	8 ± 3.2										0.0	2.0	0.04
Su	8 (3)	83 ± 29.2	82 ± 31.4	0										0.0	0.0	0.00
Um	4 (4)	1376	1041 ± 179.5	0				11 ± 2.1						0.5	0.0	0.00
Ap	3 (3)	10,456 ± 2002.4	11,351 ± 2025.5	831 ± 88.0										0.0	3.7	0.07

TABLE 2. CONTINUED

Plant species	N	Glucose	Fructose	Sucrose	Sorbitol	Mannitol	Ribose	Melibiose	Maltose	Stachyose	Arabinose	Mannose	Lactose	Trehalose	Unknown sugars	% Contribution to the total sugars		S: (G + F) (in weight)
		(nmol flower^{-1})														Minor sugars	Sucrose	
Dp	10 (3)	13 ± 6.7	76 ± 51.7	798 ± 35.0		4 ± 1.0							2 ± 0.7		5 ± 1.4	1.3	88.8	17.05
Ng	3 (3)	3 ± 1.0	27 ± 7.2	287 ± 55.7	3 ± 1.2	2 ± 0.9										1.4	89.1	17.89
Ra	5 (3)	76 ± 31.7	75 ± 32.4	643 ± 102.9												0.0	80.9	8.06
Re	5 (3)	457 ± 80.3	436 ± 69.2	390 ± 73.4												0.0	30.4	0.83
Rg	8 (3)	1146 ± 301.3	2047 ± 704.2	225 ± 65.6					26 ± 9.3						62 ± 17.9	2.5	6.4	0.13
Th	10 (2)	2 ± 1.3	2 ± 0.8	4 ± 1.5												0.0	47.2	1.70

N is the total number of flowers analyzed by HPLC, the number of runs given in parentheses. Plant abbreviations are given in Table 1.

[a] Data columns are amounts of different nectar sugars (nmol flower^{-1} ± SE); contribution (%) of sucrose and of the minor sugars (=all but the "big three") in the nectar (in nmol); sucrose/hexose ratio (calculated on a weight basis).

TABLE 3. SUMMARY VALUES OF THE PLANT FAMILIES IN THE COMMUNITY[a]

	N	% Sucrose over total sugars (nmol)	S/(G + F) (in weight)
High sucrose families			
Lamiaceae	11	62.5 ± 3.76	3.8 ± 0.65
Ranunculaceae	3	86.3 ± 2.68	14.3 ± 3.15
Fabaceae	5	65.6 ± 13.67	28.6 ± 23.41
Families with mixed floral nectars			
Asteraceae	12	23.9 ± 8.84	2.1 ± 1.25
Boraginaceae	5	45.6 ± 12.41	2.8 ± 1.51
Other	21	18.0 ± 3.78	0.6 ± 0.14
Dipsacaceae	3	20.3 ± 4.79	0.5 ± 0.15
Low sucrose families			
Apiaceae	4	8.8 ± 3.49	0.2 ± 0.08
Liliaceae	9	9.4 ± 4.60	0.3 ± 0.13

[a] Data columns are: number of plant species per family (N); % sucrose content calculated on the basis of nmoles contained in the nectar; and sucrose/hexose ratio (mean±SE) calculated on the basis of sugar (S, G, F) weights.

significant differences between these two groups in both sucrose/hexose ratio (M–W $U_{(51,22)} = 366.0$; $P = 0.019$) and sucrose content (M–W $U_{(51,22)} = 367.0$; $P = 0.017$). Among the "spring–summer" species, 60.8% have "high sucrose" nectars vs. 39.2% with "high hexose" nectars (Tables 1 and 2). The picture is

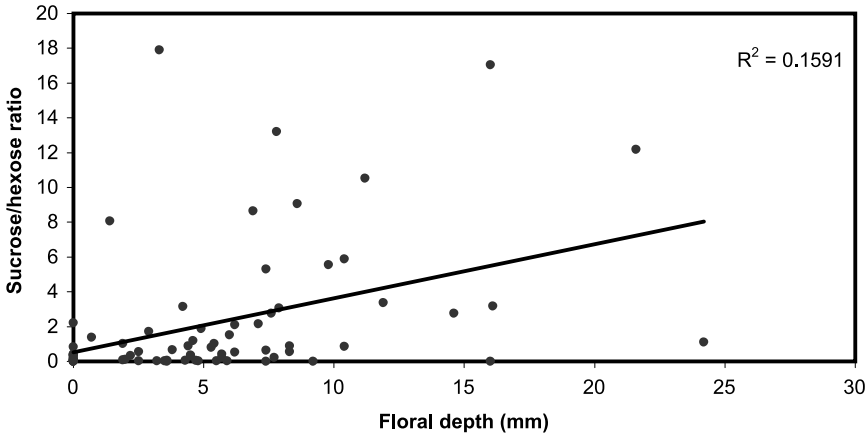

FIG. 1. Sucrose/hexose ratio represented against floral depth of the plant species studied. Best-fitted line and R^2 value are displayed on the chart. *Anthyllis hermanniae* (ratio outlier) and *Crocus cancellatus* (depth outlier) were excluded.

reversed during the wet period of the year, with 36.4% of the "winter" plants having "high sucrose" and 63.6% having "high hexose" nectars. There was no difference in sucrose/hexose ratio among plants of different life habit (K–W $H_{(3,72)} = 6.454$; $P = 0.092$).

The response of pollinator groups to nectar sucrose/hexose ratios is summarized in Tables 4 and 5. The tables give the average value of sugar ratios calculated over the species of plants visited by all insect species on the basis of all interactions observed between them in the community. Among all major insect groups, bees and wasps (aculeates) show the highest preference for high-sucrose nectars. Lepidoptera, a rather heterogeneous group, scored between bees (high) and flies (low). Among bee families, the greatest preference for high-sucrose nectar was shown by the Megachilidae, then by the Anthophoridae, followed by a third group encompassing Halictidae, Colletidae, and Apidae, and finally the Andrenidae family, which has the lowest preference. There was a significant difference in the preference for sugar profiles of nectar among the major insect groups at the level of superfamilies–orders (Table 4; ANOVA, outlier *Anthyllis* excl.: $F_{(6,1917)} = 15.63$; $P < 0.001$). The difference was equally high when major groups are broken down into families of high relevance to pollination (Table 5; $F_{(16,1907)} = 10.64$; $P < 0.001$). *A posteriori* tests (Tukey HSD test) showed that the difference between superfamilies–orders were due to bees (Apoidea–Coleoptera: $P < 0.001$; Apoidea–Diptera: $P < 0.001$; Apoidea–Lepidoptera: $P = 0.048$; Apoidea–Syrphidae: $P < 0.001$) and to aculeates (Aculeata–Coleoptera: $P = 0.001$; Aculeata–Diptera: $P < 0.001$; Aculeata–

TABLE 4. SUCROSE/HEXOSE RATIOS OF THE FLORAL NECTARS VISITED BY THE MAJOR INSECT POLLINATOR GROUPS IN PHRYGANA[a]

	Number of			Sucrose/hexose ratio: S/(G + F)	
Pollinator group	Pollinator species	Plant species visited	p–p interactions	Average value on the basis of p–p interactions	SE
Aculeata	49	29	112	2.85	0.532
Apoidea	224	69 (70)	859 (861)	2.58 (2.86)	0.133 (0.236)
Coleoptera	60	46 (47)	248 (249)	1.28 (1.77)	0.178 (0.515)
Diptera (-Syrphidae)	119	53	288	1.06	0.130
Lepidoptera	30	41 (42)	153 (156)	1.69 (4.00)	0.233 (1.344)
Other	48	27	83	1.43	0.395
Syrphidae	46	39	181	0.66	0.108
All community	576	96 (97)	1924 (1930)	1.90 (2.27)	0.080 (0.172)

Numbers in parentheses are with the outlier *Anthyllis* wherever this is visited.

[a] The preference is given as average S/(G + F) ratio calculated over all interactions of an insect group with the plants visited within the community.

TABLE 5. SUCROSE/HEXOSE RATIOS OF THE FLORAL NECTARS VISITED BY THE POLLINATOR GROUPS BROKEN DOWN TO FAMILIES IMPORTANT FOR POLLINATION IN PHRYGANA[a]

Pollinator group	Number of			Sucrose/hexose ratio: S/(G + F)	
	Pollinator species	Plant species visited	p–p interactions	Average value on the basis of p–p interactions	SE
Aculeata	50	30	113	2.82	0.528
Andrenidae	38	33	94	1.02	0.239
Anthophoridae	55	46	232	2.42	0.214
Apidae	3	60 (61)	73 (74)	1.88 (3.50)	0.370 (1.661)
Colletidae	11	15	30	2.05	0.846
Halictidae	43	49	187	2.19	0.286
Megachilidae	73	51 (52)	242 (243)	3.94 (4.42)	0.297 (0.568)
Symphyta	7	7	10	1.01	0.787
Other Hymenoptera	16	13	28	1.22	0.638
Bombyliidae	39	40	116	1.68	0.285
Anthomyid flies	55	26	118	0.56	0.085
Syrphidae	46	39	181	0.66	0.108
Other Diptera	25	26	54	0.81	0.211
Coleoptera	60	46 (47)	248 (249)	1.28 (1.77)	0.178 (0.515)
Lepidoptera	30	41 (42)	153 (156)	1.69 (4.00)	0.233 (1.344)
Hemiptera	23	22	43	1.70	0.617
Neuroptera	2	2	2	0.52	0.490
All community	576	96 (97)	1924 (1930)	1.90 (2.27)	0.080 (0.172)

Numbers in parentheses are with the outlier *Anthyllis* wherever this is visited.

[a] The preference is given as average S/(G + F) ratio calculated over all plant–pollinator interactions within the community. The group "anthomyid flies" encompasses the families Anthomyidae, Muscidae, Calliphoridae, Rhinophoridae, Sarcophagidae, Scatophagidae, and Tachinidae.

Syrphidae: $P < 0.001$). Similarly, the Tukey *a posteriori* HSD test showed that Megachilidae was the only insect family that was distinguished by its preference for high sucrose/hexose ratios, contrasting to Syrphidae and "anthomyid flies" preferring low sugar ratios (Tables 5 and 6).

DISCUSSION

The analysis of the phryganic nectars produced no surprises: they contained the most common sugars known for nectars: sucrose, glucose, and fructose (Wykes, 1952; Percival, 1961; Baker and Baker, 1983). Among the remaining minor sugars, none was consistently found within a group or a family of plants, such as

TABLE 6. SUMMARY RESULTS OF THE POLLINATOR RESPONSES TO NECTAR SUCROSE/HEXOSE RATIOS IN PHRYGANA[a]

	P			
	Aculeata	Anthophoridae	Halictidae	Megachilidae
Andrenidae	0.015			< 0.001
Anthophoridae				< 0.001
Apidae				< 0.001
Halictidae				< 0.001
Other Hymenoptera				< 0.006
Bombyliidae				< 0.001
Anthomyid flies	< 0.001	< 0.001	0.004	< 0.001
Syrphidae	< 0.001	< 0.001	0.002	< 0.001
Other Diptera	0.032			< 0.001
Coleoptera	0.007	0.024		< 0.001
Lepidoptera				< 0.001
Hemiptera				< 0.007

[a] *P* (Tukey HSD posthoc test) shows the difference between the sugar ratios of the nectars preferred by either insect groups. *P* of empty cells or not appearing pairs was NS. The outlier *Anthyllis* was not considered in the calculations.

xylose was in the nectars of the South African *Protea* and *Faurea* (Nicolson and Van Wyk, 1998). Sugar profiles of the phryganic species are commonly found in nature, with a sugar composition similar to that of most other plants.

When focusing on the two plant groups distinguished by their sucrose content and sucrose/hexose ratios (Table 3), the number of species in "high sucrose" (sucrose-dominant to sucrose-rich, according to the terminology by Baker and Baker, 1983) families exceed those of "low sucrose" (hexose-dominant to hexose-rich) families (59% vs. 41%). Although "high sucrose" species make up only 53.5% of the plant species in the community, it is interesting that most of the species flowering in spring–summer had "high sucrose" vs. "high hexose" nectars (60.8% vs. 39.2%, respectively), whereas the opposite holds for winter (36.4% vs. 63.6% for "high sucrose" and "high hexose," respectively). These differences suggest that under the hot and dry Mediterranean conditions "high sucrose" nectars may be selected against "high hexose" ones. Three explanations seem likely. (1) Hexoses, mostly products of postsecretory phenomena of sucrose hydrolysis, may result in osmotic uptake of water throughout anthesis in order to decrease nectar concentration (% w/w) (Pate et al., 1985; Nicolson, 2002). Therefore, nectars rich in hexoses need more water than nectars rich in sucrose for the same amount (weight) of sugars contained (Nicolson, 1998). This may result in water loss for nectars rich in hexoses, and although nectar volumes in the Mediterranean are generally small, the total mount of water loss per plant bearing hundreds of ephemeral, and

generally open, flowers may be appreciable. Under the extreme water limitations characterizing Mediterranean systems, hexose-rich nectar could be an inappropriate solution. (2) From the calorific point of view, nectar with a high sucrose ratio utilizes less water for the same carbohydrate bait offered to pollinators as reward, therefore contributing to water economy in the system (Nicolson, 2002). What is important for honeybees is calorific value of the reward, not the type of sugars (mono-, disaccharides) in nectars of equal calorific value (Wells et al., 1992). (3) The prevalence of "high hexose" nectars in the "winter" flowering species may be related to adaptation of the insect diet to multiple sugar types. Among the nectar sugars dealt with, only sucrose needs digestion (hydrolysis), whereas monosaccharides and water are rapidly absorbed across the midgut (Nicolson, 1998). It may not be by mere coincidence that insects such as syrphids, anthomyid a.o. flies, and beetles find monosaccharide uptake easier compared to sucrose as a quick drink or as a normal meal (Table 6). Hence, adaptation to easy-to-digest monosaccharides may constitute a differential advantage of hexose-nectars for attracting an extensive array of pollinators, which to a large extent are nonspecialized and most of which are active in wintertime.

Deep flowers have been associated mostly with pollination by specialized pollinators and protection from nectar thieves, enabling the preservation of nectar *quantity* (Baker and Baker, 1983, 1990). What has been underestimated so far is the protection of nectar *quality*, which can result by either open contact with air (evaporation, oxidation) or with a continuous contact with many non-legitimate yeast- or bacteria-bringing insects (fermentation). I argue that deep flowers are the most convenient places for nectars to be preserved. Unless protected, nectar tends to equilibrate with ambient humidity, its concentration being determined by both chemical effects and microclimatic gradients (Corbet et al., 1979; Nicolson, 1998, 2002). On the other hand, deep and closed flowers are efficient in protecting the nectar so that unwanted insects have limited access. In this respect, numerous hairs and stamens are as important as long corollas in restricting air movement and excluding insects, such as *Cistus parviflorus*, *C. salvifolius*, and *Capparis spinosa*, all key species in phrygana, comparable to the South African Proteaceae (Petanidou and Ellis, 1996; Nicolson, 2002). The presence of honey leaves or honey pockets (i.e., petal scales where nectar is accumulated) in bowl-shaped flowers in some phrygana species, probably plays a similar nectar-protective role (e.g., *Nigella arvensis*, *Ranunculus sprunerianus*).

As shown, "high sucrose" nectars prevail in deep flowers vs. "high hexose" nectars that are frequent in open flowers. Thus, plants bearing deep flowers with "high sucrose" nectars are most successful during the difficult period of the year, i.e., between April and mid-September when evapotranspiration exceeds rainfall (Petanidou and Ellis, 1993), allowing open or bowl-shaped "high hexose" flowers to thrive in fall through winter. Yet, this seasonal shift in

flowering time may constitute a potential trade-off for many, albeit unspecialized and illegitimate flower visitors (Petanidou et al., 1995).

Phylogenetic Constraints. That sucrose/hexose ratios were associated with plant family membership in the phrygana community studied was not a surprising result. This confirms previous conclusions by Percival (1961), Baker and Baker (1983), and Stiles and Freeman (1993) that the primary constraints responsible for shaping sugar profiles in the floral nectars of plants are phylogenetic. This is interesting, bearing in mind that Mediterranean communities may differ greatly from other continental communities in this respect. For instance, phylogenetic constraints were not found to play a decisive role in determining plant flowering time in phrygana, as seen elsewhere (Kochmer and Handel, 1986; Petanidou et al., 1995).

Lamiaceae, a key family of the phrygana, is also the top sucrose rewarding plant family in this community (Petanidou and Vokou, 1993; Petanidou, 1996; Petanidou and Ellis, 1996; Petanidou et al., 2000). Interestingly, in all earlier studies, Lamiaceae have been pinpointed for their high rate of sucrose-dominant to sucrose-rich nectars (Percival, 1961; Baker and Baker, 1983), although only in phrygana do all Lamiaceae species have "high sucrose" nectars. This high rate can be explained as an effect of other overwhelming constraints in the Mediterranean, such as climate or a diverse bee fauna (Michener, 1979). With high values in sucrose content, Ranunculaceae follows the Lamiaceae, probably due to its small plant number (3) and the ultra conservative *posthoc* test applied. These findings agree with Baker and Baker (1983), who found sucrose-dominant or sucrose-rich nectars in Ranunculaceae, but not with Percival (1961). Finally, Fabaceae, with very high sucrose values (Table 3) occupies an inferior position, also probably due to the heterogeneity within this group, as confirmed by Percival (1961) and Stiles and Freeman (1993). Apiaceae and Liliaceae, the "low sucrose" families of the community, lie on the opposite side of the scale as found by Percival (1961) and Baker and Baker (1983). It should be noted that all the above families are reasonably phylogenetically independent, being placed far apart in the evolutionary tree (Dodd et al., 1999), which makes convergence/divergence in their sugar ratios meaningful. The results allow for the conclusion that phryganic nectars are, to a large extent, shaped by phylogenetic constraints as found in earlier studies.

Ecological Constraints. That nectar composition (as sucrose/hexose ratio and % sucrose content) is not associated with the plant life cycle, a character resulting from complex ecological factors, is surprising. This, together with the finding that the sugar ratio does not depend on flowering time, may lead one to the conclusion that ecological constraints do not appear to have a detectable effect in shaping nectar composition in the Mediterranean communities. However, when the period of actual water deficit in the system (April–mid-September) is considered, it emerges that plants flowering in the dry period do

differentiate significantly in nectar composition from those flowering in the wet period. This result is notable because it underlines the importance of time, as an expression of water availability within the system, in effectively shaping many pollination-related attributes found in other studies, such as flowering (Petanidou et al., 1995), corolla size of flowers, as well as some nectar and nectary attributes (Petanidou et al., 2000). This study confirms that time constitutes a critical parameter in the Mediterranean, because of the overriding effect of the summer drought that characterizes these communities.

Coevolutionary Constraints. The results also provide evidence of a significant partitioning of nectar resources by the existing pollinator guilds within the community, based on the sugar profiles of the nectars. This confirms Baker and Baker (1983, 1990), but their results were derived from "pollination syndromes" and predominant pollinators alone. My results, in contrast, are based on all plant–pollinator interactions observed in the entire community throughout the year, and they consider not only predominance, but also statistical variation.

The highest preference for nectars of high sucrose content in the phrygana is shown by wasps (aculeates) and bees. The differential preference of bees for visiting flowers with high-sucrose nectars is not only in contrast to beetles, hoverflies, and other flies (as is also the case for wasps), but also includes butterflies, a heterogeneous group (Tables 4 and 5). In this respect, my conclusions do not support those of Baker and Baker (1983) that butterflies prefer sucrose-dominant to sucrose-rich nectars as long-tongue bees do. On the other hand, my data show that bees are a heterogeneous group too, with nectar preferences varying from low- and medium-sucrose (e.g., Andrenidae; Apidae, Halictidae, and Anthophoridae) to high sucrose (Megachilidae; cf. Table 6). The tendency of Megachilidae to exploit such high-sucrose nectars is certainly related to their long-tongue morphology allowing them to obtain nectar from deep flowers. Like bees, wasps are the only group in the community showing a differential preference (vs. beetles, hoverflies a.o. flies, as well as Andrenidae) to visit flowers with "high sucrose" nectars (Tables 4–6). This finding shows that wasps are important as reward consumers and probably as pollinators in these semiarid environments.

Megachilidae are the only group showing a high preference for sucrose-nectars. This family is diverse within the Mediterranean Basin (Michener, 1979), by far the most species-rich in phrygana [32% of the bees and 13% of the anthophilous insect fauna according to Petanidou and Ellis (1993)], and a key family in Mediterranean communities (Petanidou and Ellis, 1996; Petanidou et al., unpublished data). Although little is known of the nectar sugar preferences of solitary bees, experiments on social bees and other animals have shown significant preference differences: honeybees showed no preference for either sugar type of equal calorific value (1 M sucrose vs. 2 M monosaccharides; Wells et al., 1992), but *Melipona beecheii* and *M. fasciata* preferred sucrose to

glucose and fructose (Biesmeijer et al., 1999). Similarly, the peacock butterfly, *Inachis io*, strongly prefered sucrose over fructose, especially over glucose (Rusterholz and Erhardt, 1997). Bearing in mind that sucrose-nectars are advantageous to plant–pollinator relationships in phrygana (see above), I argue that the high rate of sucrose-nectars in the phryganic communities may constitute an ecophysiological response to water constraints, and could be the main driver for floral preferences by their pollinator mutualists. By being the most numerous and representative group in phrygana, the long-tongue Megachilidae can respond to the conditions set above (sucrose-nectars in deep flowers), hence they probably represent the main selecting pollinator group for "high sucrose" nectars in the Mediterranean region.

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NEST PAPER ABSORBENCY, TOUGHNESS, AND PROTEIN CONCENTRATION OF A NATIVE VS. AN INVASIVE SOCIAL WASP

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Abstract—The amount of proteinaceous food that was allocated to nest construction by a native wasp (*Polistes fuscatus*) vs. an invasive wasp (*Polistes dominulus*) in North America was examined following a field experiment under *natural* and *surplus prey* foraging conditions. Wasps of the *surplus prey* foraging conditions were provided with prey *ad libitum* within an enclosed area, while wasps of the *natural* treatment foraged in an adjacent field-woodland site. At the end of the field experiment, each nest was tested for water absorbency, toughness, and protein concentration. The hypotheses were: (1) When all nests are equally sheltered, the invasive *P. dominulus* (PD) allocates less protein to nest paper construction (for waterproofing and strengthening) and more protein to developing larvae than the native *P. fuscatus* (PF). (2) Nests of *P. dominulus* are more absorbent (less waterproof) and less tough than nests of *P. fuscatus*. Results indicate that *P. fuscatus* nests from surplus prey foraging conditions were more absorbent (less waterproof) to artificial rain drops than *P. dominulus* nests. The toughness of nests was similar between wasp species. However, nests from the natural treatment were tougher than those from the surplus prey treatment. Nests from the natural foraging conditions had half as much protein as those from surplus prey foraging conditions. There was no correlation between nest protein concentration and the number of prey taken, the number of cells, the number of adult offspring produced, or the total wasp biomass produced per colony. For PF under surplus prey conditions, protein concentration and absorbency were negatively correlated, but for PD the correlation was positive. In conclusion, when prey were scarce, *Polistes* wasps allocated less protein to nest construction. Also, the introduced *P. dominulus* may increase production of

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offspring by allocating less to nest construction than that of the native *P. fuscatus*, and so more protein to offspring production.

Key Words—Introduced species, proteinaceous food, nest construction, *Polistes fuscatus*, *Polistes dominulus*.

INTRODUCTION

In the construction and maintenance of a nest, many social wasps use an oral secretion to cement together nest material, e.g., plant fibers (McGovern et al., 1988; Espelie and Himmelsbach, 1990; Singer et al., 1992). Nest paper and the nest pedicel are primarily composed of cellulose, derived from plant material and nitrogen most likely derived from the oral secretions of the wasps (Espelie and Himmelsbach, 1990). The protein secreted by wasps may give added strength to the nest, and so the nest pedicel tends to have a higher proportion of protein than the nest paper (Singer et al., 1992). Specifically, because proline is a dominant amino acid in structural proteins, the high proline content of the pedicel protein may help to provide the structural strength required in that part of the nest (Espelie and Himmelsbach, 1990; Singer et al., 1992; Kudo et al., 2000).

The production of a proteinaceous oral secretion for nest construction may be costly, especially for a single foundress, because the foundress has multiple uses for protein: the production of oral secretion, nourishment of her brood, and her own metabolism (Kudo et al., 1998). Kudo et al. (1998) found that 10–20% of total protein resources brought in during the nest founding phase may be devoted to the production of oral secretion. This diversion of protein may prolong the larval period and, thus, the time of emergence of the first workers. Furthermore, the amount of protein allocated to nest construction may depend on environmental conditions. For example, the amount of oral secretion added to paper wasp nest material is positively correlated with exposure to rainfall (Yamane and Itô, 1994; Kudo et al., 1998; Kudo, 2000), and suggests that, in addition to nest strengthening, protein is also a mechanism for nest waterproofing. Nest paper is water-resistant (McGovern et al., 1988). In addition, foundresses that nested at exposed sites produced significantly smaller and fewer immatures than those nesting at sheltered sites (Kudo, 2000), which may reflect a tradeoff between the need for greater waterproofing and the rate of offspring production. Another cost to foraging is that the process requires extensive foraging for prey tissue (Kudo et al., 1998). Specifically, foraging by a lone foundress is risky, for herself and for her unprotected brood (Gibo, 1974, 1978).

A European paper wasp (*Polistes dominulus*) was accidentally introduced to the United States about 20 yr ago (Hathaway, 1981). Since its introduction,

P. dominulus has spread throughout most of the Northeast, seemingly at the expense of the native species, *Polistes fuscatus* (Judd and Carpenter, 1996) and recently has been found on the west coast (Cervo et al., 2000; Pickett, personal communication). Studies in North America have determined that *P. dominulus* has a higher productivity compared to native *Polistes*, including *P. fuscatus* (Pickett and Wenzel, 2000; Armstrong and Stamp, 2003; Gamboa et al., 2004). This higher productivity reflects in part earlier emergence of the first brood.

But other factors may contribute to the differential productivity and, thus, to the success of *P. dominulus* as an introduced species (Cervo et al., 2000; Pickett and Wenzel, 2000). For example, it has been suggested that *P. dominulus* may invest less to waterproof their nests because of this species' long coexistence with human structures in Europe. Specifically, *P. dominulus* has had the opportunity to adapt to nesting in protected human structures for centuries longer than have North American *Polistes* (Pickett and Wenzel, 2000). Correspondingly, *P. dominulus* may allocate less proteinaceous material to nest construction and more to their brood, thereby contributing to increased productivity.

The goal of this study was to compare the absorbency, toughness, and protein concentration of nests of *P. dominulus* (invasive species) and *P. fuscatus* (native species) when wasps had either surplus prey foraging conditions (provided food *ad libitum*) or natural foraging conditions (foraged for themselves in a natural area). We also determined how nest protein concentration corresponded to the number of prey taken, the number of cells in nests, the number of offspring produced, and the total biomass produced per colony.

METHODS AND MATERIALS

Experimental Design. Nests were collected following a 2×2 factorial experiment that was conducted using two levels for wasp species, *P. fuscatus* (PF) and *P. dominulus* (PD), and two levels of foraging conditions, designated *surplus prey* and *natural*. In the field experiment, each nest was placed within a screen cage (dimensions: $1.8 \times 1.8 \times 1.8$ m). The *surplus prey* treatment was supplied with prey (waxworms) *ad libitum*, and foraging was limited to within the cages. Waxworms (*Galleria mellonella*) were obtained from Carolina Biological Supply (Burlington, NC, USA) and reared on an artificial diet. Wasps of the natural treatment, in which cage doors were tied open, had to forage for prey within the adjacent field-woodland area. Within each cage in both foraging treatments, nests were housed in wooden boxes, and the cage frames also provided a supply of wood for making nests. For further details, see

Armstrong and Stamp (2003). Twenty-six nests (8 PD—optimal, 6 PD—natural, 8 PF—optimal, 4 PF—natural) were tested for absorbency, toughness, and protein concentration.

Absorbency. All nests were dried at 50°C to a constant weight and then weighed. Then 10 ml of water were dispensed one drop at a time (to represent rain drops) on the top of each nest (evenly around the pedicel). The nest was suspended so that it was not sitting in the water that had run off the top. After 30 sec, the nest was reweighed. Absorbency was calculated as the amount of mass gained by each nest following the artificial rain divided by the initial mass. Three trials were performed for each nest and the mean determined. Nests were redried between each trial. As nest size varied, the absorbency for each nest was divided by the mass of that nest, and the proportion was multiplied by 100 to obtain percent value. Thus, the *percent absorbency* was used for statistical analysis.

Toughness. Nest toughness was examined using a penetrameter (Sands and Brancatini, 1991). The procedure consisted of determining the grams of sand added to a container attached to a pin that caused the pin to penetrate the nest paper. The procedure was repeated at five equal-spaced locations on each nest, all approximately 1 cm from the pedicel, and mean was taken.

Protein Concentration. A protein microassay (Jones et al., 1989; Bio-Rad, Richmond, CA) was used to measure the concentration of protein for each nest. To avoid protein contamination from wasp silk, exuviae, and/or meconia in the nest protein analysis, nest material was taken from the midsection of the outermost whole cells that had never been used to hold larvae or pupae.

For each nest, 5 mg of nest material were homogenized with 0.1 N NaOH. Then 64 μl of each sample solution were diluted with 96 μl of nanopure water, and 40 μl of dye reagent (Coomassie Brilliant Blue G-250) were added. The absorbency was measured at 595 nm with a Biolinx program (Dynatech Laboratories Inc., version 2.20) on an automated spectrophotometer (MRX, Dynatech Laboratories Inc., Chantilly, VA, USA). The standard was Bovine gamma globulin.

Data Analysis. Two-way ANOVAs were performed using SYSTAT® (version 10) on absorbency, toughness, and protein concentration. The assumptions of ANOVA were met. Tukey tests were used to distinguish between species-foraging condition combinations. In addition, regression analyses were performed on protein concentration vs. nest absorbency, the total number of prey taken (for the surplus prey foraging colonies), the total number of cells, the total number of offspring produced, and the total biomass produced per colony. Data for prey, cells, offspring, and biomass are taken from Armstrong and Stamp (2003). The regression for protein concentration and total number of prey taken was only for the surplus prey foraging condition because under the natural condition we could not determine the number of prey collected.

RESULTS

Absorbency and Toughness. Although there was no effect of species or foraging conditions on nest absorbency (species: $df = 1, 22$, $F = 1.08$, $P = 0.31$; conditions: $df = 1, 22$, $F = 0.61$, $P = 0.44$), *P. fuscatus* (PF) nests from surplus prey foraging conditions were more absorbent (less waterproof) than *P. dominulus* (PD) nests from the same treatment (Tukey test, $P < 0.05$) (Figure 1). Nests from natural foraging conditions were tougher than nests from surplus prey foraging conditions ($df = 1, 22$, $F = 10.42$, $P = 0.004$) (Figure 2). However, there was no effect of wasp species on nest toughness ($df = 1, 22$, $F = 0.51$, $P = 0.48$) (Figure 2).

Protein Concentration. Overall, nests built under surplus prey foraging conditions had a greater concentration of protein in the nest material, about twice that of nests built under natural foraging conditions ($df = 1, 20$, $F = 42.27$, $P < 0.001$). There was no effect of wasp species on the amount of protein allocated to nest construction ($df = 1, 20$, $F = 1.12$, $P = 0.30$). However, PF and PD differed in the amount of protein allocated to nest construction in natural foraging conditions, such that PF nests of that treatment had a nest protein concentration twice that of PD nests (Tukey test, $P < 0.05$) (Figure 3).

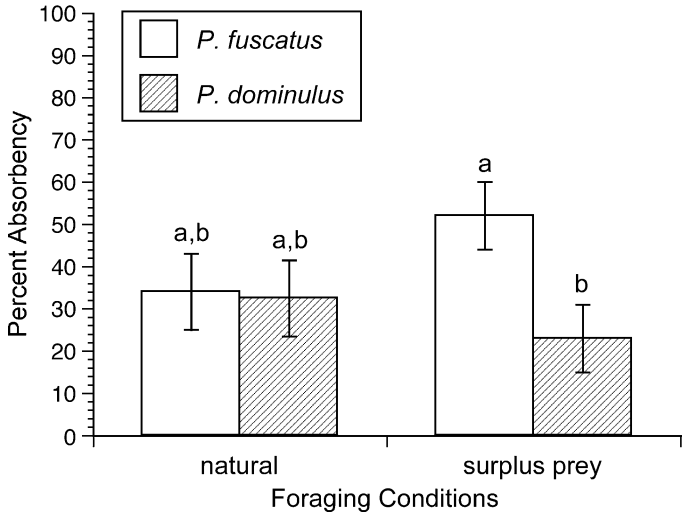

FIG. 1. The percent absorbency of nests for *P. fuscatus* and *P. dominulus* in natural *versus* surplus prey foraging conditions. Means ± SE are shown.

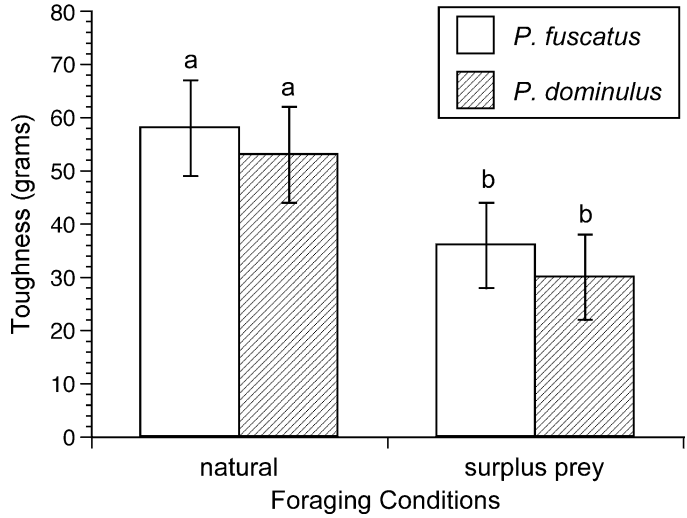

FIG. 2. The toughness of nests of *P. dominulus* and *P. fuscatus* in natural *versus* surplus prey foraging conditions. Means ± SE are shown.

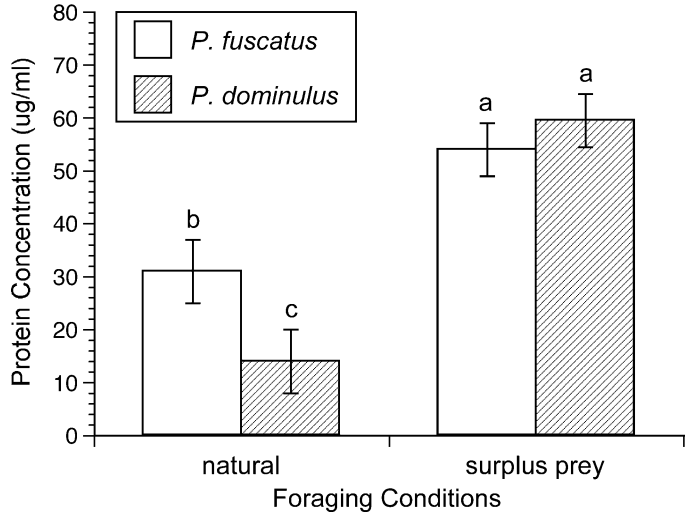

FIG. 3. The concentration of protein in nests of *P. fuscatus* and *P. dominulus* in natural *versus* surplus prey foraging conditions. Means ± SE are shown.

For PF colonies of the surplus prey treatment, there was a *negative* correlation between protein concentration vs. absorbency, such that as protein concentration increased, absorbency decreased (Table 1, Figure 4). In contrast, for PD colonies of the surplus prey treatment, there was a *positive* correlation between protein concentration vs. absorbency, such that as protein concentration increased, absorbency also increased (Table 1, Figure 4). For both PF and PD of the natural treatment, there was no correlation between nest protein concentration and nest absorbency (Table 1).

There was no correlation for either species in either treatment for protein concentration vs. the number of cells per nest, the total number of adult offspring produced per nest, the total biomass produced per nest, or the total number of prey taken (surplus prey colonies only) per nest (Table 1).

TABLE 1. REGRESSION ANALYSES FOR NEST PROTEIN CONCENTRATION VS. ABSORBENCY OF NEST MATERIAL, THE TOTAL NUMBER OF PREY TAKEN (SURPLUS PREY COLONIES ONLY), THE TOTAL NUMBER OF CELLS, THE TOTAL NUMBER OF ADULT OFFSPRING PRODUCED, AND THE TOTAL BIOMASS PRODUCED PER COLONY

	df	*F*	*P*	r^2
Protein vs. absorbency				
PF, natural	1,2	1.49	0.34	0.43
PD, natural	1,4	0.16	0.71	0.04
PF, surplus prey	1,4	12.61	0.02*	0.76
PD, surplus prey	1,6	10.93	0.02*	0.65
Protein vs. number of prey taken				
PF, surplus prey	1,4	1.17	0.34	0.23
PD, surplus prey	1,6	1.09	0.33	0.15
Protein vs. total number of cells				
PF, natural	1,2	0.31	0.63	0.14
PD, natural	1,4	0.63	0.47	0.14
PF, surplus prey	1,4	0.89	0.40	0.18
PD, surplus prey	1,6	0.39	0.55	0.06
Protein vs. total number of offspring				
PF, natural	1,2	0.56	0.53	0.22
PD, natural	1,4	0.35	0.58	0.08
PF, surplus prey	1,4	2.33	0.20	0.37
PD, surplus prey	1,6	1.50	0.27	0.20
Protein vs. total biomass produced				
PF, natural	1,2	0.63	0.51	0.24
PD, natural	1,4	0.19	0.68	0.05
PF, surplus prey	1,4	2.58	0.18	0.39
PD, surplus prey	1,6	1.20	0.31	0.17

*Statistical significance, $\alpha = 0.05$.

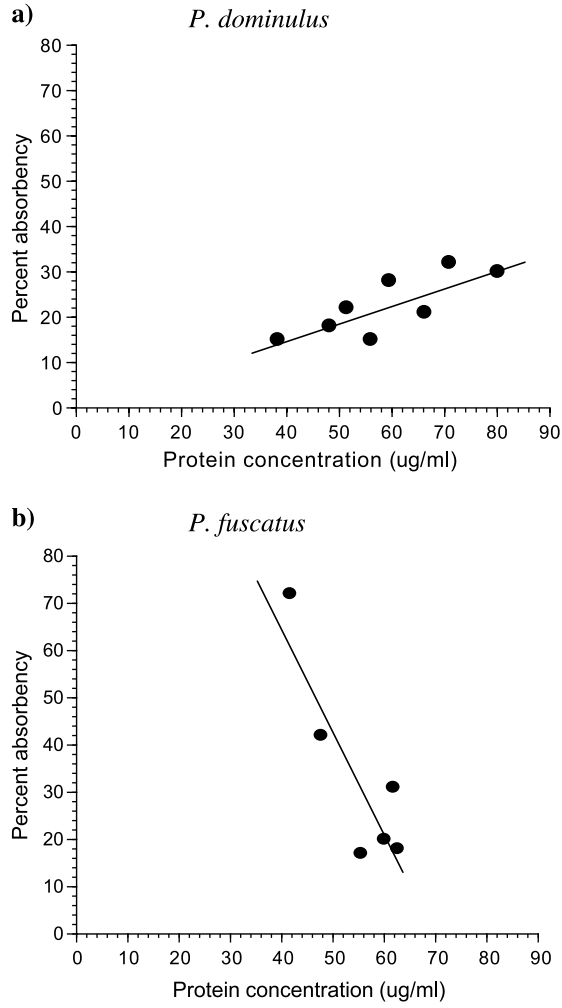

FIG. 4. Regressions for nest protein concentration *versus* percent absorbency of nest material for *P. dominulus* and *P. fuscatus* of the surplus prey treatment only.

DISCUSSION

Although wasps of the natural treatment had to forage for prey in a field-woodland site, they were able to maintain a productivity level similar to wasps of the surplus prey treatment, in terms of the number of eggs, larvae, pupae, and

adult offspring produced (Armstrong and Stamp, 2003), and thus, the lower concentration of protein in nest paper of nests of the natural treatment indicates a tradeoff. Wasps in the natural foraging conditions apparently allocated a greater proportion of proteinaceous material to the brood than to nest construction compared to those in the surplus prey foraging conditions (Figure 3). That nests of the surplus prey foraging conditions had a concentration of protein that was twice that of nests under natural conditions suggests that the wasps of the surplus prey foraging conditions could afford to use more protein for oral secretions. So, in addition to exposure to rainfall influencing protein concentration of nest material (Yamane and Itô, 1994; Kudo et al., 1998; Kudo, 2000), food abundance affects it as well.

If it is not a tradeoff, an alternative explanation for these results is that wasps of the natural treatment were successful at allocating resources to both brood and nest, while wasps of the surplus prey treatment were simply depositing excess protein into their nests. However, the behavioral data from this study suggest that the tradeoff explanation is more likely. For example, wasps of the natural treatment were less often observed checking cells or provisioning larvae and more often foraging than wasps of the surplus prey treatment (Armstrong and Stamp, 2003). These results indicate that wasps of the natural treatment spent a considerable amount of time and energy in the location and capture of prey compared to wasps of the surplus prey treatment to produce a similar brood output. Furthermore, given the substantial predation risks and energetic costs involved in leaving the nest (i.e., foragers may be located by natural enemies that respond visually to moving prey) (Reeve and Gamboa, 1987), it is unlikely that wasps of the surplus prey treatment continued to search for prey after the routine protein requirements of the brood and nest were met.

Under natural foraging conditions, PF wasps used more protein for nest construction than PD wasps. These results suggest that when prey are difficult to find (which they presumably were under the natural foraging conditions in this experiment) and when their nests are similarly sheltered (as in this study), PD uses less protein for oral secretion and so has more for the developing brood than PF. Allocation of more proteinaceous material to the brood would contribute to more offspring and more rapid development of offspring for PD compared to PF.

Based on previous studies (McGovern et al., 1988; Yamane and Itô, 1994; Kudo et al., 1998), we expected a correlation between protein concentration and nest paper absorbency. In the surplus prey foraging conditions, protein concentration of PF nests was negatively correlated with nest absorbency; however, protein concentration of PD nests was *positively* correlated with nest absorbency. Furthermore, in the natural foraging conditions, there was no correlation between protein concentration and nest paper absorbency for either species. These results may reflect in part how environmental conditions influence the

location on the nest where secretion is applied and the amount of secretion applied or reapplied. Also, as colony size increases, there are more workers to apply secretion and also more nest area to maintain, but the timing of brood emergence affects the relationship of those variables, such that there may be more construction at some times and more maintenance (and so, more secretion applied) at other times. Certainly, some nests, especially the top half, look quite "shellacked" compared to others.

The protein secreted by wasps may strengthen nests (Espelie and Himmelsbach, 1990; Singer et al., 1992). However, the results of this study do not support that hypothesis. Probably where protein contributes the most to strengthen the nest is at the pedicel, the stalk that attaches the nest to a surface, which we did not examine. In addition, the oral secretions seem to act as a glue, attaching the pedicel to a surface, yet allowing some elasticity.

For both wasp species, nests of the natural foraging conditions were tougher than nests of the surplus prey foraging conditions. It is possible that wasps of the natural foraging conditions used a different combination of wood types to construct their nests. Wasps of the surplus prey foraging treatment were restricted to using weathered pine for nest construction (from the cage frames and nest boxes). Wasps of the natural foraging treatment, in addition to having the pine in the cages, also had access to other wood types for nest construction, because they foraged freely. Wasps use weathered fibrous material from woody plants for nest paper (Raveret Richter, 2000). Although hardwoods and softwoods differ in composition (e.g., softwoods have a higher lignin content while hardwoods have a higher xylan content), wasps make no distinction between the two (McGovern et al., 1988). The weathering of hardwoods and softwoods for 25–30 yr has been found to result in an 80% loss of lignin and, thus, a loss in fiber connection (Browne, unpublished data). Consequently, nest toughness is likely a function of several factors, including protein concentration, source of plant fiber, and the age or decomposition state of the fiber used. Also, nests built where exposure to weather is greater may be constructed with greater toughness. Because all nests in this study were sheltered in boxes, that was not a source of variation here. However, colonies with abundant food may respond by gathering as much food as possible (Wenzel and Pickering, 1991) and, correspondingly, building cells as quickly as possible, with the result that nest paper is not as tough.

In summary, differences in protein allocation to nest construction between the invasive *P. dominulus* and the native *Polistes* may be a factor in the success of PD as an invasive species. When prey are scarce, PD appears to use less protein for oral secretion for nests and so may have more for the developing brood, thereby contributing to more offspring and more rapid development of offspring for PD compared to native *Polistes*. These results alone do not explain the success of PD; however, it is likely one of several contributing factors.

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ELECTROPHYSIOLOGICAL AND OLFACTOMETER RESPONSES OF TWO HISTERID PREDATORS TO THREE PINE BARK BEETLE PHEROMONES

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Abstract—We measured electrophysiological responses in the antennae of two predaceous hister beetles, *Platysoma parallelum* and *Plegaderus transversus*, exposed to racemic mixtures of primary aggregation pheromones of scolytid bark beetle prey, ipsenol, ipsdienol, and frontalin. No significant differences were found for either histerid species between male and female antennal responses to any of the three pheromones. Measurement of antennal threshold responses indicated that *Pla. parallelum* has increasing antennal sensitivity to ipsdienol, ipsenol, and frontalin. In contrast, *Ple. transversus* exhibited similar detection thresholds to all three pheromones. *Pla. parallelum* antennae exhibited different response amplitudes to the three pheromones at quantities above the detection threshold, while *Ple. transversus* had similar responses to each. Behavioral responses to the same three pheromones were evaluated for both histerid species using pedestrian olfactometer bioassays. Both species were attracted to frontalin and ipsenol, but not ipsdienol. *Pla. parallelum* was significantly more attracted to frontalin than ipsenol, while *Ple. transversus* showed no significant preference for either compound. Our results suggest that histerids that prey upon pine bark beetles may have different host or host habitat preferences, which could reduce interspecific competition.

Key Words—Scolytidae, Histeridae, *Dendroctonus*, *Ips*, *Platysoma*, *Plegaderus*, bark beetle electrophysiology, hister beetle predators, host location, kairomone, attraction, frontalin, ipsenol, ipsdienol.

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INTRODUCTION

Hister beetles (Coleoptera: Histeridae) are important natural enemies of pine bark beetles (Coleoptera: Scolytidae). They comprise approximately 6–7% of total predator abundance for southern pine beetle, *Dendroctonus frontalis* Zimmermann, and *Ips* spp. in the southern United States (Berisford, 1980; Kulhavy et al., 1989). Histerid adults and larvae feed primarily on the early life stages of bark beetles within their galleries, mined into the inner bark of pines (Kovarik and Caterino, 2000; WPS personal observations). They also facultatively prey upon secondary gallery fauna and are considered generalists within a specialized habitat (Erbilgin and Raffa, 2001). Histerids typically arrive at trees within 1 wk of bark beetle colonization (Shepherd and Goyer, 2003). The sympatric histerids, *Platysoma parallelum* (Say) and *Plegaderus transversus* (Say), are associated with both *D. frontalis* and *Ips* spp. infestations in the southeastern United States (Overgaard, 1968; Moser et al., 1971; Stein and Coster, 1977; Dixon and Payne, 1979; Goyer et al., 1980; Riley and Goyer, 1986; Shepherd and Goyer, 2003). These previous studies did not indicate any apparent host specificity for either histerid species.

Histerids exploit bark beetle aggregation pheromones as kairomonal attractants to locate host habitats (Dixon and Payne, 1980; Turnbow and Franklin, 1981; Payne, 1989; Shepherd and Goyer, 2003). Trapping studies have shown that histerids are attracted to the major aggregation pheromones of bark beetles of the southern United States, including frontalin (*D. frontalis*), ipsenol [*Ips grandicollis* (Eichhoff)], and ipsdienol [*Ips avulsus* (Eichhoff) and *Ips calligraphus* (Germar)] (Dixon and Payne, 1980; Turnbow and Franklin, 1981; Shepherd and Goyer, 2003). Variation in responsiveness to prey kairomonal odor cues may indicate that sympatric histerids use different strategies for host location. This could separate arrival times and locations on bark beetle-infested trees and thus reduce interspecific competition.

Our objective in this study was to determine differences in attraction to host pheromones between *Pla. parallelum* and *Ple. transversus* adults. We conducted both electrophysiological and behavioral assays with frontalin, ipsenol, and ipsdienol.

METHODS AND MATERIALS

Insects for Laboratory Assays. We collected adult *Pla. parallelum* and *Ple. transversus* predators from under the bark of loblolly pine, *Pinus taeda* L., logs naturally infested by *Ips* spp. at the Louisiana State University AgCenter, Idlewild Research Station, East Feliciana Parish, LA, USA. Histerids were maintained at room temperature (ca. 23°C) in glass petri dishes lined with moist

filter paper and were fed to satiation with *Ips* spp. larvae twice weekly. We used histerids in experiments up to 60 d after collection.

Electroantennogram (EAG) Recordings. For these analyses, EAG techniques were modified from those used by Visser (1979) and Scholz et al. (1998), and the equipment used was identical to that described in Asaro et al. (2004). We mounted intact head preparations of *Pla. parallelum* and *Ple. transversus* between two glass micropipette/gold electrodes filled with Beadle–Ephrussi Ringer solution and 0.02% v/v Triton X-100 surfactant (Union Carbide, Midland, MI, USA), which improved electrical contact between the antenna tip and the electrode saline. The reference electrode was inserted into the base of the excised head, and the tip of the recording electrode was touched to the intact club of one antenna. The head preparation was enclosed within a brass Faraday cage.

We tested five dilutions (0.0001, 0.001, 0.01, 0.1, and 1 μg/μl) of synthetic racemic [50(+)/50(−)] ipsenol, 97% purity (Bedoukian Research, Inc., Danbury, CT, USA), ipsdienol, 95% purity (Borregaard, Sarpsborg, Norway), and frontalin, 97% purity (BASF, Ludwigshafen, Germany) in redistilled hexane. Dilution series of a single pheromone were delivered in random order to individuals of each sex and species: ipsenol (*Pla. parallelum*—13 M/22 F, *Ple. transversus*—18 M/10 F); ipsdienol (*Pla. parallelum*—16 M/12 F, *Ple. transversus*—18 M/8 F); and frontalin (*Pla. parallelum*—12 M/17 F, *Ple. transversus*—12 M/18 F). The number of replicates differed due to availability of vigorous histerids with undamaged antennae. In addition to the pheromone samples, we puffed a hexane-only control and standard solution (frontalin, *endo*-brevicomin, and verbenone at 0.1 μg/μl in hexane) before and after each puff of a sample dilution. We used a multiple-component standard mixture of *D. frontalis* pheromones because electrophysiological and behavioral responses by *Pla. parallelum* and *Ple. transversus* to individual compounds were not known. This standard elicited consistent, strong EAGs in both sexes and species in pilot trials.

Test solutions (10 μl) were applied to 10 × 0.5-cm strips of Whatman No. 1 filter paper inside glass Pasteur pipettes. We positioned the pipette tip 2 cm upwind of the head preparation in a continuous stream (400 ml/min) of humidified, charcoal-filtered air. Puffs of air (30 ml/min; 3-sec duration) were delivered from a Syntech (Hiversum, The Netherlands) CS-05 stimulus control unit. We recorded the peak voltage amplitude during the puff delivery of each stimulus as the antennal response. An interval of 1 min between puffs was found to be sufficient for complete antennal recovery in both species. We determined the sex of each beetle by dissecting the genitalia.

Y-Tube Olfactometer Bioassays. We tested short-range anemotaxic responses of *Pla. parallelum* and *Ple. transversus* adults in pedestrian bioassays, using a Y-tube olfactometer as described in Sullivan et al. (2000). Individual histerids were

introduced into the stem of a glass Y-tube (6-mm i.d., stem 7 cm, branches 7 cm, and 135° to the stem), and a choice was scored when a beetle walked 5 cm down one branch within 8 min of introduction to the Y-tube. Filtered, humidified air (30 ml/min, 23–24°C, 50–70% RH) carried odors from two bait-holding tubes to each branch of the Y-tube.

Baits were 100 μg racemic ipsenol, ipsdienol, and frontalin in hexane (10 μl) applied to filter paper squares (9 cm^2). The solvent was allowed to evaporate for 20 sec before papers were placed inside the sample tubes. Papers with ipsenol, ipsdienol, frontalin, and hexane only (control) were tested against each other in all possible binary combinations.

We used a total of 60 individual histerids of each species in each test and did not reuse them. Beetles were starved for 5 d prior to introduction to the olfactometer. Between trials, we replaced Y-tubes with clean ones and swapped bait treatments to opposite branches to eliminate directional bias.

Statistical Analysis. We calculated net EAGs by subtracting the mean responses to the controls introduced before and after the sample or standard from the actual sample and standard responses (Scholz et al., 1998). EAG data were standardized by calculating the percentages of the net EAGs relative to the standard solution (Payne, 1975; Dickens, 1978).

We used a Wilcoxon paired signed rank test to compare antennal responses to pheromone dilutions to the average of the contiguous control responses. Detection thresholds were calculated as the lowest concentration of pheromone producing significantly greater responses than the control. We compared histerid EAGs at and above the threshold for each species for sexes combined with a Kruskal–Wallis test and a Dunn's multiple comparison test (SAS Institute, 2001). A *G*-test for goodness of fit with William's correction for small samples was used to identify significant preferences for one olfactometer branch in the Y-tube tests (Sokal and Rohlf, 1995). We set significance levels at $\alpha = 0.05$ for all tests.

RESULTS

EAG Recordings. Mean net responses (±SE) of *Pla. parallelum* to the standard mixture were 2.01 ± 0.07 mV for males ($N = 41$) and 2.49 ± 0.05 mV for females ($N = 51$). For *Ple. transversus*, the mean net responses (±SE) to the standard were 5.45 ± 0.23 mV for males ($N = 48$), and 6.35 ± 0.21 mV for females ($N = 36$). No significant differences were found for either histerid species between male and female antennal responses to the three pheromones at any concentration. Thus, we combined male and female EAG data at and above the detection threshold for each species. *Pla. parallelum* and *Ple. transversus* both exhibited

TABLE 1. EAG DETECTION THRESHOLDS TO SERIAL DILUTIONS OF RACEMIC IPSENOL, IPSDIENOL, AND FRONTALIN FOR *Platysoma parallelum* AND *Plegaderus transversus* HISTERID BEETLES (SEXES COMBINED)

Species	Pheromone	Detection threshold (μg on filter paper)[a]	*P*-value
Pla. parallelum	Ipsenol	1	<0.001
Pla. parallelum	Ipsdienol	10	<0.001
Pla. parallelum	Frontalin	0.1	<0.001
Ple. transversus	Ipsenol	1	<0.001
Ple. transversus	Ipsdienol	1	<0.001
Ple. transversus	Frontalin	1	<0.001

[a]Lowest concentration of pheromone that elicited a significantly greater EAG than average of contiguous control responses ($P < 0.05$: Wilcoxon paired signed rank test).

significant antennal responses to racemic ipsenol, ipsdienol, and frontalin (Table 1). *Pla. parallelum* detection thresholds differed, with antennae exhibiting increasing sensitivity for ipsdienol, ipsenol, and frontalin (Table 1). In contrast, *Ple. transversus* detection thresholds were the same for all three pheromones (Table 1).

Mean percent EAGs for *Pla. parallelum* sexes combined were significantly higher for frontalin than ipsenol ($P < 0.001$) and ipsdienol ($P < 0.001$) and higher for ipsenol than ipsdienol ($P < 0.04$) at both 1- and 10-μg concentrations (Figure 1). For *Ple. transversus* sexes combined, there were no significant

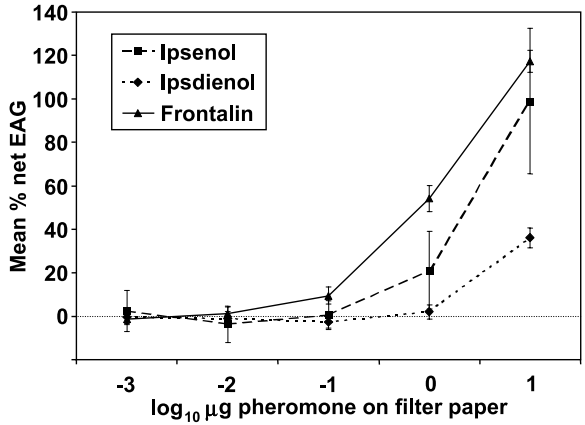

FIG. 1. Mean percent EAGs (±SE) from *Pla. parallelum* adults (sexes combined) to ipsenol ($N = 35$), ipsdienol ($N = 28$), and frontalin ($N = 29$), relative to the standard mixture of *D. frontalis* pheromones.

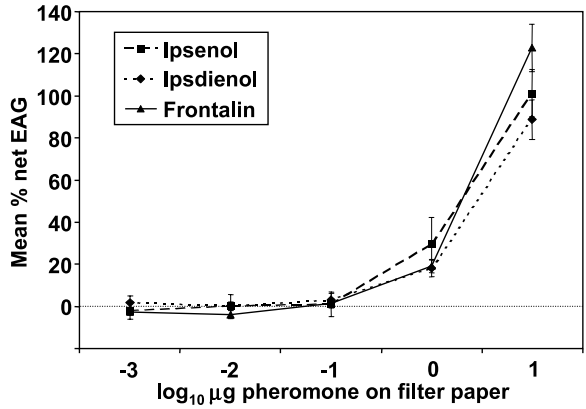

FIG. 2. Mean percent EAGs (±SE) from *Ple. transversus* adults (sexes combined) to ipsenol (N = 28), ipsdienol (N = 26), and frontalin (N = 30), relative to the standard mixture of *D. frontalis* pheromones.

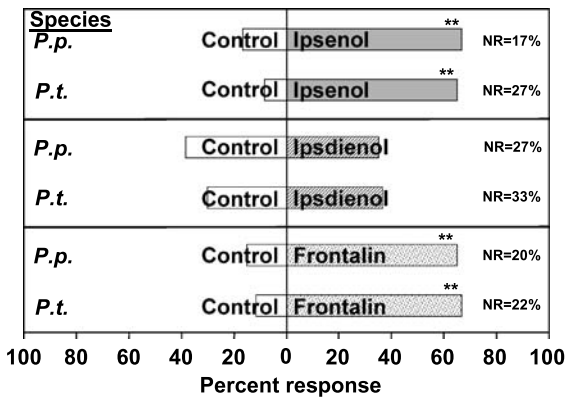

FIG. 3. Percentage of *Pla. parallelum* and *Ple. transversus* adults that walked toward either 100 µg of the pheromone sample or the hexane-only control in six paired choice tests using a Y-tube olfactometer. Asterisks indicate a significantly greater response toward one of the two choices using *G*-tests with William's correction for small samples ($P < 0.001$). *P.p.* = *Pla. parallelum*. *P.t.* = *Ple. transversus*. NR = Percentage of histerids in each test that chose neither the pheromone sample nor the control within 8 min of introduction.

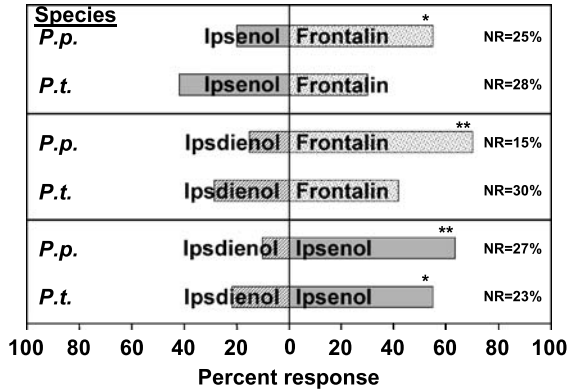

FIG. 4. Percentage of *Pla. parallelum* and *Ple. transversus* adults that walked toward either of two pheromone samples (100 μg each) in six paired choice tests using a Y-tube olfactometer. Asterisks indicate a significantly greater response toward one of the two choices using *G*-tests with William's correction for small samples (*$P < 0.01$; **$P < 0.001$). *P.p.* = *Pla. parallelum*. *P.t.* = *Ple. transversus*. NR = Percentage of histerids in each test that chose neither the pheromone sample nor the control within 8 min of introduction.

differences in responses to the three pheromones at or above the detection threshold (Figure 2).

Y-Tube Olfactometer Bioassays. Both *Pla. parallelum* and *Ple. transversus* were significantly more attracted to frontalin and ipsenol than the control (Figure 3). There was no significant attraction to ipsdienol for either species. Responses to the paired pheromone offerings differed for each species (Figure 4). *Pla. parallelum* was more strongly attracted to frontalin than ipsenol and ipsdienol and to ipsenol than ipsdienol. In contrast, *Ple. transversus* showed no significant preference for either frontalin vs. ipsenol or frontalin vs. ipsdienol when offered as paired choices, but preferred ipsenol over ipsdienol.

DISCUSSION

Different electrophysiological and behavioral responses to three primary bark beetle aggregation pheromones suggest that *Pla. parallelum* and *Ple. transversus* utilize different strategies for host or host habitat finding. The antennae of *Pla. parallelum* responded with increasing sensitivity to ipsdienol, ipsenol, and frontalin, indicating an ability to detect *D. frontalis* attack sites, from which frontalin odor plumes emanate, at greater distances than those of

Ips spp., and *I. grandicollis* colonizations at greater distances than those of *I. avulsus* and *I. calligraphu*s. This histerid likely has a larger antennal receptor population for frontalin than ipsenol and ipsdienol, as we recorded higher intensity antennal responses to frontalin for quantities above the detection threshold (Payne, 1975). We also recorded significantly higher intensity responses to ipsenol than ipsdienol for quantities above the detection threshold, providing evidence for a larger ipsenol receptor population (Payne, 1975). In contrast, *Ple. transversus* exhibited similar detection thresholds and EAGs above the detection threshold for all three kairomones and, thus, may have similarly sized receptor populations for these compounds. Electrophysiological studies of other bark beetle predators found that the clerids, *Thanasimus dubius* (F.) and *Thanasimus formicarius* (L.), also responded to multiple kairomones produced by different prey species (Hansen, 1983; Payne et al., 1984; Tommeras, 1985).

Since both histerid species were attracted to ipsenol and frontalin, but not ipsdienol in the olfactometer bioassays, they may preferentially orient toward portions of trees containing *D. frontalis* or *I. grandicollis*, rather than *I. avulsus* and *I. calligraphus*. The olfactometer preferences of *Pla. parallelum* were mirrored by greater electrophysiological responsiveness to frontalin and ipsenol. Attractive responses and the presence of a larger antennal receptor population suggest specialization for these kairomones. This histerid may be more attracted to trees or portions of trees containing *D. frontalis* than those with only *Ips* spp. In contrast, *Ple. transversus* had fewer odor preferences, differentiating only between ipsenol and ipsdienol when more than one kairomone was offered. Similar to its antennal responses, its attraction to prey kairomones appears less specific than those of *Pla. parallelum*. It may not distinguish between sites colonized by either *D. frontalis* or any of the *Ips* spp.

Differences in attraction patterns between these histerid predators may facilitate niche partitioning, reducing interspecific competition via spatial and temporal separation at sites infested with multiple bark beetle species. These histerids are generalist predators that have not been shown to associate preferentially with any of the four sympatric pine bark beetle species. Different kairomone response profiles may indicate previously unrecognized host preferences, and they may serve to stagger arrival times and separate landing sites at trees infested with multiple host species. In our study, both electrophysiological and behavioral data suggest that *Pla. parallelum* has a preference for frontalin over *Ips* spp. pheromones, while *Ple. transversus* exhibits little or no distinction among these compounds.

Complicating the interactions between these histerid species are the effects of various enantiomeric ratios of prey kairomones on behavior. Studies of histerids associated with *Ips pini* (Say) in Wisconsin have shown that *Platysoma cylindrica* (Paykull) was most attracted to traps baited with 25(+)/75(−)

ipsdienol (Raffa and Klepzig, 1989), and that *Pla. cylindrica* and *Pla. parallelum* were most attracted to traps baited with 3(+)/97(−) ipsdienol (Aukema et al., 2000a,b). In addition to bark beetle pheromones, histerids may use a variety of volatile odor cues derived from other potential prey, host trees, and microorganisms to locate their prey. Any of these compounds, individually or in combination, may provide optimum attractiveness to searching histerid predators.

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GENETIC BASIS TO DIVERGENCE OF SEX PHEROMONES IN TWO CLOSELY RELATED MOTHS, *Ostrinia scapulalis* AND *O. zealis*

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Abstract—Crossing experiments between two closely related moths, *Ostrinia scapulalis* and *O. zealis*, were conducted to gain insight into the genetic basis of the divergence of female sex pheromones. The sex pheromone of *O. scapulalis* comprises (*E*)-11- and (*Z*)-11-tetradecenyl acetates (E11 and Z11), and distinct genetic variation is found in the blend of components. This variation is largely controlled by a single autosomal locus with two alleles, $A^{E(sca)}$ and $A^{Z(sca)}$. E-type ($A^{E(sca)}A^{E(sca)}$) females produce a pheromone with a mean E11:Z11 ratio of 99:1, whereas Z-type ($A^{Z(sca)}A^{Z(sca)}$) and I-type ($A^{E(sca)}A^{Z(sca)}$) females produce a pheromone with a mean of 3:97 and 64:36, respectively. *O. zealis* is distinctive in that it has a third pheromone component, (*Z*)-9-tetradecenyl acetate (Z9), in addition to E11 and Z11, and the typical blend ratio is 60:35:5 (Z9:E11:Z11). Our study revealed that Z9 production in *O. zealis* is mainly regulated by an autosomal recessive gene $phr^{(zea)}$, which is suggested to be involved in the chain-shortening of a pheromone precursor fatty acid, and linked to $A^{E(zea)}$, a gene corresponding to $A^{E(sca)}$ in *O. scapulalis*. A few mutations in a gene involved in pheromone production could explain the dramatic shift between a two-component pheromone communication system in *O. scapulalis* and a three-component system in *O. zealis*.

Key Words—Genetics, sex pheromone, reproductive isolation, *Ostrinia scapulalis*, *Ostrinia zealis*, *Ostrinia nubilalis*, communication divergence.

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INTRODUCTION

The evolution of animal communication systems is one of the most intriguing themes in the field of evolutionary biology. Communication between individuals is established only when signals from the senders are recognizable by the receivers. Therefore, evolution in the communication traits of senders and receivers must be coordinated (Butlin and Ritchie, 1989). The communication system for mate recognition is a key to premating isolation, and can be an important factor at the beginning of species divergence. Hence, studies on the genetic basis of such communication systems would give novel insights into the mechanism of divergence in communication traits and subsequent species divergence (Monti et al., 1997).

In many moths, mate-finding communication is mediated by sex pheromones produced by females (Cardé and Baker, 1984). The pheromone components and their blend ratios are species-specific, and male response is tuned to the blend of conspecific females (Phelan, 1997). Nevertheless, hybridization between some closely related species does occur under natural or laboratory conditions (for example, Hendrikse, 1988; Foster et al., 1997; Gadenne et al., 1997; Monti et al., 1997). An examination of the sex pheromones produced by hybrid offspring should furnish important information on the divergence of pheromone communication systems.

In the present study, we dealt with two closely related *Ostrinia* species, the adzuki bean borer moth *Ostrinia scapulalis* (Walker), and the burdock borer moth *O. zealis* (Guenée) (Lepidoptera: Crambidae). *O. scapulalis* is found in central Russia, northern India, and eastern Asia, while *O. zealis* is found in eastern Asia, through Japan and China to northern India (Mutuura and Munroe, 1970; Frolov, 1998). *O. scapulalis* is polyphagous and a pest of adzuki bean *Phaseolus angularis* (Leguminosae), and hop *Humulus lupulus* (Moraceae). In contrast, *O. zealis* is oligophagous, specializing in a few Compositae plants such as thistles *Cirsium* spp. and burdock *Arctium lappa* (Ishikawa et al., 1999a). Despite these clear differences in host plant preference, these two species occur sympatrically in several localities in Japan. The sex pheromones, therefore, should play a critical role in premating reproductive isolation.

The sex pheromone of *O. scapulalis* is a mixture of (*E*)-11- and (*Z*)-11-tetradecenyl acetates (E11 and Z11), and three phenotypes have been identified in this species, i.e., E type (96–100% of E11; mean in a typical laboratory culture, 99%), I type (33–95%; mean 64%), and Z type (0–31%; mean 3%) (Huang et al., 1997, 2002; Tabata et al., 2003; Takanashi et al., 2005). These three types are mainly determined by one autosomal locus with two alleles, $A^{E(sca)}$ and $A^{Z(sca)}$: E-type, Z-type, and I-type females have the genotype $A^{E(sca)}A^{E(sca)}$, $A^{Z(sca)}A^{Z(sca)}$, and $A^{E(sca)}A^{Z(sca)}$, respectively (Takanashi et al., 2005).

The sex pheromone of *O. zealis*, on the other hand, is characterized by the presence of a third component, (*Z*)-9-tetradecenyl acetate (Z9), in addition to E11 and Z11 (Ishikawa et al., 1999b). The typical blend ratio is 65:30:5 (Z9:E11:Z11), and no large blend variation has been found to date.

We conducted laboratory crossing experiments using these two *Ostrinia* species, and investigated the female sex pheromone composition in hybrid F1 individuals and F2 or backcrossed progeny. Based on the findings, we discuss the genetic basis of pheromone communication systems in *Ostrinia*, and argue that a few mutations in a gene involved in pheromone production can explain the dramatic shift between a two-component pheromone communication system in *O. scapulalis* and a three-component system in *O. zealis*.

METHODS AND MATERIALS

Insects. Diapausing *O. zealis* larvae were collected from thistles (*Cirsium* sp.) at Irago Promontory (34°34′N, 137°01′E), Aichi Prefecture, Japan on October 24, 2001. To terminate diapause, the larvae were maintained at about 5°C and in darkness for more than 3 mo. These larvae were moved to a rearing room (60% relative humidity; 16 hr light–8 hr dark; 23°C) to facilitate post-diapause development. One to three d after emergence, moths were transferred to a screen cage (20 × 20 × 20 cm) with water-soaked cotton and allowed to mate over a period of 3 d. Mated females were individually housed in 90-ml clear plastic cups with a cotton pad soaked with water, and allowed to lay eggs on the surface of the cups. *O. scapulalis* female moths were collected in Iruma city (35°50′N, 139°23′E), Saitama Prefecture, Japan, on September 8–15, 2001. The field-collected female moths, usually mated, were housed in 90-ml plastic cups individually and allowed to lay eggs. A group of eggs from each female, which we call a "family" in the present article, was reared on an artificial diet (Silk mate™; Nosan Corp., Yokohama, Japan) in a plastic bottle (8 cm diam, 12 cm height), and maintained in the rearing room described above. After pupation, the pupae were sexed, and females and males were separately maintained in 430-ml clear plastic cups.

Crossing Experiments for Genetic Analysis of Pheromone Production. *O. scapulalis* cultures homogeneous in terms of E- and Z-type pheromone production were established from wild populations as follows. The pheromone types of at least 10 female samples per family were determined by gas chromatography as described below, and monomorphic (E or Z type) families were selected. The E or Z cultures of *O. scapulalis* were considered to be established when a monomorphic state continued for three successive generations. Crossing experiments for the analysis of pheromone production were

conducted using the *O. scapulalis* E culture (Sca^E) and Z culture (Sca^Z), and *O. zealis* (Zea). Ten pairs of 1- to 3-d-old moths were housed in a screen cage (20 × 20 × 20 cm) for 3 d with water-soaked cotton. The following crosses (female × male) were carried out: Zea × Sca^E and Zea × Sca^Z to yield F1 hybrids (F1 hybrids are expressed as $ZeaSca^E$ and $ZeaSca^Z$, respectively), $ZeaSca^E$ × $ZeaSca^E$ and $ZeaSca^Z$ × $ZeaSca^Z$ to yield F2 progeny, and eight backcrosses (Zea × $ZeaSca^E$, $ZeaSca^E$ × Zea, $ZeaSca^E$ × Sca^E, Sca^E × $ZeaSca^E$, Zea × $ZeaSca^Z$, $ZeaSca^Z$ × Zea, $ZeaSca^Z$ × Sca^Z, and Sca^Z × $ZeaSca^Z$). In a reciprocal cross for F1 hybrids (Sca^Z × Zea), no fertile mated females could be obtained from more than 50 pairs of Sca^Z × Zea, and we abandoned crossing in this direction.

Sex Pheromone Analysis. The abdominal tip, which includes a sex pheromone gland, was excised from 2- or 3-d-old virgin females during the last 3 hr of scotophase, and tips were immersed individually in hexane (10 μl/female; residual pesticide analysis grade, Wako Pure Chemicals, Kyoto) containing 20 ng of tridecyl acetate (Sigma, St. Louis, MO, USA) as an internal standard. The tip was removed after 30 min, and the extract was kept at −20°C prior to analysis. A gas chromatograph GC-14B (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector, a split/splitless injector, an automatic injector (AOC-20, Shimadzu), and a DB-Wax column (30 m × 0.25 mm i.d.; J & W Scientific, CA) was used for the analysis of extracts. Nitrogen was used as the carrier gas. The injector and detector temperatures were 230 and 270°C, respectively. The column oven temperature was maintained at 120°C for 2 min, then raised to 150°C at a rate of 5°C/min, to 200°C at 3°C/min, and finally to 240°C at 5°C/min. Aliquots (2 μl) of samples were injected into the GC with a splitless time of 1.5 min. The pheromone components were quantified by comparing their GC peak area with that of the internal standard. The authentic Z9, E11, and Z11 were purchased from Pherobank (Wageningen, The Netherlands).

RESULTS

Sex Pheromone Blend Ratios Produced by the F1 Hybrid Females. Sex pheromone compositions of Sca^E, Sca^Z, Zea, and their F1 hybrids ($ZeaSca^E$ and $ZeaSca^Z$) were analyzed based on the proportions of Z9 and E11 in the total (Z9 + E11 + Z11) (Figure 1a and b). Sca^E and Sca^Z produced no Z9, as expected. All of the Sca^E females ($N = 25$) produced only E11, and no Z11 was detected. Sca^Z females ($N = 47$) produced a pheromone with 0–10% E11. Zea females ($N = 36$) produced three components in their pheromone: % Z9 ranged from 50 to 75, and % E11 from 20 to 40 (Figure 1a). It is readily noticeable that proportions of Z9 and E11 are negatively correlated in Zea ($r = -0.99$). In other

words, the proportion of the minor component Z11 was almost fixed (5.97 ± 0.80; mean % ± SD). F1 hybrids ($ZeaSca^E$ and $ZeaSca^Z$; Figure 1b) did not produce Z9 except for two samples, both of which belonged to $ZeaSca^Z$, and the proportion of Z9 was low (<8%). $ZeaSca^E$ produced an E11-dominant blend (>85%), i.e., an Sca^E-like pheromone blend, whereas $ZeaSca^Z$ produced both E11 and Z11, like *O. scapulalis* I-type (Sca^I).

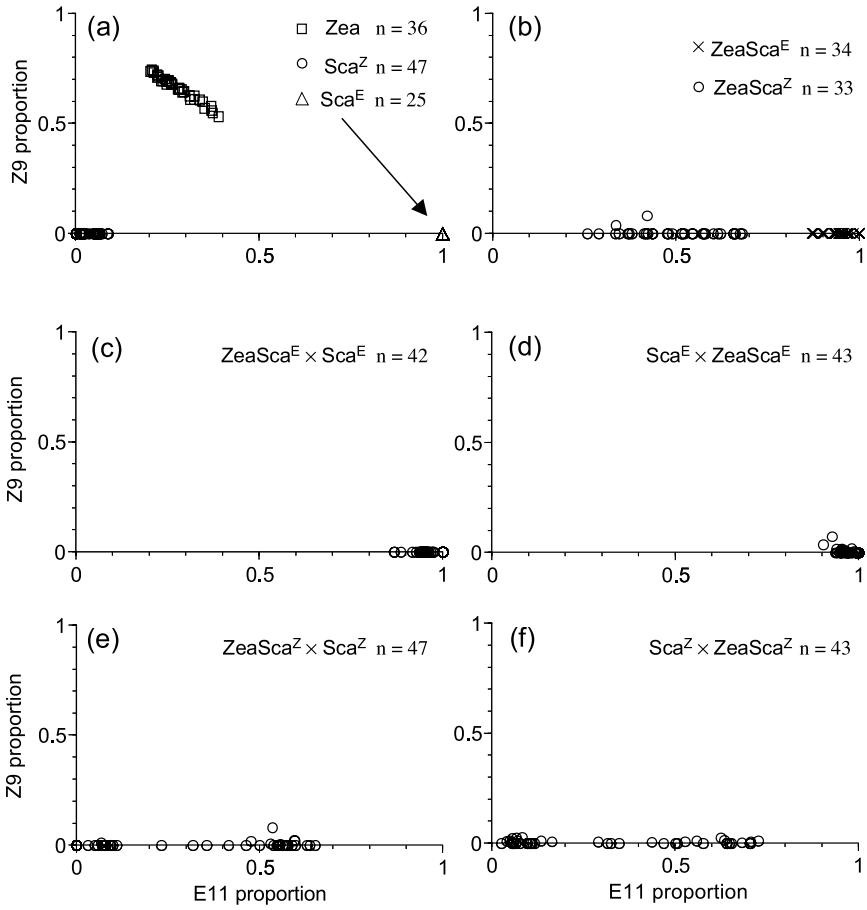

FIG. 1. Distributions of sex pheromone blends in the female progeny of *O. zealis*, E-type *O. scapulalis*, and Z-type *O. scapulalis* (a), their F1 hybrids (b), backcrosses (c–j), and F2 hybrids (k–l). Z: *O. zealis*, SE: E-type *O. scapulalis*, SZ: Z-type *O. scapulalis*. E11: (*E*)-11-tetradecenyl acetate, Z11: (*Z*)-11-tetradecenyl acetate, Z9: (*Z*)-9-tetradecenyl acetate. Abscissa: E11 / (Z9 + E11 + Z11), Ordinate: Z9 / (Z9 + E11 + Z11).

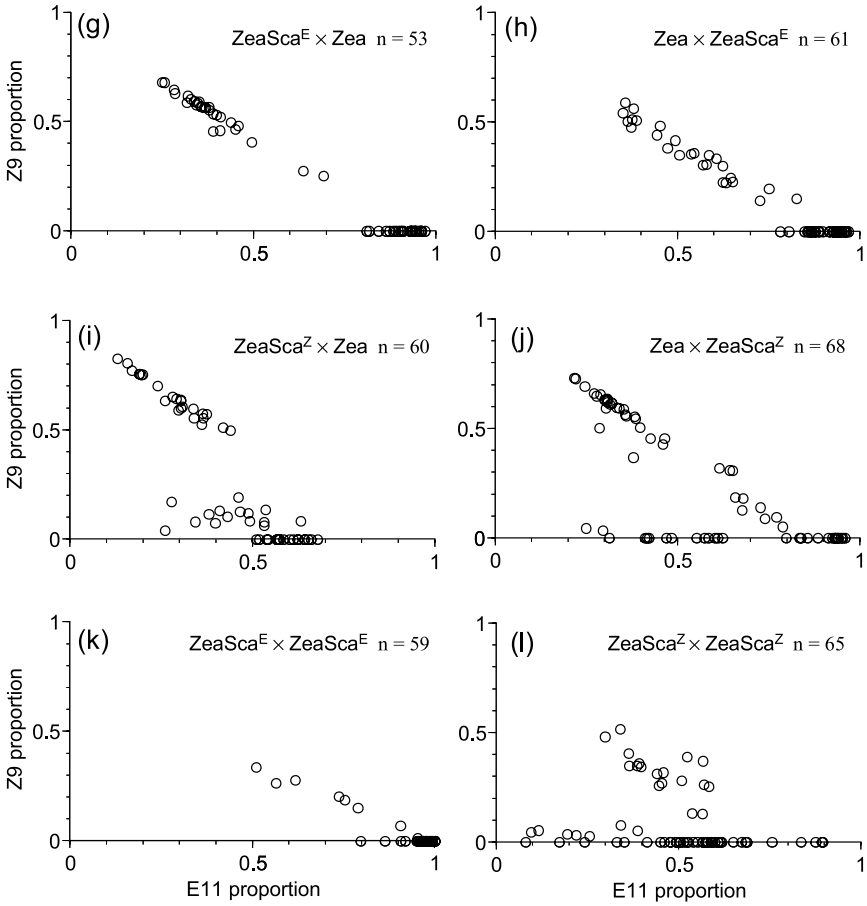

FIG. 1. CONTINUED.

Sex Pheromone Blend Produced by the Backcross and F2 Progeny. The sex pheromone blends of the progeny backcrossed to Sca^{E}, Sca^{Z}, and Zea are shown in Figure 1c–j. The backcrosses to *O. scapulalis* (Sca^{E} and Sca^{Z}), ZeaSca^{E} × Sca^{E}, Sca^{E} × ZeaSca^{E}, ZeaSca^{Z} × Sca^{Z}, and Sca^{Z} × ZeaSca^{Z} yielded five, four, five, and four families, and 42, 43, 48, and 43 female samples, respectively (Figure 1c, d, e, f). Almost all of them produced a two-component pheromone without Z9. The distributions of pheromone blend ratios were unimodal around the Sca^{E}-like blend in the backcross to Sca^{E} (Figure 1c, d), whereas they were bimodal around the Sca^{Z}-like and Sca^{I}-like blends in the backcross to Sca^{Z} (Figure 1e, f). The distributions of E11 proportion in

reciprocal crosses were not significantly different in the Sca^E and Sca^Z directions ($P = 0.31$ and 0.33, respectively; Wilcoxon test).

Two- and three-component-type pheromones were observed in the backcrosses to *O. zealis* (Zea). In the crosses of $ZeaSca^E$ to Zea ($ZeaSca^E \times$ Zea and Zea $\times$ $ZeaSca^E$; total $N = 53$ from two families and $N = 61$ from four families, respectively; Figure 1g, h), the two-component type progeny showed a Sca^E-like pheromone blend (without Z9) in both reciprocal crosses ($N = 25$ in $ZeaSca^E \times$ Zea, and $N = 35$ in Zea $\times$ $ZeaSca^E$), whereas three-component type progeny ($N = 28$ in $ZeaSca^E \times$ Zea, and $N = 26$ in Zea $\times$ $ZeaSca^E$) showed some differences in the proportion of Z9 between the reciprocal crosses ($P < 0.01$; Wilcoxon test). In Zea $\times$ $ZeaSca^E$, the distribution of Z9 proportion was relatively broad (14–59%) in the three-component type progeny, but narrow (41–68%, with two outliers, 21% and 27%) and mostly Zea-like in the reciprocal cross, $ZeaSca^E \times$ Zea. The ratios of Z9 and E11 were negatively correlated in the sex pheromone of the three-component type progeny (Figure 1g, h), as observed in their parent, Zea.

More complex patterns were observed in the backcrosses of $ZeaSca^Z$ to Zea ($ZeaSca^Z \times$ Zea and Zea $\times$ $ZeaSca^Z$; $N = 60$ from two families and $N = 68$ from five families, respectively) (Figure 1i, j). In the cross Zea $\times$ $ZeaSca^Z$, four patterns were clearly distinguishable, i.e., a three-component type high in Z9 (Zea-like type; $N = 29$), a three-component type low in Z9 and high in E11 ($N = 10$), a two-component type with an intermediate proportion of E11 (Sca^I-like type; $N = 14$), and a two-component type high in E11 (Sca^E-like type; $N = 15$). In the reciprocal cross $ZeaSca^Z \times$ Zea, in contrast, only three patterns were observed, i.e., the Zea-like type ($N = 24$), the Sca^I-like type ($N = 21$), and the three-component type low in Z9 and with an intermediate proportion of E11 ($N = 15$). No Sca^E-like type was observed.

In F2 hybrids from the cross of $ZeaSca^E \times$ $ZeaSca^E$ ($N = 58$ from three families), the three-component type had relatively low Z9 (10–37%; $N = 8$), and the two-component type showed a Sca^E-like blend (80–95% E11; $N = 50$) (Figure 1k). On the other hand, the pheromone blend of F2 hybrids from the cross of $ZeaSca^Z \times$ $ZeaSca^Z$ ($N = 65$ from four families) distributed in a scattered manner (Figure 1l). The three-component type could be further divided into a low Z9 type (3–8% Z9; $N = 7$) and an intermediate Z9 type ($N = 18$). The two-component type appeared to be a mixture of Sca^E, Sca^I, and Sca^Z-like blends (8–89% E11; $N = 40$).

DISCUSSION

Genetic Basis of Variations in Pheromone Composition in O. scapulalis and O. zealis. All hybrid F1 females except a few in $ZeaSca^Z$ produced sex

pheromones lacking Z9. This finding indicates the existence of chromosomal recessive genetic factor(s) for the production of the third component, Z9, in *O. zealis*. The involvement of cytoplasmic factors can be excluded, because hybrids did not produce Z9 despite the fact that all the F1 hybrids examined had the cytoplasm of maternal Zea (*O. zealis*), which produces Z9. Moreover, ZeaSca^{E} females produced Sca^{E}-like, and ZeaSca^{Z} females produced Sca^{I}-like pheromone. This suggests that *O. zealis* should have a gene ($A^{E(zea)}$) corresponding to $A^{E(sca)}$ in *O. scapulalis*. The backcross data for Sca^{E} and Sca^{Z} (*O. scapulalis*) support this idea; distributions of the pheromone blend ratios in ZeaSca^{E} × Sca^{E} and Sca^{E} × ZeaSca^{E} are unimodal and Sca^{E}-like, whereas those in ZeaSca^{Z} × Sca^{Z} and Sca^{Z} × ZeaSca^{Z} are bimodal and involve Sca^{Z}- and Sca^{I}-like blends.

The existence of major recessive genes that control production of Z9 is further supported by the finding that the frequencies of two patterns (three-component type and two-component type) were about 1:1 in both reciprocal backcrosses of ZeaSca^{E} to Zea (28:25, in ZeaSca^{E} × Zea; 26:35 in Zea × ZeaSca^{E}; $\chi^2 = 0.08$, $df = 1$ and $\chi^2 = 1.05$, $df = 1$, respectively; chi-square test). It was also demonstrated that these major genes are not linked to the sex chromosome (Z chromosome); the Z chromosomes of the female progeny of these backcrosses differ between the reciprocal crosses (derived from Zea in ZeaSca^{E} × Zea, and from Sca^{E} in Zea × ZeaSca^{E}) because females are heterogametic (ZW) in Lepidoptera. In addition to the major genes, the presence of genetic modifiers for Z9 production was suggested by the difference in distributions of Z9 proportions between the three-component-type females of ZeaSca^{E} × Zea and those of Zea × ZeaSca^{E}. Some of these genetic modifiers appear to exist on the Z chromosome; females of ZeaSca^{E} × Zea, which produced a pheromone high in Z9, have the Z chromosome from Zea, whereas those of Zea × ZeaSca^{E}, which produced a pheromone low in Z9, have that from Sca^{E}.

Significant differences were observed between the reciprocal crosses of the ZeaSca^{Z} hybrid to Zea. The most remarkable difference is the occurrence of the Sca^{E}-like blend (two-component and high E11 ratio) in only one direction of the crosses; 15 samples were Sca^{E}-like in cross Zea × ZeaSca^{Z} ($N = 68$), whereas none was observed in the reciprocal, ZeaSca^{Z} × Zea ($N = 60$). This finding can be explained if the major gene producing Z9, which was shown to be recessive (designated as $phr^{(zea)}$, $PHR^{(sca)}$ being the allele), and the genes regulating the E11 and Z11 ratios in *O. scapulalis* ($A^{E(sca)}$ and $A^{Z(sca)}$) and in *O. zealis* ($A^{E(zea)}$) are on a homologous chromosome (Table 1). When we consider the recombination that specifically occurs in males in Lepidopteran species (Tazima, 1964), the occurrence of two phenotypes (Zea-like and Sca^{I}-like) out of three in ZeaSca^{Z} × Zea, and four phenotypes (Zea-like, Sca^{I}-like, and Sca^{E}-like, and a three-component type with an intermediate E11/Z11) in Zea × ZeaSca^{Z} could be explained based on this hypothesis (Table 1).

TABLE 1. AN EXPLANATION OF THE GENETICS OF FEMALE SEX PHEROMONE PRODUCTION IN THE HYBRIDS OF *Ostrinia zealis* AND *O. scapulalis*

Cross[a]	Phenotype		Number of progenies		Inferred genotype
	Components	Blend type	Observed	Expected[b]	
Zea	3	Zea	36	36	$phr^{(zea)}A^{E(zea)}/phr^{(zea)}A^{E(zea)}$
Sca^E	2	Sca^E	25	25	$PHR^{(sca)}A^{E(sca)}/PHR^{(sca)}A^{E(sca)}$
Sca^Z	2	Sca^Z	47	47	$PHR^{(sca)}A^{Z(sca)}/PHR^{(sca)}A^{Z(sca)}$
Zea × Sca^E	2	Sca^E-like	34	34	$phr^{(zea)}A^{E(zea)}/PHR^{(sca)}A^{E(sca)}$
Zea × Sca^Z	2	Sca^I-like	33	33	$phr^{(zea)}A^{E(zea)}/PHR^{(sca)}A^{Z(sca)}$
$ZeaSca^E$ × Zea	3	Zea-like	28	26.5	$phr^{(zea)}A^{E(zea)}/phr^{(zea)}A^{E(zea)}$
	2	Sca^E-like	25	26.5	$PHR^{(sca)}A^{E(sca)}/phr^{(zea)}A^{E(zea)}$
Zea × $ZeaSca^E$	3	Zea-like	26	30.5	$phr^{(zea)}A^{E(zea)}/phr^{(zea)}A^{E(zea)}$ $phr^{(zea)}A^{E(zea)}/phr^{(zea)}A^{E(sca)}$ [c]
	2	Sca^E-like	35	30.5	$phr^{(zea)}A^{E(zea)}/PHR^{(sca)}A^{E(sca)}$ $phr^{(zea)}A^{E(zea)}/PHR^{(sca)}A^{E(zea)}$ [c]
$ZeaSca^Z$ × Zea	3	Zea-like	24	30	$phr^{(zea)}A^{E(zea)}/phr^{(zea)}A^{E(zea)}$
	3	Atypical	15	0	Unexplainable
	2	Sca^I-like	21	30	$PHR^{(sca)}A^{Z(sca)}/phr^{(zea)}A^{E(zea)}$
	2	Sca^E-like	0	0	
Zea × $ZeaSca^Z$	3	Zea-like	29	34	$phr^{(zea)}A^{E(zea)}/phr^{(zea)}A^{E(zea)}$
	3	Atypical	10		$phr^{(zea)}A^{E(zea)}/phr^{(zea)}A^{Z(sca)}$ [c]
	2	Sca^I-like	14	34	$phr^{(zea)}A^{E(zea)}/PHR^{(sca)}A^{Z(sca)}$
	2	Sca^E-like	15		$phr^{(zea)}A^{E(zea)}/PHR^{(sca)}A^{E(zea)}$ [c]

[a] Zea, Sca^E, and Sca^Z represent *O. zealis*, E-type *O. scapulalis*, and Z-type *O. scapulalis*, respectively. For further details, see text.

[b] The number of phenotypes was expected from our single-major-gene ($phr^{(zea)}/PHR^{(sca)}$) model for Z9 production.

[c] Recombination of chromosomes occurs only in males in *Lepidopteran* species (Tazima, 1964).

In summary, the present study suggests that the production of Z9, the major pheromone component of *O. zealis*, is regulated by a recessive autosomal gene $phr^{(zea)}$and additional genetic modifiers. The $phr^{(zea)}$ gene is suggested to be linked to $A^{E(zea)}$, a gene on the same locus as $A^{E(sca)}$ (or $A^{Z(sca)}$), which controls the blend ratio of E11 and Z11 in *O. scapulalis*.

Biosynthesis of Sex Pheromones in O. scapulalis and O. zealis. Moth sex pheromones are biosynthesized through a relatively simple cascade. Typically, C_{18} or C_{16} saturated fatty acids derived from the fatty acid synthesis cycle are modified by chain-shortening, desaturation, and reduction, and the resultant unsaturated fatty alcohols are further modified to acetate or aldehyde (for review, see Tillman et al., 1999). The pheromones, therefore, are likely to be relatively easily changed by a few mutations in genes coding the enzymes involved in pheromone biosynthesis (Roelofs and Rooney, 2003).

Although few studies have been carried out on pheromone biosynthetic pathways in *O. scapulalis* and *O. zealis*, both the genetics and biosynthesis of

sex pheromone are well understood in a congener, *O. nubilalis*, which is phylogenetically very close to *O. scapulalis* (Kim et al., 1999) and shows pheromone polymorphism (E- and Z-races) similar to *O. scapulalis* in terms of both pheromone components and their blend ratios (Roelofs et al., 1987, 2002; Zhu et al., 1996b). In *O. nubilalis*, two alleles at an autosomal locus, $A^{E(nub)}$ and $A^{Z(nub)}$, are concerned with the regulation of pheromone blend (Roelofs et al., 1987). Zhu et al. (1996b) showed that the large difference in pheromone blend is attributable to the fatty acyl reduction step in the biosynthetic pathway (cf. Figure 2), and suggested that $A^{E(nub)}$ and $A^{Z(nub)}$ are involved in the control of the stereospecificity of fatty acyl reductase. $A^{E(sca)}$ and $A^{Z(sca)}$ in *O. scapulalis* and $A^{E(zea)}$ in *O. zealis* are inferred to be also involved in the control of the specificity of reductase (Figure 2).

In *O. nubilalis*, Z11-14:Acyl and E11-14:Acyl, but not Z9-14:Acyl, are found in the pheromone gland, being consistent with production of Z11 and E11, and nonproduction of Z9. Here, a curious finding was the accumulation of a large amount of apparently unused Z11-16:Acyl in the gland (Linn and Roelofs, 1995; Ma and Roelofs, 2002). Recently, a functional assay of the Δ11-desaturase gene of *O. nubilalis*, *Onu-Z/E11*, using a yeast pYES2 expression system, showed that the desaturase enzyme produces mainly Z11-16:Acyl, along with a mixture of Z11- and E11-14:Acyls (Roelofs et al., 2002). Thus, it appears that in the pheromone gland of *O. nubilalis*, Z11-16:Acyl is produced as a major "byproduct" of Δ11-desaturase (cf. Figure 2). In the present study, production of Z9 in *O. zealis* was suggested to be controlled by a recessive gene, $phr^{(zea)}$. Control of Z9 production by $phr^{(zea)}$ and its allele $PHR^{(sca)}$ can be explained if we assume that this locus is involved in the blockage of chain-shortening reaction of Z11-16:Acyl to Z9-14:Acyl (Figure 2). In *Ostrinia* with a three-component pheromone (*O. zealis*, which carries $phr^{(zea)}$), the chain-shortening reaction is actively occurring, while in species with a two-component pheromone (*O. scapulalis* and *O. nubilalis*, which carry $PHR^{(sca)}$ or its equivalent), Z9-14:Acyl, and hence Z9, is not produced due to blockage of this reaction. Chain-shortening steps in the biosynthetic pathway were demonstrated to be affected in a pheromone mutant strain of *Trichoplusia ni* (Jurenka et al., 1994), and can be important in causing divergence in the pheromone composition.

Divergence of Sex Pheromone Communication Systems in O. scapulalis and O. zealis. For the divergence of communication systems to occur in a species, the signal senders and receivers have to coevolve in the same direction. This is a difficult process because the signal senders' traits and receivers' traits are rarely genetically coupled (Boake, 1991). Sexual communication systems are under strong stabilizing selection (Butlin and Trickett, 1997); individuals who send an outlying signal will be selected away rapidly because no conspecific receivers can recognize it and they fail to mate and reproduce. In

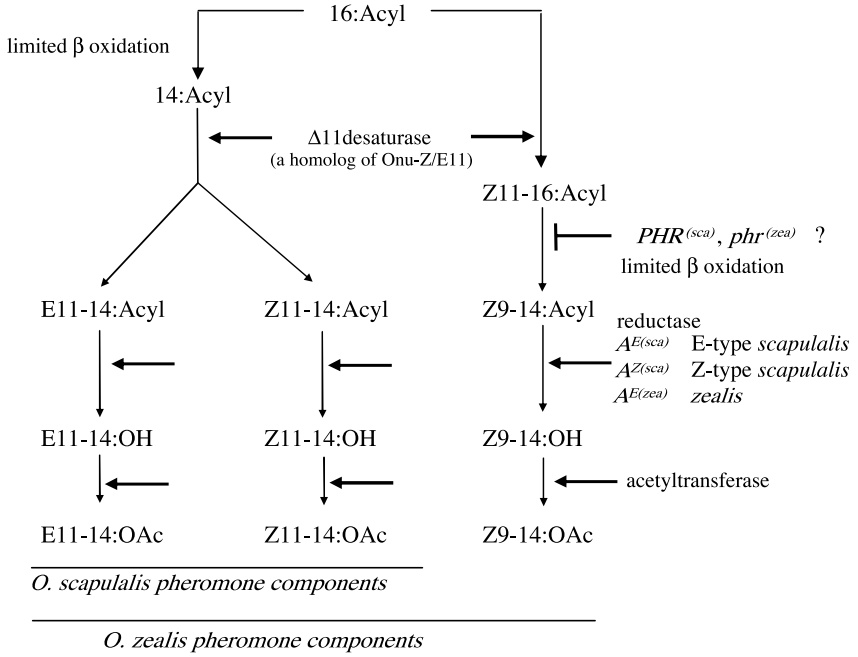

FIG. 2. Biosynthetic pathway and enzymes (genes) involved in the production of pheromone components in *O. scapulalis* and *O. zealis* inferred from the findings of the present study and information available on pheromone biosynthesis in a congener *O. nubilalis* (Roelofs et al., 1987, 2002; Linn and Roelofs, 1995; Zhu et al., 1996b; Roelofs and Rooney, 2003). The symbol resembling a rotated "T" indicates inhibition. The chemical formulae are symbolized as follows: Z = (*Z*)-configuration, E = (*E*)-configuration, the number following Z or E = the position of the double bond, the number before the colon = carbon chain length, Acyl = carboxyl, OH = alcohol, OAc = acetate of alcohol.

this regard, *O. nubilalis*, which shows a distinct sex pheromone variation within a species, has been a good model for studying the evolution of pheromone communication systems (Zhu et al., 1996a; Butlin and Trickett, 1997; Roelofs et al., 2002). Based on detailed genetic analyses of pheromone production in *O. nubilalis*, Zhu et al. (1996a) found that a part of the variations in the pheromone production are hidden due to canalization, and they suggested that these hidden variations played an important role in the divergence of a pheromone communication system.

In the present study, a recessive genetic factor ($phr^{(zea)}$) that alters the signal, sex pheromone composition, was discovered. Recessive gene mutations on signal traits may be able to alleviate selection pressures at a low frequency, because the mutated gene will remain mostly cryptic (Falconer, 1989). If a new

signal trait can be sustained in a population for an extended period, albeit at a low frequency, there would be a chance for this trait to be tracked by receivers with variant preference. Therefore, $phr^{(zea)}$ may explain the divergence of the sex pheromone communication system between *O. scapulalis* and *O. zealis*, i.e., a two-component system (E11 and Z11) and a three-component system (Z9, E11, and Z11). Regarding the male response, our preliminary study showed that variations in male response behaviors in hybrids between Sca^{E} and Zea and their backcrossed progenies could be largely explained by an autosomal locus that determines preference for, or rejection of, Z9 (J. Tabata, unpublished data). Further studies on the genetics of male responses are necessary to clarify the mechanism underlying the divergence of pheromone communication systems in *Ostrinia*.

Despite intensive phylogenetic analyses based on mitochondrial gene sequences, the phylogeny of *Ostrinia* species is not completely determined (Kim et al., 1999; S. Hoshizaki et al., unpublished data). At present, therefore, we cannot conclude the direction of the change, namely, which of the two systems (two-component or three-component) is more ancestral. The genus *Ostrinia* comprises 21 species worldwide (Mutuura and Munroe, 1970; Ohno, 2003). Studies on the sex pheromones of seven species inhabiting Japan have shown that the number of pheromone components alone varies from one to three (Fu et al., 2004). More extensive studies on the sex pheromones of *Ostrinia*, along with studies on the phylogeny of this genus, are needed to clarify the evolution of sex pheromone communication systems in *Ostrinia*.

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COMPARATIVE INVESTIGATION OF THE VOLATILE URINARY PROFILES IN DIFFERENT *Phodopus* HAMSTER SPECIES

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Abstract—Stir bar sorptive extraction method was used for investigation of the urinary volatile profiles in male and female *Phodopus campbelli* and *Phodopus sungorus* hamsters. Additionally, female *Phodopus roborowsky* urinary profiles were characterized. A quantitative analytical approach allowed comparisons of 17 selected compounds in urine. Results showed that *campbelli* and *sungorus* species show similar urinary volatile profiles for males and females. Differences appeared only in concentrations. Several unique compounds, such as pyrazine derivatives, were found to be gender- and age-specific. *P. roborowsky* females exhibited a completely different urinary volatile profile from *campbelli* and *sungorus* females, featuring a unique set of substituted quinoxalines.

Key Words—Pheromone candidates, hamster urine, urinary volatiles, *Phodopus campbelli*, *Phodopus sungorus*, *Phodopus roborowsky*, stir bar, sorptive extraction, gas chromatography–mass spectrometry.

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INTRODUCTION

Urinary scent marking is an important means of chemical communication in mammals. Examples include rodents (Ralls, 1971; Novotny et al., 1990), canids (Jorgenson et al., 1978; Raymer et al., 1984), and red deer (*Cervus elaphus*) (Bakke and Figenschou, 1990), among others. Urinary scent marks may convey multiple messages, ranging from the species chemosignals and individual-recognition compound patterns to aggression and dominance signaling to various primer pheromone activities. Chemical identification of various scent constituents has become essential in elucidating the physiological and behavioral responses pertaining to olfaction. Such activities have thus far been most successful with investigations in the house mouse (*Mus domesticus*) where distinct chemical compounds have been linked to endocrinology and behavior (for review, see Schwende et al., 1986; Novotny et al., 1990), whereas their direct action on the vomeronasal neurons has also been verified (Leinders-Zufall et al., 2000; Sam et al., 2001).

Hamsters have been yet another frequently studied rodent in terms of olfaction. Here most studies have centered on investigations of flank gland markings (in both genders) and female vaginal secretions. Gland marking behavior of the Syrian golden hamster (*Mesocricetus auratus*) has been well established (Johnston, 1975, 1985), whereas the dense and viscous urine of this desert animal is generally viewed as less important in terms of chemical communication. Vaginal secretions of the Syrian golden hamster are also involved in a distinct communication function (Johnston, 1974; Singer et al., 1976) containing aphrodisin (a protein of the lipocalin family). However, it is improbable that aphrodisin serves a direct pheromonal function (Singer et al., 1987; Singer and Macrides, 1990; Vincent et al., 2001); it is more likely that it functions as a pheromone carrier (Briand et al., 2000).

Among the additional members of the hamster family, such as *Phodopus sungorus* (*P. sungorus*), *P. campbelli*, and *P. roborowsky*, fewer studies have accumulated to date. Secretions of the ventral gland and supplementary sacculi have recently been characterized chemically (Burger et al., 2001a,b). Additional glands and secretions may also be used in chemical communication. Unlike with the Syrian hamster, the *Phodopus* hamsters seem to involve urine odors (Lai et al., 1996). In a recent study of the effects of cross-fostering between *P. campbelli* and *P. sungorus* (Vasilieva et al., 2001), the young hamster appeared to learn quickly the olfactory cues from family members. Whereas we do not know currently the origin of the scent cues used in the adaptation process, the urinary odor (if composed of chemically distinct molecules) would be suggested. Earlier studies (Laska and Hudson, 1995) demonstrated that—at least in the squirrel monkey (*Saimiri sciureus*)—urine contains a considerable amount of information of a potential signal value. Consequently, the purpose of this study

was to characterize the volatile urinary constituents of *P. campbelli* and *P. sungorus* with respect to gender and age. In addition, due to the availability of a few *P. roborowsky* urinary samples, a comparison was also made with this species.

Whereas species and individual recognition may involve quantitative arrays of different chemosignals, it was deemed necessary to employ a highly quantitative technique for the extractions of urinary organic constituents. Based on the previously described stir bar extraction procedure for aqueous media (Baltussen et al., 1999, 2002), we have recently adapted this methodology to quantitative profiling of volatile and semivolatile compounds in biological media (Soini et al., 2005). Its excellent reproducibility has permitted reliable determinations of differences in volatile urinary profiles of different hamster species in this study. Numerous chemically distinct profile constituents were subsequently identified through a combined capillary gas chromatography–mass spectrometry and quantified with an element-specific detector.

METHODS AND MATERIALS

Animals and Age Groups. All three species of the genus *Phodopus* (*P. campbelli*, *P. roborowsky*, and *P. sungorus*) were available; gender, ages, and number of animals are listed in Table 1. Animals were born and raised in captivity and kept in indoor rooms in solid-bottom, polycarbonate cages (30 × 15 × 15 cm) with wood-chip bedding material. For all animals, the bedding material was identical (autoclaved, natural wood chips from pinewood). Bedding material was not subjected to chemical analyses. Since *Phodopus* hamsters are social

TABLE 1. CODES AND AGES FOR THE *Phodopus* HAMSTER GROUPS

Species	Subject groups	Age (months)	Number of animals
Phodopus campbelli female	cf4	4	3
	cf9	9	7
P. campbelli male	cm4	4	4
	cm9	9	3
	cm14	14	3
Phodopus sungorus female	sf1	1	3
	sf10–11	10–11	7
P. sungorus male	sm1	1	4
	sm10–11	10–11	5
	sm14	14	1
Phodopus roborowsky female	rf12	12	4

animals, they were kept in groups (three to five animals) of the same sex or in family units (a pair and its litter) for several weeks. The colony was maintained on a 14-hr light/10-hr dark light cycle with lights off at 10:00 hr; temperature was 21 ± 1°C. All animals had free access to hamster chew and water. Sunflower seeds, fruits, and lettuce were occasionally provided as a dietary supplement.

In *P. campbelli* and *P. sungorus*, the males and females represented different age groups. In *Phodopus* species, sexual maturation is reached between postnatal days 30 and 45. During the first 4 mo of life, animals may be regarded as young adults; animals between 9 and 14 mo-old are in their prime. Life span lasts for up to 2 yr.

Sample Collection. Urine was collected from all three hamster species (University of Tübingen, Germany). To collect urine, animals were removed from colonies and kept individually in small metabolic cages until they produced about 1 ml of urine, or up to 4 hr. If an animal did not produce enough urine in one sampling session, the procedure was repeated the following day. Estrous female hamsters were not subjected to urine collection (estrous state was checked regularly). Samples were kept frozen until analyzed.

Sample Preparation. All glassware was washed with distilled water and acetone and dried at 80°C in the oven. Volatile and semivolatile compounds were extracted from 1.0 ml of undiluted urine by sorptive extraction with a Twister PDMS polymer-coated stir bar (10 mm, 0.5-mm film thickness, 24 µl PDMS volume, Gerstel GmbH, Mülheim an der Ruhr, Germany) for 60 min. Stirring speed was 800+ rpm on the Variomag Multipoint HP 15 stirplate (H+P Labortechnic, Oberschleissheim, Germany). After 60-min extraction time, a stir bar was rinsed with a small amount of distilled water, dried gently on the paper tissue, and was placed in the glass injector liner for mass spectrometry (MS) identification or in the TDSA autosampler tube for a gas-chromatographic (GC) quantification.

Mass Spectrometry. A Finnigan MAT Magnum ion trap gas chromatograph–mass spectrometer (GC-MS) system was used for the compound identification (Finnigan MAT, San Jose, CA, USA). The system was provided with a DB-5 capillary column (30 m × 0.25 mm, i.d., 0.25-µm film thickness, J&W Scientific, Folsom, CA, USA). Helium carrier gas head pressure was 12 psi. At the beginning of the column, a loop of uncoated deactivated silica tubing (30 cm × 0.25 mm, i.d.) was attached by using a universal Press-Tight Connector (Restek Corporation, Bellefonte, PA, USA) as described earlier (Ma et al., 1999). The loop was cooled with liquid nitrogen, while the Twister stir bar was held in the injector liner for 15 min at 250°C for the thermal desorption of the analytes. Subsequently, the desorbed compounds were cryotrapped into the liquid nitrogen cooled loop. After removing liquid nitrogen cooling, the GC temperature was held at 40°C for 5 min and increased to 200°C at the rate of 2°C/min. The final temperature was held for 10 min. The manifold and transfer line temperatures were 220 and

300°C, respectively. The ion trap was operating in the positive electron ionization mode. Spectra were scanned from 40 to 350 msu (1 scan/sec).

Gas Chromatography. Gas-chromatographic (GC) equipment for the quantitative analysis consisted of an Agilent GC Model 6890 with an Atomic Emission Detector (AED) Model G2350A (Agilent Technologies Inc., Wilmington, DE, USA) and a Thermal Desorption Autosampler (TDSA, Gerstel GmbH). The separation capillary was DB-5 (30 m × 0.25 mm, i.d., 0.25-μm film thickness from J&W Scientific Folsom, CA, USA). Samples were thermally desorbed in a TDSA automated system, followed by injection into the column with a cooled injection system CIS-4. TDSA operated in a splitless mode. Temperature program for desorption was 20°C (0.5 min), then 60°C/min to 280°C (10 min). Temperature of the transfer line was set at 280°C. CIS was cooled with liquid nitrogen to −60°C. After desorption and cryotrapping, CIS was heated at 12°C/sec to 280°C with the holding time of 10 min. Temperature program in the GC was 40°C for 5 min, then increasing to 200°C at the rate of 2°C/min. The final temperature was held for 10 min. Carrier gas head pressure was 14 psi (flow rate 1.2 ml/min). The GC unit was operated in the constant flow mode. The emission lines for carbon (193-nm), sulfur (181-nm), and nitrogen (174-nm) were monitored in the atomic plasma emission detection.

Statistical Analysis. Pirouette Lite (Infometrix, Inc., Woodinville, WA, USA) was used for exploratory multivariate analysis to obtain hierarchical cluster patterns for classification of analysis data and for establishment of chemical relations within the subject groups. When appropriate, group comparisons were calculated using the nonparametric Mann–Whitney *U* test.

RESULTS AND DISCUSSION

According to the literature (Sokolov and Vasilieva, 1993; Vasilieva et al., 1990) and the results presented here, *P. campbelli* and *P. sungorus* seem closely related. Yet, they are widely considered as two distinct species. Therefore these two species were compared against each other. *P. roborowsky* will be discussed separately.

I. Campbelli and Sungorus Groups. Campbelli and *sungorus* hamster groups each consisted of 10 males and females (Table 1). Preliminary screening showed that both groups exhibited relatively similar chemical constituents in their profiles. Differences were seen mainly in the levels of compounds within the same age and gender groups. Gender and age inflicted certain profile differences, which will be discussed in more detail below.

The compounds identified by GC-MS in the adult animals are shown in Table 2A. A short list of compounds (numbers 1–17) in Table 2B indicates

TABLE 2A. IDENTIFIED AND PARTIALLY IDENTIFIED COMPOUNDS IN URINE OF MALE *P. sungorus* AND *P. campbelli* HAMSTERS (AGE 10–11 MO)

Compound	GC-MS (min)	RI[a]	Compound	GC-MS (min)	RI[a]
Acetone	1.00	760	An alkenylpyrazine (C-4)	24.48	1165
Butanone	1.42	766	A terpene	24.88	1173
2-Pentanone	1.87	773	2-Nonanol	25.10	1177
3-Pentanone	2.45	781	Phenylethylamine	25.58	1186
3-Hexanone	5.05	819	Phenylacetone	26.55	1204
4-Heptanone	8.82	868	Phenylacetonitrile	27.20	1217
2,6-Dimethylpyridine	9.27	876	A dimethylpropylpyrazine	30.17	1273
2-Heptanone	10.30	896	An ethylpropylpyrazine	30.53	1280
Heptanal	10.33	897	An alkenylpyrazine (C-5)	30.80	1285
2,5-Dimethylpyrazine	10.85	906	Methylsalicylate (food)	31.33	1295
3-Hepten-2-one	12.80	943	A butylmethylpyrazine	31.58	1300
Benzaldehyde	14.08	968	Decanal	32.62	1319
1-Methylpiperidine	15.03	986	Formanilide	32.80	1323
6-Methyl-1-hepten-2-one	15.68	998	Benzothiazole	33.15	1329
Octen-2-ol	16.30	1010	Quinoline	34.08	1347
6-Methyl-5-hepten-2-one	16.43	1012	An alkylpyrazine (C-6)	35.87	1381
Phenol	16.85	1020	Geraniol	36.33	1390
An alkylpyrazine (C-3)	17.20	1027	An alkenylpyrazine (C-6)	36.43	1392
A trimethylpyrazine	17.37	1030	An alkylpyrazine (C-6)	37.02	1403
A propylpyrazine	17.68	1036	2-Undecanol (branched)	37.92	1420
Acetophenone	21.82	1114	Indole	38.42	1429
Methylaniline	22.38	1125	Undecanal	38.67	1434
4-Nonanone	22.82	1133	2-Undecanol (branched)	39.42	1448
1-Octanol	23.03	1138	An alkylpyrazine (C-8)	47.05	1593
6-Methyl-2-octanol	23.82	1152	Geranylacetone	49.20	1634
A methylpropylpyrazine	24.05	1157	An alkylpyrazine (C-8)	50.38	1656
			Vitamin K (menadione from food)	52.47	1696

[a]Retention index on DB-5 column phase.

substances that have been used for quantitative measurements by TDSA-GC-AED. A typical MS total ion chromatogram (TIC) of the male hamster urine is shown in Figure 1. Peak numbers refer to Table 2B.

Compound identification was made by GC-MS based on retention times, spectra, and known standard compounds. Based on the total ion chromatogram (TIC) profiles (as shown in Figure 1), the compound profiles were quantitatively compared with the GC-AED profiles, whereas the peak identities were assigned for these measurements. Figure 2 shows a typical GC-AED compound profile (carbon 193-nm line) for a male *campbelli* hamster.

TABLE 2B. COMPOUNDS QUANTIFIED BY GC-AED

Compound no.	(min)	Compound name
1	8.82	4-Heptanone
2	10.30	2-Heptanone
3	10.85	2,5-Dimethylpyrazine
4	17.68	A propylpyrazine
5	22.38	Methylaniline
6	22.82	4-Nonanone
7	24.05	A methylpropylpyrazine
8	24.48	An alkenylpyrazine (C-4)
9	25.10	2-Nonanol
10	27.20	Phenylacetonitrile
11	30.17	A dimethylpropylpyrazine
12	30.53	An ethylpropylpyrazine
13	32.80	Formanilide
14	36.33	Geraniol
15	36.43	An alkenylpyrazine (C-6)
16	37.02	An alkylpyrazine (C-6)
17	37.92	2-Undecanol (branched)

Within the complex urinary compound profiles, average levels were calculated for 17 identified compounds (Table 2B). Averages were grouped pertaining to the different species/gender/age groups. Calculations were based on the corresponding integrated peak areas for each compound. Logarithmic ($\log_{10}$) transformations log(peak area + 1) were used to normalize the graphs due to large numerical values of the integrated peak areas (Zar, 1999).

In general, male hamster urine contained higher levels of all compounds in both *campbelli* and *sungorus* species when compared with females. Typically, urinary profiles in *campbelli* and *sungorus* males were closely related. Figure 3 shows average levels of 17 compounds (from Table 2B) for *campbelli* females (9 mo) and males (9 mo) and *sungorus* females (10–12 mo) and males (10–11 mo).

Several quantified compounds were either gender- or age-specific in *campbelli* and *sungorus*. Also, some of the compounds were typical for the particular hamster species. Hamster groups *campbelli* females (cf), *campbelli* males (cm), *sungorus* females (sf), and *sungorus* males (sm) were further divided into subgroups based on their ages. The group codes and number of individuals in each group are shown in Table 1. Summary of the identified species/gender/age-specific compounds is shown in Table 3.

Multivariate hierarchical cluster analysis (HCA) in Figure 4 shows "similarity degrees" of different hamster groups based on the averages of 17 quantified compounds. Clusters connected closest to 1.0 on the *X*-axis mark

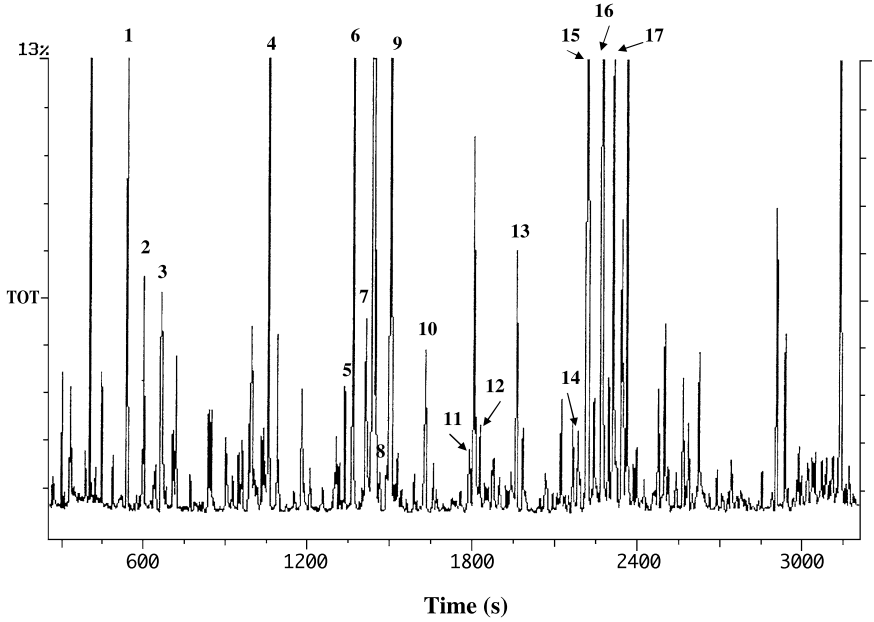

FIG. 1. A GC-MS total ion chromatogram (TIC) of the SBSE-extracted urine of a male *P. sungorus* hamster. Analytical conditions are described in the text. Numbers refer to identified compounds in Table 2B used for quantification by GC-AED.

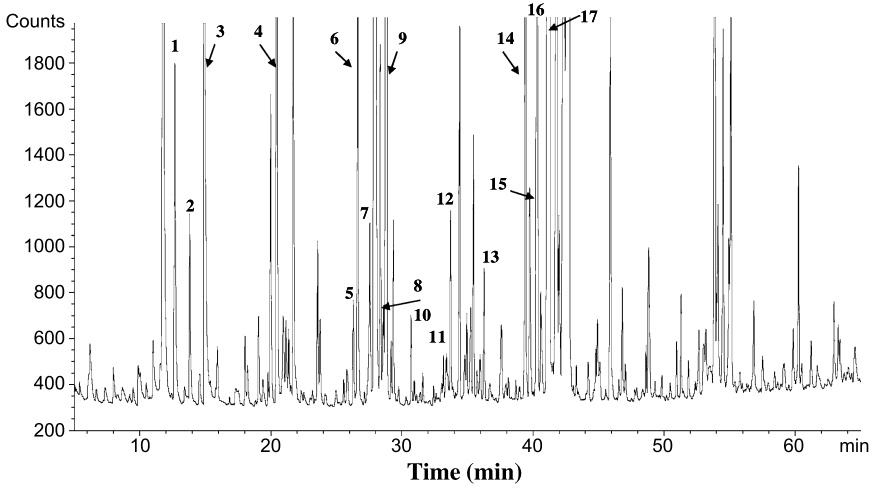

FIG. 2. A urinary volatile profile of a male *P. campbelli* hamster (cm-9) by GC-AED, carbon line 193-nm. Separation conditions are described in the text.

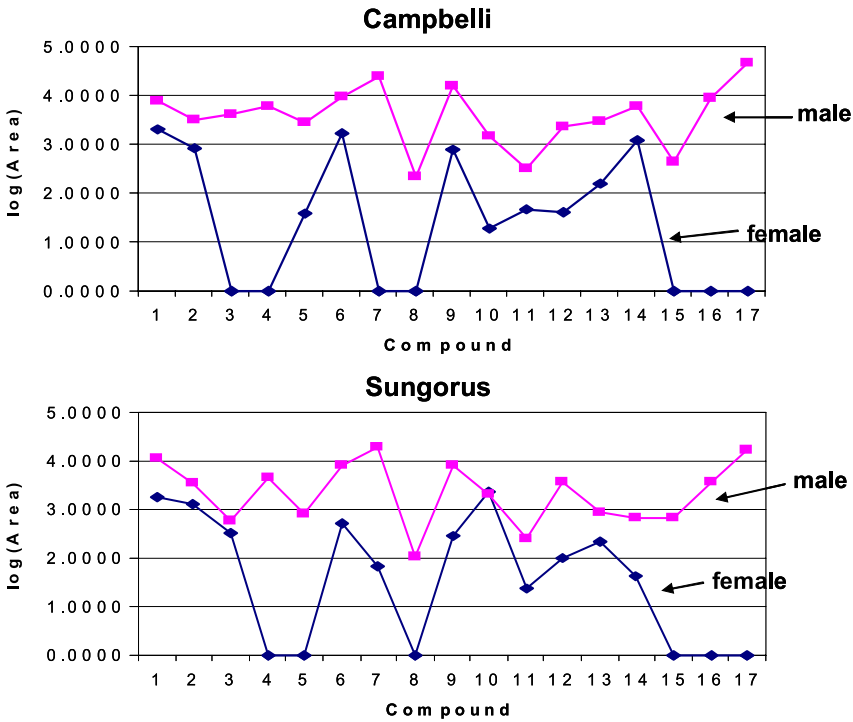

FIG. 3. Averages of levels for compound numbers 1–17 for mature female and male *campbelli* and *sungorus* hamsters. Logarithmic values for peak areas were used [log(peak area + 1)].

the most similar group properties. As shown in Figure 4, volatile profiles of male *campbelli* and *sungorus* hamsters for 17 identified compounds relate closely to each other. Within the corresponding female profiles, relation is not so clear.

Additional comparative qualitative information was obtained from compound profiles by GC-AED sulfur 181-nm line chromatograms (Figure 5). GC-AED facilitated detection of sulfur compound profiles at trace picogram levels (Soini et al., 2005). Characterization by GC-MS was not successful for these compounds due to low levels. As noted for the GC-AED carbon 193-nm line chromatograms (average results in Figure 3), also sulfur-containing, yet unidentified compounds appeared at higher levels in male hamster urine than in female urine.

Method Precision. Reproducibility of extraction and the GC analysis was investigated with the pooled male *campbelli* hamster urine. Four parallel

TABLE 3. SUMMARY OF GENDER- AND AGE-SPECIFIC COMPOUNDS AND THEIR AVERAGE[a] VALUES FOR *P. campbelli* AND *P. sungorus* GROUPS QUANTIFIED BY GC-AED

Compound		*P. campbelli*				*P. sungorus*			
		Adult		Juvenile		Adult		Juvenile	
No.	Name	Male (9)	Female (9)	Male (4)	Female (4)	Male (10–11)	Female (10–11)	Male (1)	Female (1)
3	2,5-Dimethylpyrazine	3.6	0	3.9	0	2.4	2.1	0	0
4	A propylpyrazine	3.8	0	3.9	0	3.6	0	0	0
5	Methylaniline	3.5	0.9	3.5	2.1	2.7	0	0	0
7	A methylpropylpyrazine	4.4	0	4.7	0	4.3	1.3	3.7	0
8	An alkenylpyrazine (C-4)	2.0	0	4.1	0	1.4	0	0	0
10	Phenylacetonitrile	3.2	0.4	0.8	0.3	3.3	3.4	0	0
14	Geraniol	3.8	0.9	1.9	0	2.4	0.9	1.7	0
15	An alkenylpyrazine (C-6)	2.3	0	3.3	0	2.6	0	0	0
16	An alkylpyrazine (C-6)	3.9	0	4.4	0	3.6	0	2.6	0
17	2-Undecanol (branched)	4.7	0	2.9	0	4.2	0	4.6	0

[a]Log (peak area + 1).

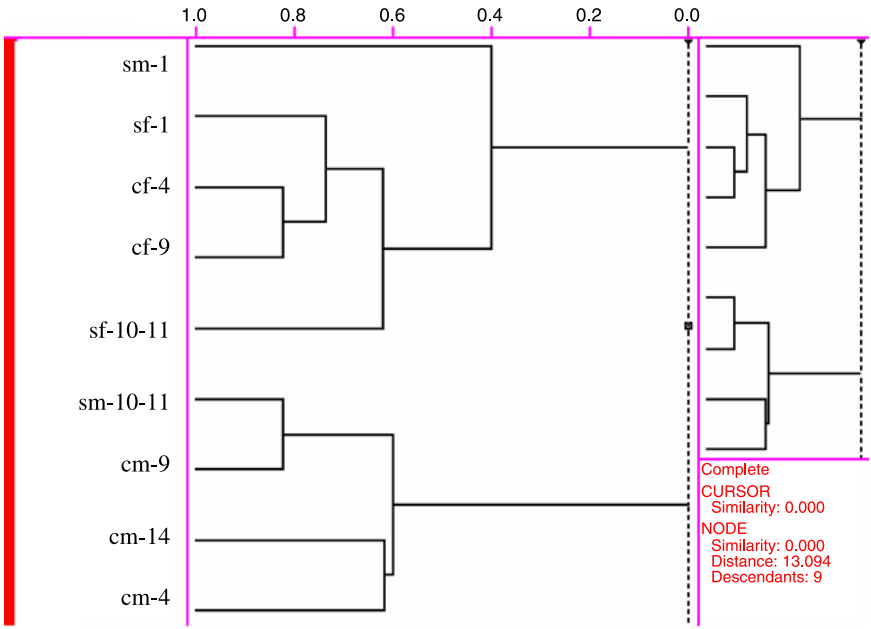

FIG. 4. A hierarchical cluster analysis (HCA) graph for all hamster groups using levels of compounds 1–17 as variables (see Table 2B).

extractions were performed, and the relative standard deviations of peak areas were calculated (RSD). Typical variability for the analytes was between 0.1 and 2.0% (RSD, $N = 4$) as shown in Table 4. A uniform sorptive polymer coating, automated thermal desorption sample introduction, and a constant flow control in the GC-AED system have all contributed to the acceptable reproducibility of the results when no internal standard was deemed necessary.

Role of Pyrazines. In all male *campbelli* and *sungorus* urinary profiles, the presence of pyrazines was a dominating factor, as seen in Table 2A. Compounds with numbers 3, 4, 7, 8, 12, 15, and 16 (Table 2B) appeared to be related to gender and age in both hamster groups. *Campbelli* males showed more *male-specific pyrazines* (2,5-dimethylpyrazine, a propylpyrazine, a methylpropylpyrazine, a C-6 alkylpyrazine, and alkenyl C-4 and C-6 pyrazines). In the *sungorus* group, female urine also contained 2,5-dimethylpyrazine and a C-4 alkylpyrazine, however, with the latter appearing at higher levels in *sungorus* males than in females ($P < 0.02$). Ethylpropylpyrazine (compound 12) levels were significantly higher in both *campbelli* ($P < 0.002$) and *sungorus* males ($P < 0.02$) compared with females. Ethylpropylpyrazine levels were also significantly higher in mature *sungorus* males (10–14 mo) compared with

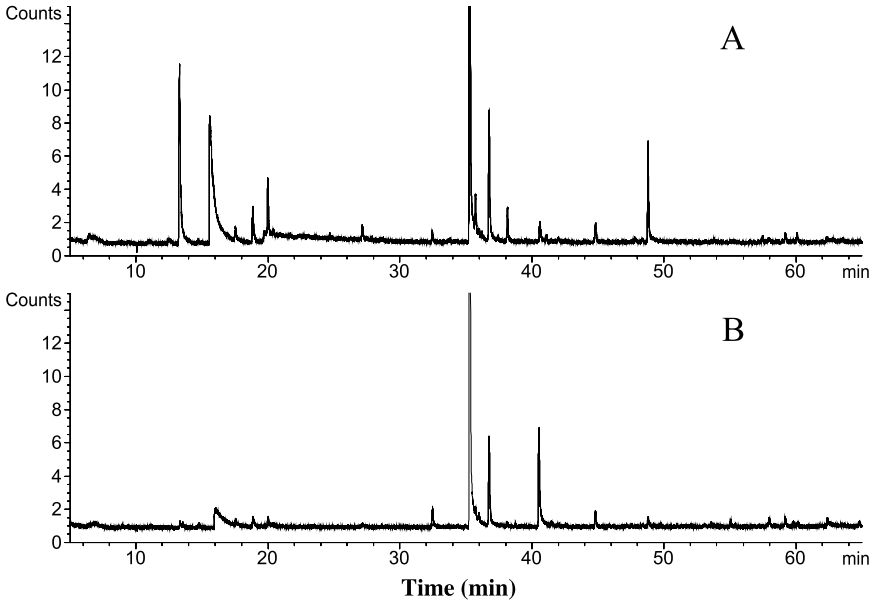

FIG. 5. Comparative urinary volatile profiles for pooled (A) male *sungorus* and (B) female *sungorus* urine by GC-AED sulfur line 181-nm. Compounds were not identified.

juvenile (1 mo) animals ($P < 0.002$). In all *campbelli* and *sungorus* females, the less volatile alkylated C-6 pyrazines (compounds 15 and 16) were absent (below detection limit). Urinary alkenyl C-4 and C-6 pyrazines (compounds 8 and 15) appeared higher in old male hamsters as well as in younger individuals, although the differences were not statistically significant. Figure 6 illustrates average levels of unsaturated alkenyl pyrazines in the *campbelli* male urinary profiles in the different age groups. Large differences in pyrazine levels within gender and age suggest that pyrazines may be under endocrinological control and may thus be an important means of chemical communication for both *campbelli* and *sungorus* hamsters.

Ketones. Relatively small differences of the levels of 4- and 2-heptanone over the age, gender, and species groups were observed. Figure 7 shows that 2-heptanone levels, as an example (compound 2), in *campbelli* (9 mo) and *sungorus* (10–11 mo) males were relatively close to each other. The same applied to *campbelli* (9 mo) and *sungorus* (10–12 mo) females (data not shown). Statistically significant higher levels of 4-nonanone were found in male urine compared with females in both *campbelli* ($P < 0.02$) and *sungorus* groups ($P < 0.002$). These findings suggest that urinary ketones may not carry specific signaling properties as pyrazines do for *campbelli* and *sungorus* hamsters,

TABLE 4. REPRODUCIBILITY OF 17 QUANTIFIED COMPOUNDS BY GC-AED EXEMPLIFIED BY DETERMINATION OF SAMPLES OF POOLED MALE *P. campbelli* URINE ($N = 4$)

Compound no.	Compound	Average log (peak area)	Standard deviation (SD) of log (peak area)	Relative standard deviation of log (peak area) (RSD %, $N = 4$)
1	4-Heptanone	3.573	0.077	2.2
2	2-Heptanone	3.123	0.040	1.3
3	2,5-Dimethylpyrazine	3.559	0.071	2.0
4	A propylpyrazine	3.548	0.035	1.0
5	Methylaniline	3.014	0.025	0.8
6	4-Nonanone	3.543	0.003	0.1
7	A methylpropylpyrazine	4.610	0.034	0.7
8	An alkenylpyrazine (C-4)	3.564	0.028	0.8
9	2-Nonanol	3.577	0.013	0.4
10	Phenylacetonitrile	Below detection limit		
11	A dimethylpropylpyrazine	3.040	0.033	1.1
12	An ethylpropylpyrazine	3.064	0.021	0.7
13	Formanilide	3.455	0.054	1.6
14	Geraniol	3.226	0.021	0.6
15	An alkenylpyrazine (C-6)	3.873	0.011	0.3
16	An alkylpyrazine (C-6)	4.523	0.014	0.3
17	2-Undecanol (branched)	4.024	0.013	0.3

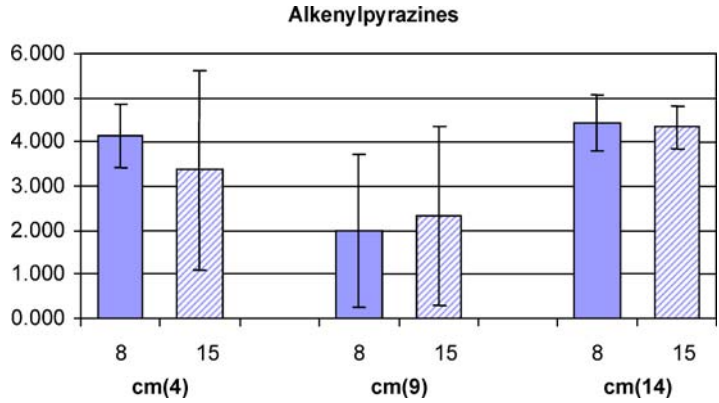

FIG. 6. Averages of alkenyl pyrazine compounds 8 and 15 (see Table 2B) in different male *campbelli* age groups (ages 4, 9, and 14 mo).

although they may be involved in the creation of the baseline scent for the species.

Alcohols. Among the identified alcohols, branched 2-undecanol (compound 17) appeared male-specific in both *campbelli* and *sungorus* species (Figure 3). Surprisingly, branched 2-undecanol levels were already high in the urinary profiles of young *sungorus* males. Levels were declining with age. Geraniol (a terpene alcohol, compound 14) levels appeared somewhat higher in male and female *campbelli* mature hamsters (not a statistically significant

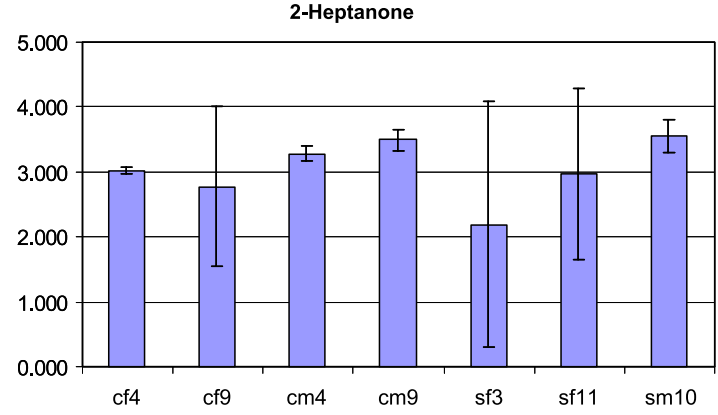

FIG. 7. Average levels of 2-heptanone (compound 2) in different hamster groups.

difference). Only in the *campbelli* group, were geraniol levels higher in males than in females ($P < 0.02$). Young females in both *campbelli* and *sungorus* groups lacked geraniol in their profiles. A branched alcohol, 2-nonanol (compound 9), was found in all age, gender, and species groups. *Sungorus* females showed the lowest levels of 2-nonanol (data not shown) from all groups.

Selected Nitrogen Compounds. Methylaniline (compound 5), phenylacetonitrile (compound 10), and formanilide (compound 13) were independent variables related to each other based on a hierarchical cluster analysis (HCA, data not shown). Within the male profiles, methylaniline was specific for the *sungorus* males only (Figure 3). In the male profiles, methylaniline was shown only in the age group of 9–11 mo (not seen in juvenile and 14-mo-old males). Phenylacetonitrile was not seen in the urine of immature female or male *sungorus* hamsters (1 mo). Among mature *sungorus* males and females, there were no statistically significant differences, whereas the levels in male mature *campbelli* were higher than in females ($P < 0.02$).

Figure 8 shows comparisons of the averages of selected compounds in *campbelli* (9 mo) and *sungorus* (10 mo) male urinary profiles. *Campbelli* average levels tended to be slightly higher than *sungorus* levels. However, the individual variation in their concentrations was clearly larger in the *sungorus* group.

II. P. roborowsky. Within the *roborowsky* group (four female subjects), a completely different urinary volatile pattern was seen. A list of identified or tentatively identified *roborowsky* female urinary compounds is shown in Table 5.

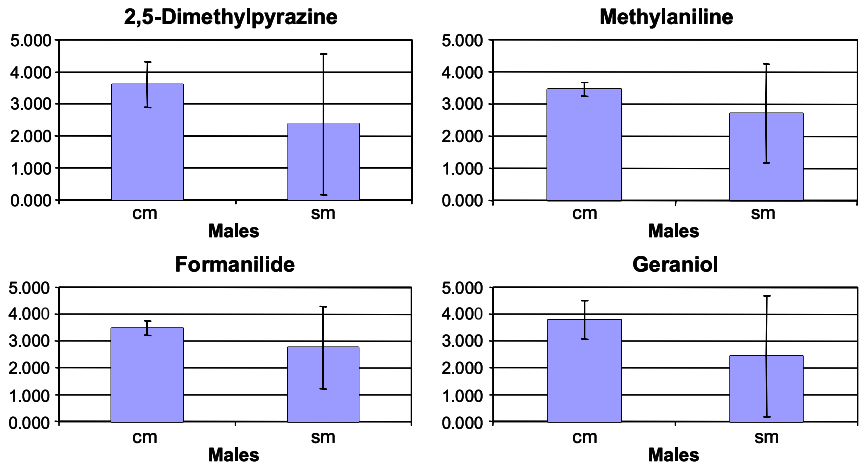

FIG. 8. Comparison of averages and standard deviations of selected compound levels in *campbelli* (cm) and *sungorus* (sm) urinary volatile profiles.

TABLE 5. IDENTIFIED AND PARTIALLY IDENTIFIED COMPOUNDS IN THE URINE OF FEMALE *P. roborowsky* (AGE 12 MO)

Compound	Rt (sec)	Rt (min)	RI[a]
4-Heptanone	544	9.07	879
2-Heptanone	607	10.12	895
3-Hepten-2-one	779	12.98	937
6-Methyl-3-heptanone	847	14.12	954
Benzaldehyde	857	14.28	956
Phenol	996	16.60	990
Acetophenone	1310	21.83	1068
1-Octenol	1356	22.60	1079
1-Octanol	1369	22.82	1082
A cresol	1393	23.22	1088
An unknown pyrazine	1422	23.70	1095
2-Nonanone	1454	24.23	1103
A ketone	1502	25.03	1115
Phenylacetonitrile	1629	27.15	1146
3-Nonen-2-one	1660	27.67	1154
An ester	1730	28.83	1172
An ethylphenol	1790	29.83	1186
1-Nonanol	1820	30.33	1193
Methylsalicylate	1875	31.25	1207
Benzothiazole	1988	33.13	1234
An ester	2020	33.67	1242
An ester	2090	34.83	1260
Geraniol	2163	36.05	1277
A branched ketone	2191	36.52	1284
4-Undecanone	2245	37.42	1298
Indole	2300	38.33	1311
4-Phenyl-3-buten-2-one	2559	42.65	1375
Eugenol	2569	42.82	1377
An alkylquinoxaline (C-3)	2853	47.55	1447
Geranylacetone	2947	49.12	1470
Ethylcinnamate	2981	49.68	1479
A tridecanone	3023	50.38	1489
Menadione	3138	52.30	1517
An alkylquinoxaline (C-4)	3186	53.10	1529
An alkylquinoxaline (C-4)	3220	53.67	1538

[a]Retention index.

Screening of urinary profiles of *P. roborowsky* revealed that few compounds were in common with *campbelli* and *sungorus* (Table 2A). A dominant array of alkyl- and alkenylpyrazines was not present in these samples. Instead, the *roborowsky* urine featured a group of higher-boiling alkyl quinoxalines. The presence of ketones, alcohols, and esters was characteristic for the group of 60

identified compounds. The origin of ketones is likely to be β-and ω-oxidation of fatty acids. This suggests that metabolic pathways in *P. roborowsky* hamsters differ markedly from *P. sungorus* and *P. campbelli* hamsters. Also, based on the large difference on the chemical constituent types in urine, the baseline scent properties of the *roborowsky* female hamster are expected to differentiate from those of *sungorus* and *campbelli* females.

In summary, quantitative data were proven highly reproducible using the stir bar sorptive extraction (SBSE) methodology. Typically, relative standard deviations (RSD, $N = 4$) were 0.1–2.0% for normalized peak areas. This analytical feature provided reliability for the urinary profile comparisons. Ultrahigh sensitivity of the atomic emission detection for sulfur-containing compounds provided extra information about comparative urinary volatile profiles.

The chemical characterization data on *P. campbelli*, *P. sungorus*, and *P. roborowsky* verified relatively similar compound profiles in *campbelli* and *sungorus* (males and females), which differed substantially from *P. roborowsky* (females only, males were not available in the screening study). This suggests that metabolic pathways in *campbelli* and *sungorus* hamsters resemble each other but differ substantially from the *roborowsky* species. In *campbelli* and *sungorus*, different substituted pyrazines dominated the urinary profiles. Several pyrazines and branched 2-undecanol were male-specific in both species. Methylaniline and phenylacetonitrile were age-specific in both male hamster species. The individual compound level variability within the *P. sungorus* was clearly larger than in the *P. campbelli* species.

The urinary profiles of *roborowsky* female hamsters were dominated by ketones, alcohols, and esters. Similar pyrazine arrays, as seen in *campbelli* and *sungorus*, were clearly not observed. Instead, low-volatility alkyl quinoxalines were detected.

One could hypothesize that urinary compound classes such as pyrazines, within a certain volatility range (early eluting pyrazines vs. later eluting pyrazines), may classify the overall perception of the urine odor so that a closely related species may learn the scent codes, as reported (Vasilieva et al., 2001) under cross-fostering conditions. The question remains which urinary compounds carry most crucial information and whether concentration level differences play a role in scent recognition between *campbelli* and *sungorus*. At this time, it is not known whether there is any effect of a seasonal variation on the urinary profiles.

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PREVALENCE OF CHEMICAL DEFENSES AMONG FRESHWATER PLANTS

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Abstract—Although macrophyte–herbivore interactions in freshwater systems were generally disregarded for many years, recent data suggest that herbivory can be intense and important in structuring freshwater communities. This has led to the hypothesis that chemical defenses should be common among freshwater plants, but few studies have reported such chemical defenses, and no previous studies have assessed the frequency of chemical defenses among a substantial number of freshwater plant species. In a study of 21 macrophyte species co-occurring with the omnivorous crayfish *Procambarus acutus* in a southeastern USA wetland environment, we found that extracts of 11 species (52%) deterred feeding by *P. acutus* when tested in artificial foods at natural concentrations. Of these 11 chemically defended species, one species, *Eupatorium capillifolium*, consistently had a more unpalatable extract following mechanical damage to plant tissue, indicative of an activated chemical defense. Because herbivores are commonly nitrogen-limited and select food based on several plant traits, including plant nutritional value, it might be expected that chemical defenses would be especially important for protein-rich plants. However, we found no relationship between soluble protein concentration and deterrence of plant extracts.

Key Words—Chemical defense, herbivory, freshwater, macrophyte, crayfish, *Procambarus acutus*.

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INTRODUCTION

Community structure and plant abundance are strongly influenced by herbivory, with plant–herbivore interactions being well investigated in terrestrial and marine ecosystems (Lubchenco and Gaines, 1981; Fritz and Simms, 1992). Such interactions have been less studied in freshwater systems because it was previously thought that herbivory had minimal impact on freshwater macrophytes (Hutchinson, 1975). However, recent studies show large impacts of herbivory in freshwater ecosystems (e.g., Cyr and Pace, 1993), and this impact should select for macrophyte defenses, as in terrestrial and marine systems (Hay and Fenical, 1988; Rosenthal and Berembaum, 1992). This reasoning led us to investigate the prevalence of antiherbivore chemical defenses among freshwater macrophytes, where only a few specific studies have been conducted, but where no general assessments of many species are available.

Chemical defenses may be constitutive (always present), induced (production up-regulated gradually), or activated (produced immediately) in response to enemy attack (Paul and Van Alstyne, 1992; Karban and Baldwin, 1997). Studies of marine algae indicate that in about 20% of species, greater levels of defense may be activated within seconds of physical damage (Cetrulo and Hay, 2000), but whether activation is rare or common is unknown for freshwater macrophytes. Newman et al. (1990, 1992, 1996) found that in damaged watercress, 2-phenylethyl glucosinolate was enzymatically converted to 2-phenylethyl isothiocyanate, deterring invertebrate feeding. Bolser et al. (1998) found that in the freshwater orchid *Habenaria repens*, the concentration of a deterrent compound, habenariol, increased threefold when cells were lysed.

In this investigation, we asked what proportion of a group of 21 freshwater macrophytes is chemically defended against a co-occurring generalist crayfish, *Procambarus acutus*. We also asked whether mechanical damage to plants led to chemical changes affecting palatability, allowing us to detect some types of activated defenses. We also hypothesized that plants of higher nutritional value to herbivores would be under greater threat of herbivory, would experience greater selection for defenses, and would, therefore, possess more frequent or more potent chemical defenses. To test this, we investigated the relationship between protein content and chemical defenses in this group of plants.

METHODS AND MATERIALS

Study Organisms. We used crayfish as feeding assay organisms because they are generalist feeders, behave well in laboratory assays, and can have great effects on macrophyte biomass in natural systems (e.g., Lodge and Lorman, 1987). The omnivorous crayfish *P. acutus*, commonly found among macro-

phytes, is native to and distributed throughout the southeastern USA, including the Chattahoochee River wetlands in Georgia (Hobbs, 1981). *P. acutus* were collected from wetlands at the Chattahoochee National Recreation Area (Cochran Shoals Unit, near Atlanta, GA, USA) from January to September 2003. Identification of crayfish was accomplished by examination of morphological traits (Hobbs, 1981). They were housed in a water table with recirculating water and were separated by plastic dividers creating 40 chambers measuring 12.5 × 12.5 cm. On days when crayfish were not being used for feeding assays, they were fed with commercial trout pellets.

Multiple sites within the Chattahoochee National Recreation Area wetlands in north Georgia were used for plant collections. Twenty-one freshwater macrophyte species were chosen for study, based on availability from May to October 2003 (see Table 1). We focused on abundant plants and avoided rare plants because of sample size limitations. Identification of macrophytes was accomplished by examination of morphological traits (Godfrey and Wooten, 1979, 1981).

Macrophytes were gently pulled up by the roots, placed in Ziploc bags, and stored on ice for transport to the laboratory at Georgia Institute of Technology. At the lab, plants were gently rinsed, patted dry, weighed, and each collection was sorted into two batches of equal wet mass and volumetric displacement in water. Pressed voucher specimens are stored at the Georgia Institute of Technology.

Chemical Methods. To mimic the shredding action of feeding crayfish and thus simulate herbivore damage, macrophytes were shredded for 45 sec in water using a blender to generate the simulated "grazed" treatment. This method has been used successfully by previous researchers examining activated defenses among macroalgae (Paul and Van Alstyne, 1992; Cetrulo and Hay, 2000). To simulate a nongrazed plant (simulated "nongrazed" treatment), plant material was placed in methanol (MeOH) before blending. This procedure should inhibit enzymatic activity within the plant and prevent activation of chemical defenses. After blending each treatment, plant material and solvent were placed in 1-l flasks, and equal amounts of the opposite solvent were added, resulting in a 1:1 blend of MeOH/H_2O. After extracting for 1 hr at 4°C, extracts were vacuum-filtered, and solvents were removed by rotary evaporation. To maximize both the yield and polarity range of compounds extracted, plant material was further extracted once each with three additional solvent systems: 100% MeOH, then 1:1 MeOH/dichloromethane, and then 1:2 MeOH/dichloromethane. These extracts were combined with the 1:1 MeOH/H_2O extract, dried by rotary evaporation, and stored at −20°C until used in feeding assays.

Feeding Assay Methods. Two types of feeding assays were conducted: (1) tests of extracts from simulated "grazed" treatments were compared to extracts from simulated "nongrazed" treatments, and (2) tests of "grazed" or "nongrazed" treatments were compared to no-extract controls. For each assay,

TABLE 1. FRESHWATER PLANT SPECIES USED IN THIS STUDY, SOME PLANT TRAITS, AND ASSESSMENT OF CHEMICAL DEFENSES FROM THE CURRENT STUDY

Plant species	Common name	Duration[a]	Origin[a,b,c]	Chemical defense	Protein	
					% of dry mass	mg per ml fresh plant tissue
Agrostis scabra	Rough bentgrass	Perennial	US native	None	4.90 ± 0.08	4.41 ± 0.08
Alisma subcordatum	American water plantain	Perennial	US native	Constitutive	Not measured	
Alternanthera philoxeroides	Alligatorweed	Perennial	S. America native	None	Not measured	
Aster sp.		Perennial		Constitutive	3.40 ± 0.13	16.3 ± 0.6
Cabomba pulcherrima	Fanwort	Perennial	US native	None	5.91 ± 0.50	2.95 ± 0.25
Cyperus odoratus	Fragrant flatsedge	Annual/perennial	US native	None	4.95 ± 0.07	8.41 ± 0.12
Eupatorium capillifolium	Dogfennel	Perennial	US native	Activated and constitutive	4.48 ± 0.29	4.48 ± 0.29
Galium tinctorium	Stiff marsh bedstraw	Perennial	US native	Constitutive	8.08 ± 0.17	11.3 ± 0.2
Hydrolea quadrivalvis	Waterpod	Perennial	US native	Constitutive	4.85 ± 0.18	2.91 ± 0.11
Hypericum mutilum	Dwarf St. Johnswort	Annual/perennial	US native	Constitutive	8.80 ± 0.49	10.6 ± 0.6

Ludwigia repens	Creeping primrose-willow	Perennial	US native	None	8.63 ± 0.26	6.04 ± 0.18
Lycopus virginicus	Virginia water horehound	Perennial	US native	None	5.79 ± 0.22	5.21 ± 0.20
Mimulus ringens	Allegheny monkeyflower	Perennial	US native	Constitutive	4.41 ± 0.39	7.93 ± 0.70
Murdannia keisak	Marsh flower	Perennial	Asia native	None	7.63 ± 0.44	2.90 ± 0.17
Myriophyllum aquaticum	Parrot-feather	Perennial	S. America native	None	10.2 ± 0.7	6.14 ± 0.42
Polygonum densiflorum	Denseflower knotweed	Annual/perennial	US native	Constitutive	2.15 ± 0.11	2.15 ± 0.11
Polygonum punctatum	Dotted smartweed	Annual/perennial	US native	None	2.47 ± 0.15	1.98 ± 0.12
Polygonum sagittatum	Arrowleaf tearthumb	Annual/perennial	US native	None	1.24 ± 0.05	2.87 ± 0.16
Sagittaria latifolia	Broadleaf arrowhead	Perennial	US native	Constitutive	7.25 ± 0.63	3.05 ± 0.27
Saururus cernuus	Lizard's tail	Perennial	US native	Constitutive	4.04 ± 0.16	2.83 ± 0.11
Utricularia sp.	Bladderwort		US native	Constitutive	8.50 ± 0.54	5.95 ± 0.38
Broccoli/lettuce control					12.4 ± 0.6	12.4 ± 0.6

Protein content data are also provided for the broccoli and lettuce mixture that was used as the experimental food.
[a] USDA (2004).
[b] Godfrey and Wooten (1979).
[c] Godfrey and Wooten (1981).

25–40 crayfish were used, and one or two assays were run with each group each day.

Feeding assay foods and experimental design followed procedures of Bolser et al. (1998). Assay food consisted of freeze-dried, finely ground broccoli and lettuce (1:1) in an agar matrix with the addition of plant extracts (either "grazed" or "nongrazed" extracts) or no extracts (no-extract control). For testing of each plant species, we used a quantity of dried broccoli/lettuce corresponding to the plant's dry mass for a 20-ml volume of fresh plant tissue. An aliquot of extract corresponding to 20 ml of fresh plant material was dissolved in acetone/methanol/ethyl ether (1:1:3) and added to the broccoli/lettuce powder. Solvents were then removed on a rotary evaporator, and the extract-coated broccoli/lettuce mixture was suspended in 7.0 ml distilled water, combined with a boiled solution of 0.37 g agar in 12.0 ml distilled water, and quickly poured into a mold beneath which lay window screen. Once cooled, the food adhered to the window screen, and each food patch was cut with a razor blade to contain a total of 63 window screen squares. No-extract control foods were prepared in the same manner, including the solvents described above, but without plant extracts.

To directly contrast the effects of extracts from "grazed" vs. "nongrazed" treatments, each crayfish was independently offered a food strip consisting of one patch of food with "grazed" extract and one patch of food with "nongrazed" extract. For tests of "grazed" or "nongrazed" treatments vs. no-extract controls, each crayfish was offered a food strip consisting of one patch of food with either "grazed" or "nongrazed" extract and one patch of no-extract control food. Each crayfish was allowed to feed until approximately half of the total amount of food on its strip was eaten, typically taking between 1–6 hr, but for some foods up to 24 hr. Once removed from crayfish, the number of food squares missing (eaten) from each treatment patch was counted.

Statistical Analyses. A Shapiro–Wilk test indicated nonnormal distribution of feeding assay results. Therefore, the nonparametric Wilcoxon signed-rank test was used to compare consumption of "grazed" treatments vs. "nongrazed" treatments and to compare consumption of "grazed" or "nongrazed" vs. no-extract controls. The nonparametric Mann–Whitney *U* statistical test was used to compare the amount consumed of the "grazed" treatment from the "grazed" vs. no-extract control assay with amount consumed of the "nongrazed" treatment from the "nongrazed" vs. no-extract control assay (Sokal and Rohlf, 1994). For a few species, uncertain interpretation of results led us to repeat feeding assays at a later date, using extracts from the initial plant collection. A Mann–Whitney *U* test was used to determine whether the data generated from these repeated assays could be combined with the data from the original feeding assays. If the two data sets were significantly different from each other, they were analyzed and presented separately. Linear regression was used to assess

whether a macrophyte's protein content was predictive of the deterrence of its extract. Extract deterrence was calculated for each replicate by subtracting the amount of "grazed" treatment eaten from the amount of the no-extract control food eaten, and dividing this by the total amount eaten, and then averaging across all replicates of one assay (consumption of "grazed" treatment rather than "nongrazed" treatment was chosen for this calculation so that extract deterrence of plants with activated chemical defenses would be evaluated at their activated levels). For plant species that were collected, extracted, and assayed twice, results from both feeding assays were averaged to generate a value of extract deterrence. An extract deterrence value of +1 indicates that only no-extract control food was consumed (i.e., strong deterrence); a deterrence value of 0 indicates that there was no preference between no-extract control food and "grazed" extract food (i.e., no chemical defense). Negative deterrence values suggest feeding stimulation by the plant extract. Data in figures are presented as means ± one standard error. In all analyses, $P < 0.05$ was considered statistically significant.

Plant Nutritional Analyses. Wet mass, dry mass, ash-free dry mass, and volumetric displacement were determined for five separate plants for each macrophyte species. All samples were patted dry and weighed, dried at 45°C until a constant dry mass was achieved (24 hr) and weighed to obtain dry mass, and placed in an oven at 450°C for 4 hr and weighed to obtain ash-free dry mass. To determine the approximate nutritional value of each plant species, soluble protein content was measured according to Bradford (1976) for 19 of the 21 plants species (two plant species were unavailable for recollection at the time of this measurement). Whole plant samples were weighed, freeze-dried, and ground. Three 5-mg samples of each plant were digested in 1.0 ml NaOH (1 mol/l) for 24 hr at 4°C, centrifuged, and 100-µl aliquots of the supernatant were added to 3.0 ml of Bradford reagent. After 15–20 min, absorbance was measured at 595 nm using a Spectronic 21D spectrophotometer against bovine serum albumin (BSA) standards. Five protein standards were used to construct a standard curve (data not shown, $r^2 = 0.997$).

RESULTS

Extracts from 11 of the 21 species tested (52%) significantly depressed crayfish feeding relative to no-extract controls (Table 1, Figures 1 and 2). These chemically defended plants include one floating macrophyte, *Utricularia* sp., and 10 emergent macrophytes: *Alisma subcordatum* (Alismataceae), *Aster* sp. (Asteraceae), *Eupatorium capillifolium* (Asteraceae), *Galium tinctorium* (Rubiaceae), *Hydrolea quadrivalvis* (Hydrophyllaceae), *Hypericum mutilum* (Clusiaceae), *Mimulus ringens* (Scrophulariaceae), *Polygonum densiflorum* (Polygonaceae),

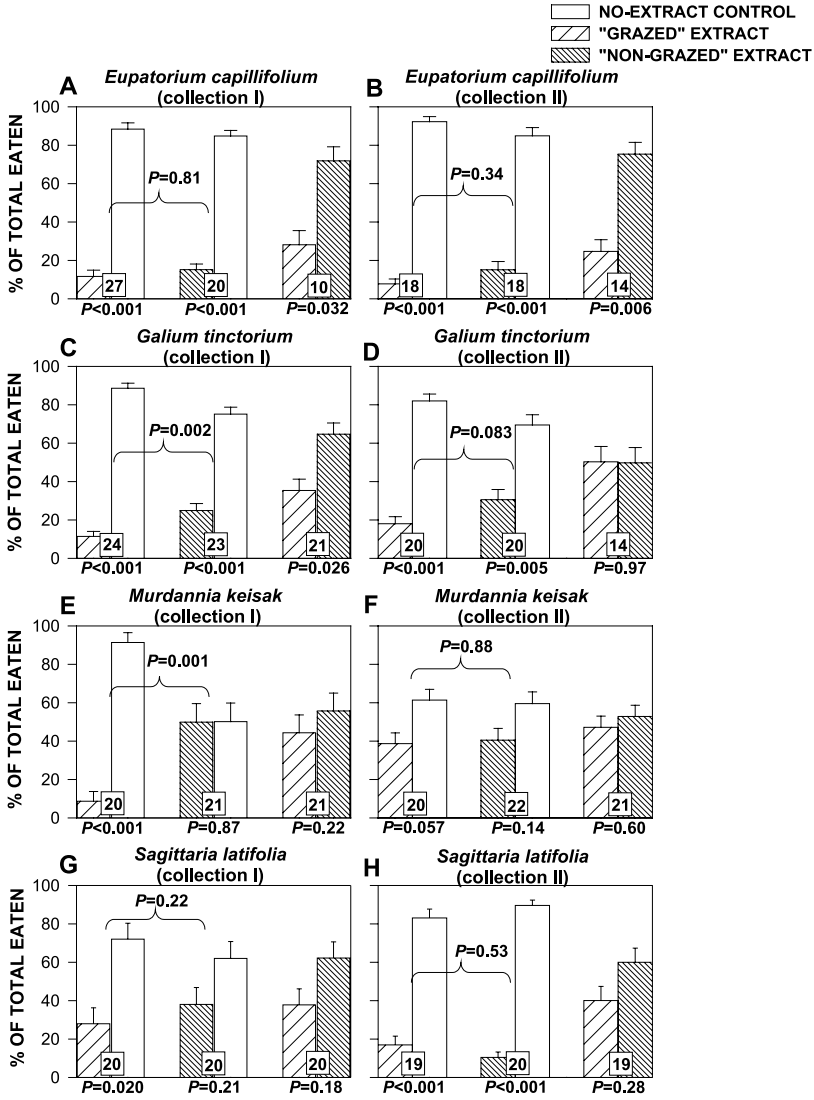

FIG. 1. Effects of simulated "grazed" and "nongrazed" freshwater plant extracts on crayfish feeding behavior: macrophytes found to have activated and constitutive chemical defenses (*E. capillifolium*) and macrophytes with initial data suggesting activated defenses, but upon repeated experiments, found to have constitutive chemical defenses (*G. tinctorium* and *S. latifolia*) or no chemical defenses (*M. keisak*). Histogram pairs represent paired experiments. Sample size is noted in a box at the base of each histogram.

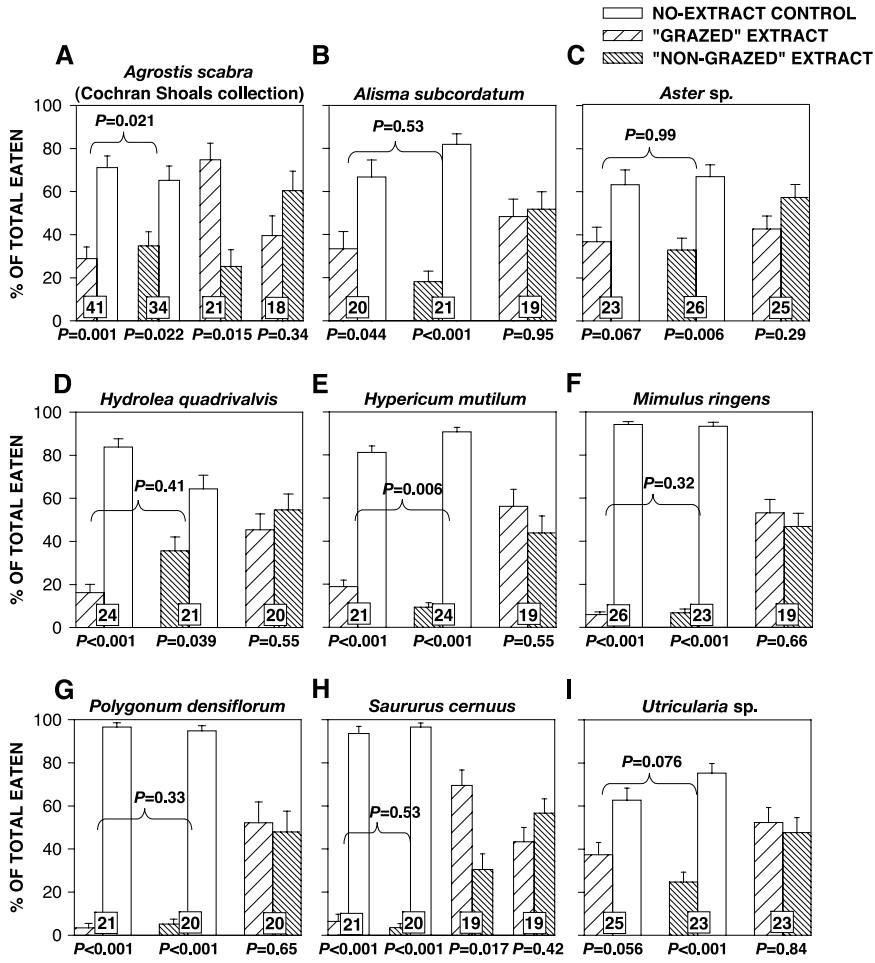

FIG. 2. Effects of simulated "grazed" and "nongrazed" freshwater plant extracts on crayfish feeding behavior: macrophytes found to have constitutive chemical defenses. Analysis and symbols as in Figure 1.

Sagittaria latifolia (Alismataceae), and *Saururus cernuus* (Saururaceae). Collections of one additional emergent species, *Agrostis scabra* (Poaceae), appeared to be chemically defended at one site (Cochran Shoals, Figure 2A), but not at a site 3 mi away (Johnson Ferry, data not shown), although collections were made within 5 wk of each other. The remaining nine macrophytes' crude extracts did not significantly deter crayfish feeding when tested at natural concentrations (data not shown). These nondeterrent plants were one submersed macrophyte

Cabomba pulcherrima (Cabombaceae) and eight emergent macrophytes: *Alternanthera philoxeroides* (Amaranthaceae), *Cyperus odoratus* (Cyperaceae), *Ludwigia repens* (Onagraceae), *Lycopus virginicus* (Lamiaceae), *Murdannia keisak* (Commelinaceae), *Myriophyllum aquaticum* (Haloragaceae), *Polygonum punctatum* (Polygonaceae), and *Polygonum sagittatum* (Polygonaceae).

When originally tested, three macrophyte species (*E. capillifolium*, *G. tinctorium*, and *M. keisak*) appeared to utilize activated chemical defenses because plant extracts generated by the simulated "grazed" treatment (i.e., mechanically damaged prior to extraction) significantly deterred crayfish feeding relative to extracts from the simulated "nongrazed" treatment (i.e., mechanically damaged after treatment with organic solvent had started) (Figure 1A, C, and E). The evidence for activated chemical defenses among these three plants came either from direct comparison of crayfish consumption of foods containing "grazed" vs. "nongrazed" extracts (*E. capillifolium*; $P = 0.032$), or indirectly by comparing data from assays testing consumption of treated foods vs. no-extract controls (*M. keisak*; $P = 0.001$), or by both direct and indirect comparisons (*G. tinctorium*; $P = 0.026$ and 0.002, respectively). Additionally, the "grazed" extract of *S. latifolia* was found to be deterrent relative to no-extract controls ($P = 0.020$), whereas its "nongrazed" extract was not ($P = 0.21$), although there was no significant difference in consumption of foods containing these extracts relative to each other ($P = 0.18$ direct comparison; $P = 0.22$ indirect), so this plant did not appear to possess an activated defense (Figure 1G).

To reassess *S. latifolia*'s mechanism of chemical defense and to test whether the activated defense results for the three plant species were reproducible, these four species were recollected, treated and extracted as before, and new feeding assays were performed. Crayfish again strongly preferred "nongrazed" over "grazed" extracts from *E. capillifolium* ($P = 0.006$; Figure 1B), indicating that this plant utilizes a chemical defense activated by physical damage, although it also possesses a constitutive defense because "nongrazed" extracts were also deterrent. Crayfish were again deterred by extracts of *G. tinctorium*, but for this assay, there was no significant difference in palatability of "grazed" and "nongrazed" extracts ($P = 0.97$ direct comparison; $P = 0.083$ indirect; Figure 1D). Thus, *G. tinctorium* has constitutive chemical defenses, but we could not consistently show activation of a chemical defense. Feeding assays of the second collection of *S. latifolia* also suggested a constitutive chemical defense; both "grazed" and "nongrazed" extracts were unpalatable to crayfish, and their effects were indistinguishable from each other (Figure 1H). Neither "grazed" nor "nongrazed" extracts of *M. keisak* significantly deterred crayfish feeding relative to no-extract controls in the second feeding assay (although nonsignificant trends toward deterrence were observed; $P = 0.057$ and 0.14, respectively, Figure 1F), and the "nongrazed" extract did not deter crayfish feeding in the first assay ($P = 0.87$, Figure 1E), leaving us unable to find

consistent repeatable effects of extracts from this species. Therefore, of the 21 macrophytes studied, only *E. capillifolium* showed consistent changes in extract palatability characteristic of a chemical defense activated by physical damage. Thin layer chromatography analysis showed no obvious chemical difference between "grazed" and "nongrazed" extracts of this plant (data not shown).

In some cases, results of initial feeding assays were difficult to interpret. For example, assays directly comparing palatability of "grazed" and "nongrazed" extracts of *A. scabra* (Cochran Shoals collection) and *S. cernuus* suggested crayfish preference for "grazed" over "nongrazed" extracts (Figure 2A and H), whereas comparisons of palatability of each of these extracts with no-extract controls indicated strong chemical defenses. Because assays directly testing "grazed" vs. "nongrazed" extracts for these plants took up to 24 hr, 4–20 times longer than typical assays, we hypothesized that both "grazed" and "nongrazed" extracts were so unpalatable as to make crayfish preferences between these extracts arbitrary. For these two plant species, feeding assays using extracts from the initial collections were repeated, and crayfish preference for "grazed" extracts was not observed the second time (Figure 2A and H data on far right).

For the 19 plant species for which total soluble protein was measured, positive relationships were identified between: (1) dry mass per volume of fresh plant tissue and soluble protein per volume of fresh plant tissue ($P < 0.001$, Figure 3A); (2) ash-free plant dry mass per plant dry mass and soluble protein per plant dry mass ($P < 0.001$, data not shown); and (3) ash-free plant dry mass per plant dry mass and soluble protein per volume of fresh plant tissue ($P < 0.001$, data not shown). Compared to the 19 plants analyzed, the broccoli/lettuce mixture used as food base for the feeding assays had the highest value of protein per dry mass and the second highest value of protein per volume of fresh plant tissue.

No significant relationship was found between the magnitude of deterrence of plant extracts and plant protein content, neither when protein was expressed as soluble protein per volume of fresh plant tissue ($P = 0.95$, Figure 3B) nor when protein was expressed as a percentage of plant dry mass ($P = 0.67$, Figure 3C). No significant relationship existed between magnitude of deterrence and protein content either when chemically defended plants were grouped together or when nonchemically defended plant were grouped together ($P > 0.13$ and $P > 0.29$, respectively, whether calculated using protein as a proportion of plant volume or of dry mass).

DISCUSSION

Despite the high rate of herbivory in freshwater systems (Cyr and Pace, 1993), and the prediction that selection for antiherbivore defenses, including

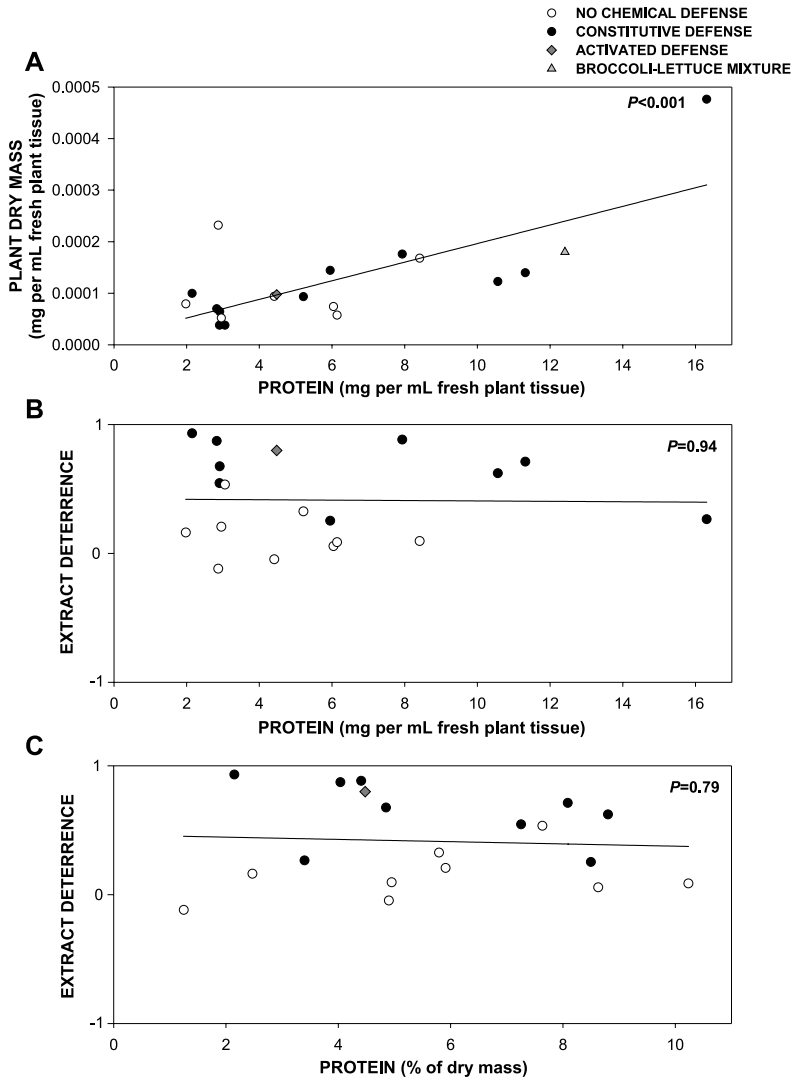

FIG. 3. (A) Relationship between soluble protein concentration and plant dry mass for 19 freshwater plant species in this study and broccoli/lettuce mixture used as experimental food (r^2 = 0.53). (B) Relationship between soluble protein concentration per volume of fresh plant tissue and deterrence of plant extracts for 19 freshwater plant species. A positive extract deterrence value indicates strong deterrence, zero indicates no deterrence, and a negative value indicates feeding stimulation by the extract. (C) Relationship between soluble protein concentration as a proportion of plant dry mass and deterrence of plant extracts.

chemical defenses, should be strong (Lodge et al., 1998), only 14 species of freshwater macrophytes had been examined for chemical defenses prior to this study. The current study provides evidence for 19 previously untested species and predicts that a majority (52% of 21 species in this study; 55% of 33 species tested overall) utilize chemical defenses against generalist herbivores (data from Newman et al., 1992; Bolser and Hay, 1998; Bolser et al., 1998; Cronin, 1998; Kerfoot et al., 1998; Kubanek et al., 2001; Cronin et al., 2002; this study). Because almost all of the freshwater macrophytes chosen for this and previous studies were selected based on availability and not because they were suspected of being chemically defended, it is likely that, in general, freshwater macrophytes are commonly defended chemically.

Studies like ours that use crude extracts to assess deterrence risk overestimating the frequency of chemical defenses, if artifacts of the extraction process contributed to deterrence. Only isolation of deterrent compounds followed by assessment of molecular structures within a biosynthetic framework can remove this risk. Of the 11 plant species predicted in this study to be chemically defended, the deterrence of only one (*S. cernuus*) has been attributed to characterized plant products, in that case, a group of lignans, neolignans, and sesquineolignans (Kubanek et al., 2000, 2001). We advocate caution in suggesting specific deterrent mechanisms for the remaining 10 species, although it is unlikely that artifacts contributed to the observed deterrence of more than a few species.

Including the one freshwater macrophyte species with a probable activated chemical defense from this study (*E. capillifolium*), and the two macrophytes, *Nasturtium officinale* and *H. repens*, found to possess activated chemical defenses in previous investigations (Newman et al., 1992, 1996; Bolser et al., 1998), a total of 9% (3 of 33) of freshwater plant species tested for chemical defenses have been found to display consistent changes in palatability characteristic of activated chemical defenses. In a study similar to ours in which marine macrophytes were sampled based on availability, Cetrulo and Hay (2000) found that 17% (7 of 42) of tropical and temperate seaweed species appeared to use activated chemical defenses. Despite this high frequency, only two seaweed-activated defense mechanisms are well understood: acrylic acid plus dimethyl sulfide, produced by enzymatic action in several seaweeds (Van Alstyne and Houser, 2003), and halimedatrial, a reactive polyaldehyde converted from the less deterrent halimedatetraacetate in *Halimeda* spp. (Paul and Van Alstyne, 1992). Overall, Cetrulo and Hay (2000) suggest that activated chemical defenses may be fairly common among marine plants. In contrast, a relatively smaller proportion of freshwater plants have been shown to possess activated chemical defenses (Newman et al., 1990; Bolser et al., 1998; this study).

In our study, the low frequency of activated chemical defenses (1 of 21 plant species) may be an underestimate of the actual frequency due to

methodological limitations. First, because our feeding assays focused on only one herbivore, we may have missed defenses that deter other herbivores. Second, plants may respond to specific cues or types of damage from herbivores rather than responding simply to physical damage. The most severe limitation of our study may be that volatile or labile activated defenses would have been missed. Our laboratory practices would have removed damage-activated volatile cues like dimethyl sulfide and acrylic acid (Van Alstyne and Houser, 2003), hydrogen cyanide (Conn, 1979), and isothiocyanates and nitriles (Newman et al., 1990). However, none of these groups of volatile compounds are known from the 14 families of plants used in our study, and dimethyl sulfide and acrylic acid are thought to be restricted to marine algae. Extremely labile compounds like oxytonin 2 (Jung and Pohnert, 2001) would have been lost by decomposition. However, some known activated defenses could have been detected in assays like ours, including habenariol (Wilson et al., 1999) and halimedatrial (Paul and Van Alstyne, 1992).

As previously reported (e.g., Cronin and Hay, 1996), we found that chemical defenses can vary by location, time, and environmental conditions. *A. scabra* appeared to be chemically defended at one site (Figure 2A) but not another (data not shown). Extracts of *A. philoxeroides* were palatable in our study (data not shown); yet Cronin et al. (2002) reported that the same species collected in North Carolina was chemically defended against a crayfish congeneric to ours. Four other macrophyte species for which initial data suggested activated defenses (Figure 1) were recollected 1–4 mo later at the same sites, and an activated chemical defense could only be reproduced for one of these (Figure 1B). Results of the second series of experiments indicated constitutive chemical defenses for two plant species (Figure 1D and H) and no chemical defense for one (Figure 1F). This raises the possibilities of type I or II experimental error, that plant defenses varied by location or season, or that defenses were occasionally activated in the field due to physical stress or herbivore attack.

Because herbivores are often nitrogen-limited (Mattson, 1980), protein content should be a good proxy of plant nutritional quality. High protein plants may be desirable to herbivores, selecting for greater defenses in these plants. However, our initial hypothesis that plants with greater nutritional value should be more strongly defended chemically was not supported. We found no relationship between deterrence of plant extracts and protein content (Figure 3).

We chose the conservative strategy of using an artificial food similar in protein content to the most protein-rich of our 21 plant species (Table 1). This avoided a potential artifact of consumers responding to weakly deterrent compounds that would be ineffective within the context of the natural nutritional value of individual plants, but with the risk that some deterrence could have been missed. However, we detected deterrence for 11 of 21 plant

extracts. The 10 species with palatable extracts included some with the highest protein levels. If the high protein content of our artificial food had masked the deterrence of some extracts, this would be expected to have disproportionately affected low protein plants, potentially contributing to a relationship between protein content and deterrence, which was not observed (Figure 3).

In summary, our finding that extracts of 11 of 21 freshwater macrophyte species investigated significantly deterred feeding by a generalist crayfish provides evidence that chemical defenses in freshwater ecosystems are probably common, although chemical defenses activated by mechanical damage were consistently observed for only one of these plant species. When combined with previous studies on freshwater plant chemical defenses, it is clear that many freshwater macrophytes use chemical defenses, particularly constitutive chemical defenses, to defend themselves against generalist herbivores.

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SPATIAL AND TEMPORAL VARIABILITY OF CYTOTOXIC METABOLITES IN POPULATIONS OF THE NEW ZEALAND SPONGE *Mycale hentscheli*

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Abstract—Intraspecific variation in the composition of three cytotoxic secondary metabolites from the New Zealand marine sponge *Mycale hentscheli* collected at two sites in central New Zealand was quantified by ^{1}H NMR techniques. A total of 275 sponges were analyzed bimonthly over 15 mo to compare intersite (~100 km) and intrasite (~100 m) spatial and temporal variations in the metabolites. Biological and physical characteristics of sponge size, morphology, depth, and temperature were recorded at each site. Metabolite concentrations were found to vary in space and time. Metabolite composition was site-specific; mycalamide A, pateamine, and peloruside A were present at Pelorus Sound, whereas pateamine was absent from sponges at Kapiti Island. Pateamine and peloruside A concentrations in sponges at Pelorus Sound varied seasonally; no such patterns were observed at Kapiti Island. Relationships of compound concentration with volume and depth were complex. High levels of peloruside A in Pelorus Sound sponges from between 8 and 10 m depth coincided with a density boundary layer and chlorophyll *a* maximum.

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Key Words—Marine sponge, Demospongiae, Poecilosclerida, *Mycale hentscheli*, cytotoxic secondary metabolites, ^{1}H NMR, spatial and temporal variation, environmental variables, New Zealand.

INTRODUCTION

The endemic marine sponge *Mycale hentscheli* Bergquist & Fromont, (Demosponigiae: Poecilosclerida: Mycalidae) has attracted considerable attention from chemical researchers because its crude extracts are usually potently cytotoxic in biological assays against mammalian cell lines (Perry et al., 1988). Three classes of biologically active compounds with pharmaceutical potential have been characterized from this species over the past 15 years (Figure 1). The mycalamides were the first group of novel cytotoxic compounds to be isolated from *M. hentscheli*. Mycalamides A (**1**) and B (**2**) were first described in 1988 from *M. hentscheli* collected in Otago Harbour on the southeast coast of the South Island (Perry et al., 1988). More recently, we reported the isolation of mycalamide D (**3**) from specimens collected from the northeast coast of the North Island (West et al., 2000a). The mycalamides are potent eukaryotic cytotoxins with IC_{50} values against mammalian cells in the low nanomolar

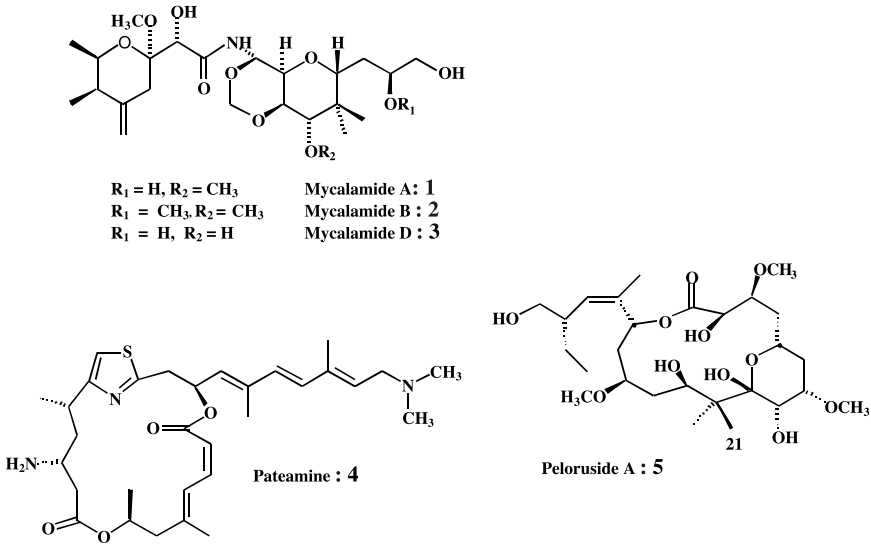

FIG. 1. Chemical structures of three classes of bioactive compounds from *M. hentscheli*: mycalamide A (**1**), B (**2**), D (**3**), pateamine (**4**), and peloruside A (**5**).

range. They have been reported to be potent protein synthesis inhibitors and have recently been found to cause apoptosis (Hood et al., 2001).

The biochemically unrelated macrolide pateamine (**4**) was isolated from *M. hentscheli* collected in Doubtful Sound on the southwest coast of the South Island. Pateamine is also a potent eukaryotic cytotoxin active in the subnanomolar range (Northcote et al., 1991). It has subsequently been found to have immunosuppressive and apoptotic properties (Romo et al., 1998; Hood et al., 2001). Interestingly, no mycalamides were detected in the specimens collected in Doubtful Sound.

The most recently discovered cytotoxic metabolite is peloruside A (**5**), which was isolated from specimens of *M. hentscheli* collected from Pelorus Sound on the north coast of the South Island (West et al., 2000b). Peloruside A is structurally unrelated to either the mycalamides or pateamine, and yet is also a potent eukaryotic cytotoxin. Recently we found that peloruside A is a microtubule stabilizer with potency and mode of action similar to Taxol® and the epothilones. All three classes of *Mycale hentscheli* metabolites show similar profiles of toxicity to a range of organisms and cell lines.

M. hentscheli is a soft, fleshy sponge, sparsely invested with skeletal spicules. It grows as massive to encrusting forms living on subtidal reefs to below 30 m throughout New Zealand (Figure 2). Large variations in relative concentrations of the three compounds are evident in collections of this species made over the past decade, from different geographic regions within New Zealand, and at different times of the year. Intraspecific variability in secondary metabolites through space and time has been studied for few sessile invertebrate taxa (Hay, 1996). Spatial variation is documented for soft corals (Maida et al., 1993; Puglisi et al., 2000), gorgonians (Harvell et al., 1993; Roussis et al., 2000), and sponges (Thompson et al., 1983, 1987; Becerro et al., 1995). Relatively few studies have, however, described long-term quantitative temporal changes in biologically active compounds. Turon et al. (1996) and Duckworth and Battershill, (2003) described such changes using biological assays in the encrusting sponge *Crambe crambe*, and on New Zealand sponges, *Latrunculia wellingtonensis* and *Polymastia croceus*, respectively. These studies measured potential synergistic effects of two or more biologically active compounds, but could not be used to quantify relative concentrations of individual compounds within an organism.

The development of marine compounds with pharmaceutical potential is, however, dependent on a sustainable supply of the desired compounds themselves (Dumdei et al., 1998). Without knowledge of the underlying patterns of production in the natural environment, sustainable supply through wild harvest or aquaculture becomes problematic (Munro et al., 1999).

The primary aim of our study was to describe spatial and temporal patterns of biologically active compounds in *M. hentscheli*.

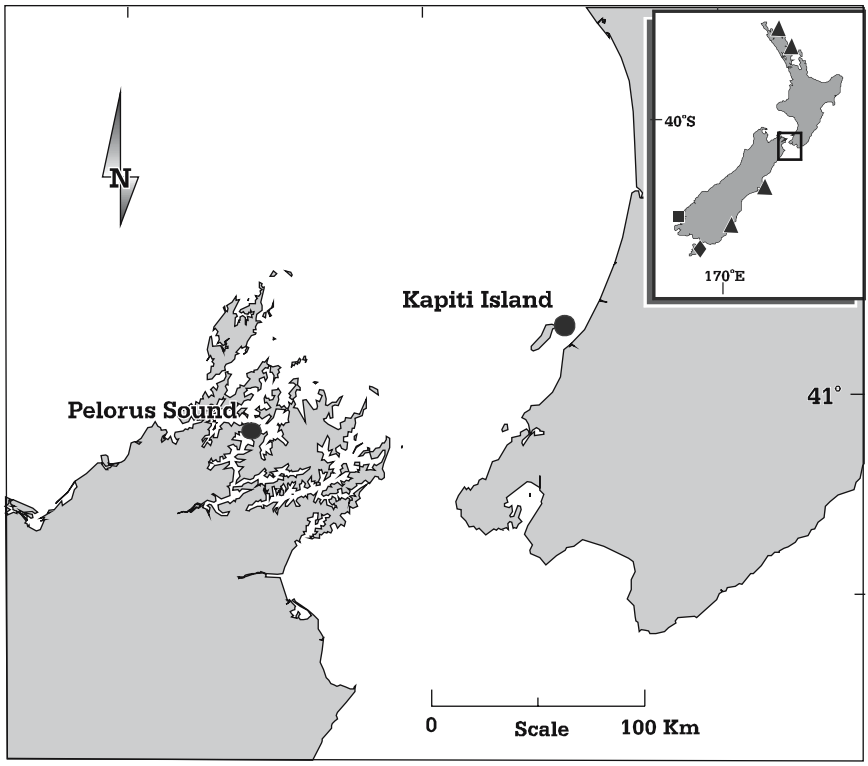

FIG. 2. Pelorus Sound and Kapiti Island study sites, central New Zealand region. The inset shows the geographic distribution of specimens of *M. hentscheli* that yielded compounds isolated in earlier studies: ▲ = mycalamides, ■ = pateamine, and ♦ = mycalamides and peloruside A.

METHODS AND MATERIALS

Study Sites. Sampling was carried out over the entire depth range of *M. hentscheli* at two sites—Pelorus Sound (Pelorus) in the Marlborough Sounds, South Island, and at Kapiti Island (Kapiti) on the southwest coast of the North Island, New Zealand—in 1999–2000 (Figure 2). Sites separated by approximately 100 km were chosen because they represented extremes in known environmental conditions for *M. hentscheli* over a relatively small latitudinal gradient; the site on Kapiti Island is a physically dynamic coastal environment exposed to moderate wave action and tidal mixing. The water is oceanic, and clarity and water quality are high, with low levels of fine suspended sediment.

The physical environment at the Pelorus site, situated in the main channel, is dominated by an almost continuous salinity or thermally induced density stratification, where low-salinity water overlies high-salinity oceanic water (Gibbs et al., 2002). Upper water column current velocities may reach 1–2 m s^{-1}, resulting in horizontal transport of nutrients and phytoplankton above the pycnocline. Suspended sediments are greatest near the seafloor where resuspension by tidal currents occurs (Gibbs, 2001). Hobo® temperature loggers (Onset Computer Corp.) were placed at the top and bottom extent of the sponge populations at each site. Temperatures were recorded at 30-min intervals for 15 mo and plotted as mean daily temperatures.

Sampling Procedure. To examine small-scale intrasite variation in compound concentrations, two populations (A and B) approximately 100 m apart were chosen from each site to contain sponges of similar size and depth ranges. Ten whole sponges were sampled bimonthly for 15 mo (seven sampling periods) from each population (20 samples per site) from August 1999 to November 2000. The sponges were sampled *in situ*, bagged separately for chemical analysis, and the bedrock scraped clean to minimize the chance of resampling the same organism. The depth and size of each sponge collected were recorded. Depth was adjusted to mean sea level (±10 cm), and volume was estimated by using a ruler to measure height, width, and length similar to methods used in other studies e.g., Fell and Lewandrowski, 1981; Meroz and Ilan, 1995.

Taxonomic Identification. Representative subsamples were taken from the 20 sponges collected at each site during December 2000 to confirm taxonomic identity and to describe the morphological characteristics of populations at each site. Sponge tissue was paraffin-embedded and histologically sectioned for light microscopy and spicule mounts prepared for morphometric analysis of mega- and microsclere dimensions, according to the method described in Kelly-Borges and Vacelet (1995).

Extraction and Isolation. Approximately 15 g of each frozen *M. hentscheli* specimen were lyophilized (48 hr) to dryness and ground to a homogenous powder. Approximately 1.5 g of the dry, powdered, sponge material were weighed and extracted with 80% MeOH/H_2O (20 ml) and MeOH (20 ml). Both extracts were loaded onto a 1.0 × 2.0 cm Amberchrom® polystyrene–divinyl–benzene column by dilution with H_2O. The column was washed with H_2O (10 ml) and eluted with 55% Me_2CO/0.2 M NH_4OAc adjusted to pH 4.0 with AcOH (10 ml) into a flask containing 0.2 M NH_4OH (20 ml). The neutralized eluent was desalted on a second Amberchrom® column, and concentrated to dryness under reduced pressure.

^1H NMR Analysis and Quantification. 30 μl (1.8 μmol) of a standard solution of trimethoxybenzene (50 mg) in $CDCl_3$ (5 ml) and 500 μl of $CDCl_3$ were added to the dry concentrated sample. The resulting solution was sonicated (3 sec) in an ultrasonic bath and transferred to an NMR tube (5 mm).

One microliter of pyridine-d_5 was added to the solvent in the NMR tube as a buffer. A ^{1}H NMR spectrum was obtained on a Varian Inova® spectrometer at 300 MHz with the following acquisition and processing parameters: temperature 25°C/298.1 K, relax delay 15.0 sec, pulse width 90.0°, acquisition time 5.0 sec, 64 repetitions, line broadening 0.5 Hz, total time 21 min and 20 sec. The integrals of resonances at δ 7.49(1H), 5.88(2H), and 4.74(1H) of mycalamide A, δ 6.98 (1H), 6.81 (1H), and 6.31 (3H) of pateamine, and δ 4.53 (1H) of peloruside A were compared to the integral of the trimethoxybenzene resonance at δ 6.10 to determine the molar quantities of the three analytes. The concentration of mycalamide A, pateamine, and peloruside A was expressed in $\mu g\ g^{-1}$ dry weight sponge. Ten replications of the analytical protocol on a single homogenous dry sponge sample gave reproducibility of ±5%.

Data Analysis. A partially hierarchical (nested) three-factor mixed-model analysis of variance (ANOVA) was used to compare differences in concentration of each compound among populations within sites and between sites at different times of the year. The factors were time (fixed), site (fixed), and population (random) nested within site. All data were square root transformed prior to analysis, as variances proved heterogeneous in residual plots. Analysis of covariance (ANCOVA) was used to analyze relationships of compound concentrations with depth and volume. Metabolite concentrations over time are described, but not compared statistically with temperature. The GLM ANOVA and ANCOVA were run using SAS statistical software (SAS/STAT, 1990). Two sample Student's *t*-tests were used to test for significant differences in sponge volume between sites. A χ^2 test was used to compare observed with expected peloruside depth–frequency distributions at Capsize Point. The expected frequency of peloruside A occurrence for each depth category was expressed as a percent of the total number ($N = 137$) of sponges sampled for the compound at that site. *t*-Tests and χ^2 tests were computed using NCSS 97 (Hintze, 1997).

RESULTS

Ecological and Environmental Variables. Sponges were distributed between 3 and 15 m at Pelorus Sound and between 10 and 26 m at Kapiti Island, with the shallowest depth at both sites corresponding to the lower extent of the brown macrophytes *Carpophyllum flexuosum* and *Ecklonia radiata*. All sponge samples examined from Kapiti Island and Pelorus Sound conformed to the description of *M.* (*Carmia*) *hentscheli* (Bergquist and Fromont, 1988). Although there was no significant difference in mean volume among sponges at each site ($t_{180} = -0.84$, $P = 0.39$), morphology and spicule size differed. Pelorus sponges had a massive growth form, with thicker subtylostyles and spicule tracts cored

by substantially more nonspecific detritus than sand, whereas Kapiti sponges had an encrusting growth form with thin megascleres, characteristic of low silica content in oceanic waters. A greater abundance of microscopic epibionts were noted on and in each Kapiti sponge, including polychaetes, filamentous algae, a chain-forming diatom, and a hydroid-like filamentous organism that is also present in the holotype from Takatu Point, Northland. Fewer such associations were evident in the Pelorus Sound specimens, but the cellular mesophyl of Pelorus sponges was more grainy and pigmented.

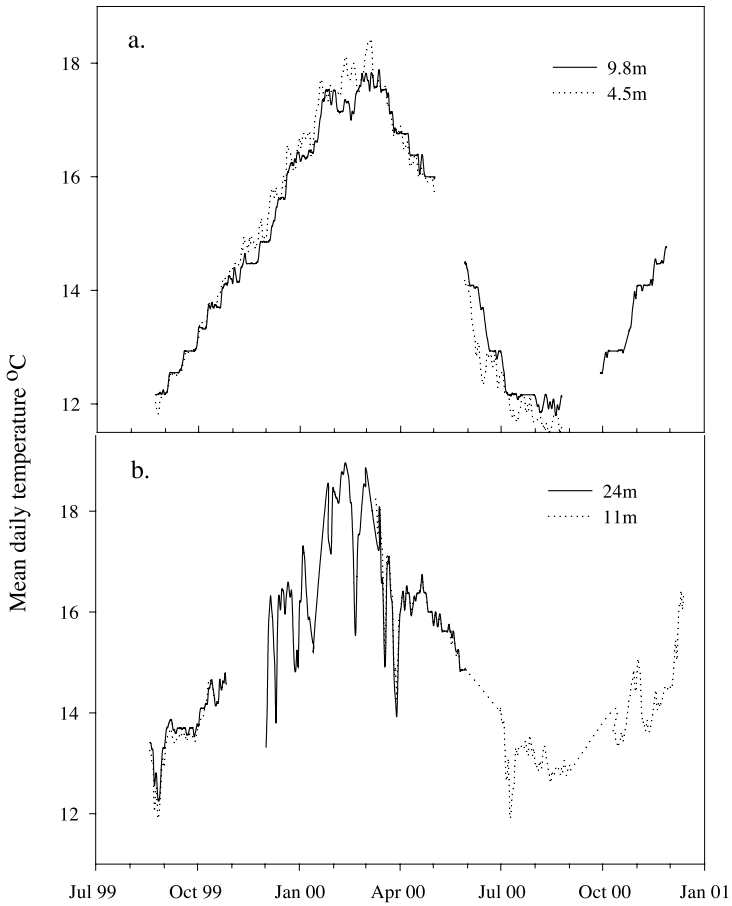

FIG. 3. Seasonal patterns in water temperature recorded by data loggers at the bottom and top extent of the depth range of *M. hentscheli* at each study site: (a) Pelorus Sound; (b) Kapiti Island.

Maximum water temperatures at both sites occurred during February–April 2000 and declined to a minimum in July–August 2000. Summer highs were similar at both sites (17.3 and 16.9°C); however, the water temperature at Pelorus was approximately 1.5°C cooler than at Kapiti Island during winter (Figure 3a and b). Temperature stratification occurred at Pelorus Sound during winter and summer (Figure 3a). Summer temperatures in shallow water were 1°C warmer than for deep water and the reverse occurred during winter.

No temperature stratification occurred at Kapiti Island (Figure 3a). Some data at Kapiti were not recovered due to lost or flooded loggers, although collectively the data showed large within-season variation in water temperatures that occurred at this site. Temperature in both seasons varied as much as ~3°C over 9 d. Both depths showed the same degree of change suggesting that water was well mixed during these events.

Spatial and Temporal Patterns

Mycalamide A. There were significant differences in the relative concentrations of mycalamide A among sites, but not among populations within sites (Table 1a). Concentrations were greater at Pelorus Sound (Figure 4a) than at Kapiti Island (Figure 4b), and differences among populations nested within sites varied over time [i.e., the population (site) × time interaction term was significant, Table 1a]. Generally, the highest concentrations of mycalamide A were recorded in sponges at Pelorus Sound during spring.

Pateamine. Kapiti Island sponges contained no pateamine throughout the study. At Pelorus Sound, however, pateamine was present in sponges, and rela-

TABLE 1A. MEAN SQUARES AND *F* RATIOS FROM A PARTIALLY HIERARCHICAL THREE-FACTOR ANOVA (TYPE III SUMS OF SQUARES) OF THE EFFECT OF TIME OF YEAR AND SITE ON THE CONCENTRATION OF MYCALAMIDE A AND PELORUSIDE A IN *M. hentscheli* SPONGES

		Mycalamide A		Peloruside A	
Source of variation	*df*	MS	*F*	MS	*F*
Time	6	82.5	2.88	36.6	9.51***
Site	1	637.9	43.1*	58.7	19.9*
Time × Site	6	64.8	2.26	42.7	11.09***
Population (Site)	2	14.8	2.70	2.9	0.48
Time × Population (Site)	12	28.6	5.30***	3.9	0.63
Error	247	5.4		6.1	

Significance of the *F* ratio is abbreviated by $*P < 0.05$ and $***P < 0.001$. All data were square root transformed.

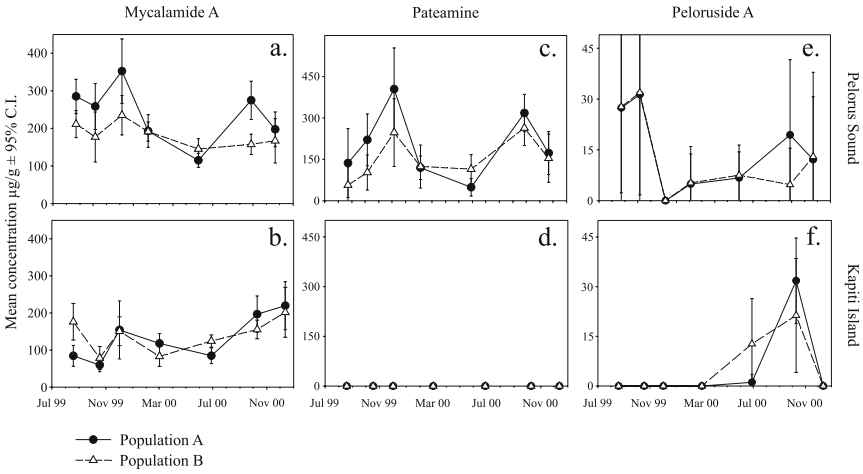

FIG. 4. Temporal patterns in mean concentration of three compounds contained in *M. hentscheli* sampled from two study sites. Circles and diamonds represent data from two populations sampled within each site (N = 10 whole sponges sampled from each population).

tive concentrations of the compound varied significantly over time, although differences among times were not consistent between the two populations within the site (Table 1b). Mean concentrations were greatest in spring, approximately two- to fourfold higher than in winter (Figure 4c). Patterns of change over time at Pelorus Sound were similar for pateamine and mycalamide A (Figure 4a and c).

Peloruside A. Relative concentrations of peloruside A varied significantly between sites, but were not consistent over time (Table 1a). In the first winter,

TABLE 1B. MEAN SQUARES AND F RATIOS FROM A TWO-FACTOR ANOVA (TYPE III SUMS OF SQUARES) OF THE EFFECT OF TIME OF YEAR AND POPULATION ON THE CONCENTRATION OF PATEAMINE IN *M. hentscheli* SPONGES

Source of variation	*df*	MS	Pateamine
Time	6	312.9	3.49**
Population	1	10.6	0.32
Time × Population	6	89.6	2.7*
Error	123	33.1	

Significance of the F ratio is abbreviated by *$P < 0.05$ and **$P < 0.01$. All data were square root transformed.

the compound was present at Pelorus Sound but not at Kapiti Island, but thereafter no clear differences between the two sites were apparent (Figure 4e and f). There was high variability among replicates at each combination of time and population; only a few sponges sampled within a population at any one time contained the compound. At Pelorus Sound, 23% ($N = 137$) of sponges sampled contained detectable amounts of the compound. Although highly variable, peloruside A concentrations appeared to be highest during spring.

Temperature, Volume, and Depth Relationships. At Pelorus Sound, metabolite concentrations increased as water temperatures rose during spring (Figure 4a, c, and e), but declined before temperatures peaked in summer (Figure 3). The timing of successive concentration maxima was not the same in consecutive

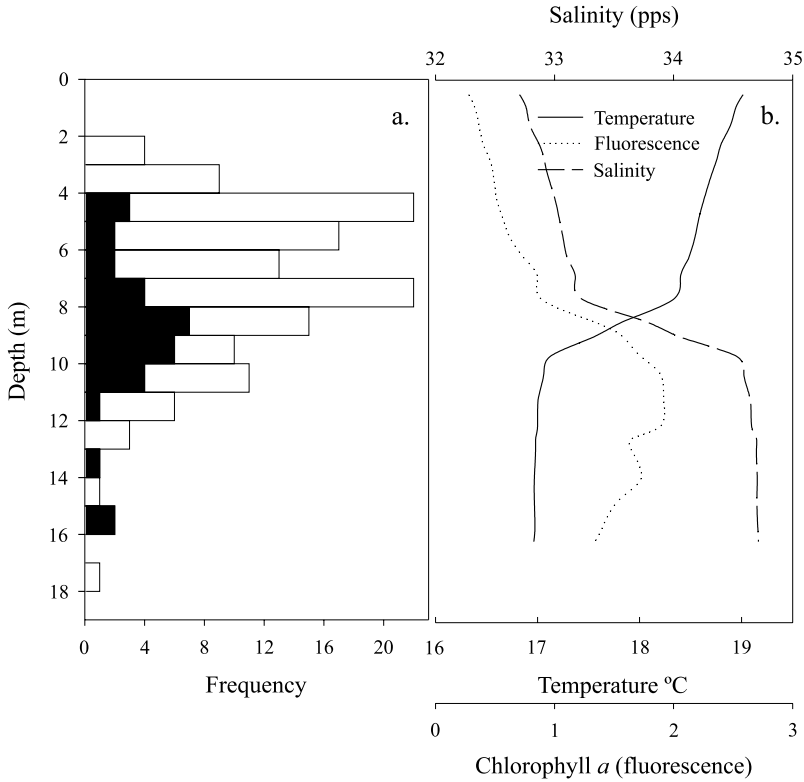

FIG. 5. (a) Frequency of sponges that contained detectable amounts of peloruside A in relation to depth at Pelorus Sound ($N = 137$): □ = total frequency of sponges sampled, ■ = frequency of sponges sampled containing peloruside A; and (b) a vertical temperature, chlorophyll *a* (fluorescence) and salinity profile from the Pelorus site taken on January 28, 2002.

years, nor was any seasonal pattern obvious at Kapiti Island where within-season temperatures were less stable.

There were significant relationships of square-root mycalamide A and peloruside A with volume in the ANCOVA, but these varied with time, i.e., high-order interactions occurred at the volume × population × time level for mycalamide A ($F_{18, 212} = 2.30$, $P = 0.003$), and peloruside A ($F_{18, 212} = 1.81$, $P = 0.025$).

Depth was not a significant covariate for mycalamide A, but there was a relationship with depth for pateamine that varied with time (depth × time, $F_{6, 109} = 2.51$, $P = 0.026$). There was also a highly significant relationship of peloruside A with depth, but this varied among populations at different times (depth × population × time, $F_{18, 219} = 4.45$, $P < 0.001$). Peloruside A at Pelorus Sound was most common in sponges at between 8 and 10 m depth (Figure 5a). No sponges collected above 4 m contained peloruside A, whereas 55% of sponges between 8 and 10 m contained the compound. However, there was no significant difference in the observed *versus* expected frequency of compound occurrence ($\chi^2_{15} = 20.6$, $0.2 > P > 0.1$). This apparent peloruside A maximum was coincident with the depth of the density boundary layer and chlorophyll *a* peak biomass at this site (Figure 5b).

DISCUSSION

The marked spatial and temporal variation in biologically active metabolites of the sponge *M. hentscheli*, as measured at two sites in central New Zealand, suggests that complex ecological and physical factors, largely undetected in this research, are operating within habitats to influence chemical variability in this species. Differences in morphology and chemistry of Pelorus Sound and Kapiti Island sponges may in part be explained by extremes in their physical environment; Kapiti Island represents a high-energy well-mixed coastal environment, whereas Pelorus Sound was a semiestuarine sheltered environment influenced by freshwater runoff. Variations in *M. hentscheli* compounds were, however, not directly correlated to water temperature, depth, or salinity.

Variations in the relative composition of the biologically active metabolites in *M. hentscheli* over time were site-specific. Compounds in sponges at the sheltered Pelorus Sound site were generally higher during spring and summer, and lower in winter. Duckworth (2000) described a similar seasonal pattern in toxicity of the New Zealand sponge *L. wellingtonensis*, suggesting that higher degrees of biochemical activity within the sponge in spring prevented fouling of the surface. Intraindividual cyclic variation in toxicity has also been described in the sponge *C. crambe*. High toxicity evident in the peripheral ectosome of

C. crambe during the boreal summer and autumn correlated with seasonal variation in competitors (Turon et al., 1996). The lack of obvious seasonal variation in *M. hentscheli* secondary metabolites at the exposed Kapiti Island site remains unexplained, and the duration of our study was too short to confirm that temporal variation in metabolite concentrations observed for sponges at Pelorus Sound has annual periodicity, and did not occur by chance alone. The temporal variations in metabolite concentrations observed could also be explained by seasonal changes in skeletal spicule mass relative to metabolite concentrations. However, the gravimetric assay is unlikely to have confounded the interpretation of seasonal patterns in *M. hentscheli*, because spicules in this species are sparse relative to tissue volume and are part of a skeleton that is identical in form in any part of the sponge, new or old growth (unpublished data).

Relatively few studies have documented depth-related variation in secondary metabolites within habitats. Harvell et al. (1993) recorded higher concentrations of diterpenes in deep colonies of the gorgonian *Briarium asbestinim*. Chanas and Pawlik (1997) suggest higher levels of chemical deterrence in deeper *Xestospongia muta* colonies are correlated to depth-specific differences in predation levels. Conversely, higher concentrations of diterpenes found in *Rhopaloides odorabile* in shallow, illuminated conditions may deter surface overgrowth by algae (Thompson et al., 1987). Our results suggest that although there is a complex relationship, depth may be an important factor for peloruside A production in *M. hentscheli* sponges in Pelorus Sound.

The ecological role of secondary metabolites in *M. hentscheli* is unknown. However, the variability of metabolite concentrations between sites and similar temporal patterns in populations within sites suggest that *M. hentscheli* sponges produce secondary metabolites in response to site-specific ecological factors, which have yet to be determined.

Aquaculture experiments on *M. hentscheli* explants transplanted into different environments (unpublished data) further support our hypothesis that the expression of biologically active compounds in this species is determined by site-specific environmental conditions. Therefore, if compounds from this species are of value to harvest then the biological factors triggering metabolite production, as well as seasonal and geographic patterns, need to be considered in the design of any aquaculture program.

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PHYTOTOXIC EFFECTS AND CHEMICAL ANALYSIS OF LEAF EXTRACTS FROM THREE *Phytolaccaceae* SPECIES IN SOUTH KOREA

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Abstract—We analyzed phenolic compounds and other elements in leaf extracts and compared morphology of three species of the Phytolaccaceae family found in South Korea. To test allelochemical effects of the three *Phytolacca* species, we also examined seed germination and dry weight of seedlings of *Lactuca indica* and *Sonchus oleraceus* treated with leaf extracts. The concentrations of total phenolic compounds were exotic *Phytolacca esculenta* (3.9 mg/l), native *Phytolacca insularis* (4.4 mg/l), and exotic *Phytolacca americana* (10.2 mg/l). There was no significant difference in concentrations between *P. esculenta* and *P. insularis*, but the concentration of total phenolics in *P. americana* was two times higher than either *P. esculenta* or *P. insularis*. Analysis of aqueous extracts by HPLC showed seven phenolic compounds (gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, *m*-hydroxybenzoic acid, *ρ*-coumaric acid, and cinnamic acid). Total phenolics in *P. americana* were eight to 16 times higher than either *P. esculenta* or *P. insularis*, respectively. *P. americana* inhibited seed germination and dry weight of the two assay species. The phytotoxic effects of the two *Phytolacca* species were different, despite the fact that *P. esculenta* and *P. insularis* had similar levels of total phenolic compounds. We also found that *P. americana* had invaded Ullung Island, which suggested that *P. americana* had excellent adaptability to the environment. The three species of Phytolaccaceae in South Korea can be distinguished by their different allelopathic potentials and morphologies.

Key Words—Allelochemical effects, Phytolaccaceae, total phenolic compounds, seed germination, morphology.

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INTRODUCTION

Many plants in the Phytolaccaceae are distributed throughout tropical and subtropical zones. Three species of Phytolaccaceae are found in South Korea: *Phytolacca esculenta*, *Phytolacca insularis*, and *Phytolacca americana* (Lee, 1985). Among these, *P. americana* is native to North America, but is distributed widely in Europe and Asia. It is a perennial weed in the central and southern provinces and Cheju Island. Since 1993, it has become an invasive exotic in South Korea and is threatening native species (Lee et al., 1997; Han et al., 1998; Kim et al., 2000), especially in polluted areas (Park, 1995a; Park et al., 1999). *P. insularis* is native to Ullung Island, and *P. esculenta* is native to China, but occurs rarely in the central province including Cheju Island in South Korea (Lee, 1985; Park, 1995b). There have been several reports of the role of allelopathy in exotic plant invasions and its defense against the spread of invaders to maintain biodiversity of natural systems, community structure, and ecosystem function (Callaway and Aschehoug, 2000; Keane and Crawley, 2002; Kennedy et al., 2002; Bais et al., 2003; Vila and Weiner, 2004). Little is known about the Phytolaccaceae except for a few papers dealing with the invasive threat of *P. americana* in South Korea (Han et al., 1998; Kim et al., 2000). Researchers in South Korea have claimed that *P. esculenta* and *P. insularis* are not morphology distinct, although their distributions are limited to different areas. The opinion of researchers is that *P. insularis* is the same species as *P. esculenta* and that there are no native species of Phytolaccaceae in South Korea. To address this issue, morphological and chemical analyses of these three species were conducted.

Allelopathy is an ecological mechanism operating in natural and managed ecosystems. In competition between exotic and native species, exotic species that produce large amounts of allelochemicals effectively repel other species, and their ability to invade plant communities increases (Pellissier, 1993; Inderjit and Dakshini, 1998; Kim et al., unpublished). There are many secondary metabolites that act as plant allelochemicals including phenolic, terpenoid, flavonoid, and alkaloids. Among these, phenolic compounds are the most abundant under field conditions and are known to affect seed germination, seedling growth, cell division, and fungal activity (Lodhi, 1976; Bhowmik and Doll, 1984; Rice, 1984, 1995; Inderjit, 2003; Kim et al., 2000).

Recently, phenolic-induced oxidative damage was found to be mediated by aluminum (Sakihama and Yamasaki, 2002) and that accumulation of such metals occurs by conjugation with phenolic compounds in various plant organs (Santiago et al., 2000; Lavid et al., 2001). Under field conditions, phenolics interfere with the growth and establishment of competing plant species by releasing water-soluble compounds into the soil. Furthermore, extracts of *P. americana* inhibit seed germination, seedling growth, and exhibit antifungal activity at low concentrations (Lee et al., 1997; Kim et al., 2000).

Phenolic compounds were not studied in the context of physiological responses associated with this family. Therefore the goal of this study was to characterize the three species located in different environments by comparing physioecology of allelochemical effects, heavy-metal accumulation, and morphology in South Korea. The objectives of the present study were to (1) determine the effect of the leaf extracts of Phytolaccaceae (*P. esculenta*, *P. insularis*, and *P. americana*) on seed germination and seedling dry weight of *Sonchus oleraceus* and *Lactuca indica* and (2) differentiate species of Phytolaccaceae according to morphology, phenolic compounds, and allelochemical effect.

METHODS AND MATERIALS

Study Areas. We examined the bioassay of seed germination and seedling growth with the extract of roots, leaves, fruits, and stems. Leaf extract had the strongest effect among these, so we used leaf extracts in this experiment. Plant extracts were made from fresh leaves of 10 individual plants per species (*P. esculenta* V. Houtte, *P. insularis* Nakai, and *P. americana* L.). Leaves were collected from Youngdong (36°10′N, 127°46′E) in Chungcheong Province (*P. esculenta*), Dodong, Ullung Island (37°28′N, 130°54′E) in Kyungpook Province (*P. insularis*), and Suwon (37°16′N, 127°00′E) in Gyeonggi Province (*P. americana*), South Korea, in August 2001. Seeds of *S. oleraceus* L. and *L. indica* Hara were collected in October and November 2000 from old fields in Seoul, South Korea (37°30′N, 127°12′E), and were used for the seed germination bioassay. Youngdong (*P. esculenta*) and Dondong (*P. insularis*) sites are located on rural hillsides with less anthropogenic pollutants. On the other hand, Suwon (*P. americana*) site is at a recently developed suburban hillside, influenced by heavy vehicle traffic. The climate is temperate, with four distinct seasons. June, July, and August, corresponding to the summer season, account for more than half of the total annual rainfall.

Analysis of Phenolic Compounds and Heavy Metals. Chemical standards used for analysis of phenolic compounds were purchased from Sigma Chemical Co. Two hundred grams of fresh leaves of Phytolaccaceae (*P. esculenta*, *P. insularis*, and *P. americana*) were extracted with 1-l distilled water at room temperature for 48 hr and then centrifuged at 15,000 rpm for 30 min (Centrikon T-1045, Kontron Co.). The supernatant was collected and stored at 4°C until used for bioassay and analysis. Phenolic compounds were purified from water extracts by adding 10 ml of saturated NaCl to 40 ml of aqueous extract in a funnel. The pH was adjusted to pH 2 with 1 N HCl. After adding 20 ml of 5% $NaHCO_3$ and mixing, we collected the $NaHCO_3$ solution. This was adjusted to pH 2 with HCl and mixed with 20 ml of ether. After mixing, the ether layer was evaporated under reduced pressure with a rotary evaporator. The residue was

dissolved into 5 ml of acetonitrile. Individual phenolics were analyzed by HPLC (Hewlett Packard Series 1050, USA), with diode-array detection (250, 254, 284 nm). Twenty microliters of extract was injected onto a μBonda-Pak C18 Radial Pak (0.8 × 10 m) column, with a mobile phase of acetonitrile and 0.02 M sodium acetate (pH 4.3) and a flow rate of 1.3 ml/min. Total phenolics were measured using the Folin-Ciocalteu phenol reagent (Swain and Hillis, 1959). Leaf extracts were analyzed for elements B, Al, Co, Cd, Cu, Fe, Mn, Ni, Pb, and As (Agricultural Improving Institute, 1988).

Morphological Comparison of Phytolaccaceae. The morphology of Phytolaccaceae was observed by anatomy microscope (36, EPT, 160T). The anther of *P. esculenta* was observed at 2.5 × 50 and the flower and the fruit at 2.5 × 8.5. The anther of *P. insularis* was observed at 2.5 × 25 and the flower and the fruit at 2.5 × 10, and the anther of *P. americana* at 2.5 × 30 and the flower and the fruit at 2.5 × 10. Those were compared between Phytolaccaceae. Photographs were taken using a Nikon F801 camera, using T-MAX 100 Kodak film (Phillips et al., 2000).

Seed Germination and Seedling Bioassay. Leaf extracts were filtered through Whatman No. 1 filter paper and then diluted to concentrations of 0, 12.5, 25, 50, 75, and 100% for bioassay. After being selected for uniform size, seeds from *S. oleraceus* and *L. indica* were sterilized for 3 min in a solution of 5% sodium hypochlorite and then rinsed with distilled water. Thirty seeds were sown in a Petri dish (diam 90 mm) on filter paper treated with leaf extract solution. The bioassay was repeated four times in a growth chamber set at a constant temperature of 26°C and 80% relative humidity, with an alternating photoperiod of 12 hr light/12 hr dark for 10 days. The germination percentage

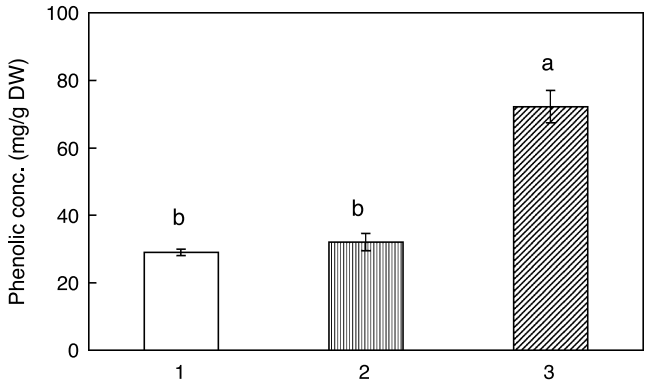

FIG. 1. Comparison of total phenolic compounds from leaf extracts of (1) *P. esculenta*, (2) *P. insularis*, (3) *P. americana*. Means (±SE, $N = 5$) with the same letter are not significantly different (Duncan's multiple range test, $P < 0.001$).

and dry weight for each seedling were measured at each concentration of extract. Seed germination was assessed daily, whereas the dry weight of seedlings was measured after 1 wk. This bioassay was modified from Lodhi (1976).

Statistical Analysis. The data were normally distributed, and significant differences at $P < 0.001$ between the total phenolic compound, treatments, and controls of the bioassay were calculated using Duncan's multiple-range test (SAS Institute 2000, Figures 1 and 4).

RESULTS

Among the species examined, *P. americana* had the highest total phenolic concentrations at 72.2 mg/l, whereas *P. esculenta* and *P. insularis* had concentrations of 29.0 and 32.1 mg/l, respectively (Figure 1, $P < 0.001$). Seven phenolic compounds were identified by HPLC analysis of Phytolaccaceae extracts: gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, *m*-hydroxybenzoic acid, *ρ*-coumaric acid, and cinnamic acid. Chlorogenic acid in *P. esculenta* and *P. americana* exhibited the highest concentration, whereas *P. insularis* appeared to have none. A small concentration of *m*-hydroxybenzoic acid appeared in *P. esculenta*, but not in *P. americana* and *P. insularis*. An unknown peak was present at Rt = 5.5 min in *P. esculenta* and *P. insularis* that was not observed in *P. americana*. Another unknown peak was observed at 14.5 min in *P. esculenta* that was not present in either *P. insularis* or *P. americana* (Figure 2).

Most of the elements (B, Al, Mn, Ni, Cu, Zn, As, Cd, Pb, except Fe) were found about 3–16 times more concentrated in the leaves of *P. americana* than those of *P. esculenta* and *P. insularis*, respectively (Table 1).

Fruit from the three Phytolacca species had different characteristics. The fruit of *P. esculenta* was berry-like with the ovary separated into eight cells. The number of stamens was eight, the anther was pink, and the stem was pale green (Figure 3A). The fruit of *P. insularis* was also berry-like, the number of stamens was eight, the anther was white, and the stem was green (Figure 3B). The fruit of *P. americana* was spherical, the number of stamens was 10, the anther was white, and the stem was green and reddish (Figure 3C).

Seed germination and dry weight of *S. oleraceus* and *L. indica* were inhibited with increasing concentrations of Phytolaccaceae extract (Figure 4). Germination rates of the two species were greatly reduced in the leaf extracts of *P. americana* in comparison to *P. insularis* and *P. esculenta*, and were slightly stimulated by 12.5 and 25% concentration of *P. insularis* and *P. esculenta* (Figure 4A and C), respectively. The dry weights of *S. oleraceus* and *L. indica* were similar to the germination result, but the dry weights of *S. oleraceus* were slightly stimulated by 12.5–75% leaf extracts of *P. insularis* and *P. esculenta* (Figure 4B). *L. indica* seedling dry weight was stimulated by 12.5–75% of

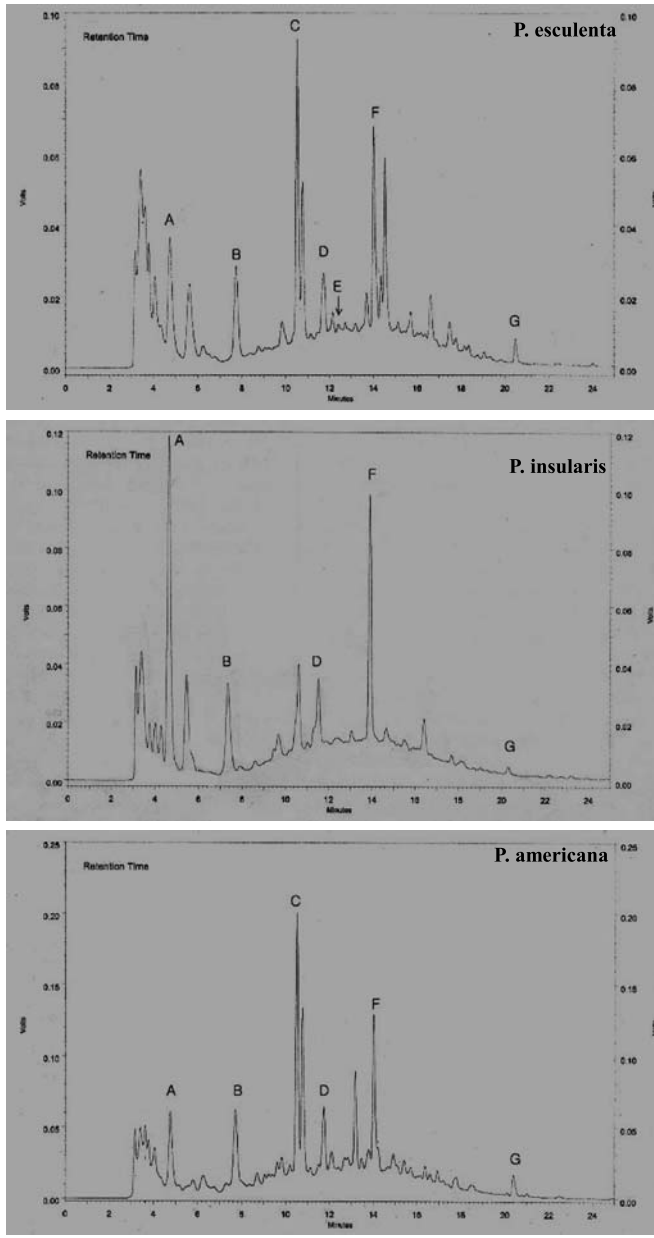

FIG. 2. Analysis of phenolic compounds in leaf extracts of *P. esculenta* (1), *P. insularis* (2), and *P. americana* (3) by HPLC. Identified phenolics are (A) gallic acid, (B) protocatechic acid, (C) chlorogenic acid, (D) caffeic acid, (E) *m*-hydroxybenzoic acid, (F) ρ-coumaric acid, and (G) cinnamic acid.

TABLE 1. HEAVY METAL AND NUTRIENT CONCENTRATIONS OF *Phytolacca* LEAF EXTRACT

	P. esculenta (mg/l)	*P. insularis* (mg/l)	*P. americana* (mg/l)
B	0.39	0.31	**0.81**
Al	1.49	1.32	**35.99**
Mn	8.11	0.42	**66.33**
Fe	2.80	**4.00**	2.32
Ni	0.0	0.0	**0.04**
Cu	0.04	0.03	**0.25**
Zn	0.72	0.05	**3.49**
As	0.04	0.04	**0.20**
Cd	0.0	0.0	**0.01**
Pb	0.0	0.0	**0.02**
Total	**13.59**	**6.17**	**109.46**

Highest concentrations of each element are in bold.

P. insularis (Figure 4D). Leaf extracts of the three species were significantly different (Figure 4, $P < 0.001$). Seed germination of *P. americana* was little affected by extracts of *P. americana* (data not shown).

DISCUSSION

Phenolic compounds have been studied as candidate allelochemicals because they are water-soluble and can complex with ions in the soil (Rice, 1984; Appel, 1993). *P. esculenta* and *P. americana* are exotic species, whereas *P. insularis* is the only native species of the Phytolaccaceae distributed in South Korea. *P. americana* is widely distributed not only in polluted areas, but also occurs in fields throughout South Korea, whereas the distribution of *P. esculenta* and *P. insularis* is limited and is decreasing in South Korea (Lee, 1985; Park, 1995a; Park et al., 1999). This phenomenon may be explained by total leaf phenolic concentration shown in the present study. *P. americana* had a much higher total phenolic concentration (72.18 mg/l) than those of either *P. esculenta* (29.04 mg/l) or *P. insularis* (32.08 mg/l) (Figure 1). The phenolic compounds in leaf extracts of *P. americana* were found to inhibit growth of 35 plant species and nine fungal species (Kim et al., 2000). The quantity and chemical nature of phenolics have important effects on the distribution of plant species and a plastic response that contributes to survival in adverse environmental conditions (Del Moral, 1972; Inderjit and Dakshini, 1998). Wu et al. (1998) isolated 14 phenolic compounds from *Buchloe dactyloides* with the most abundant phenolic compounds being salicylic and cinnamic acids. Among seven phenolic compounds analyzed, chlorogenic acid was the most abundant compound in the exotic species, *P. americana* and *P. esculenta*, whereas the

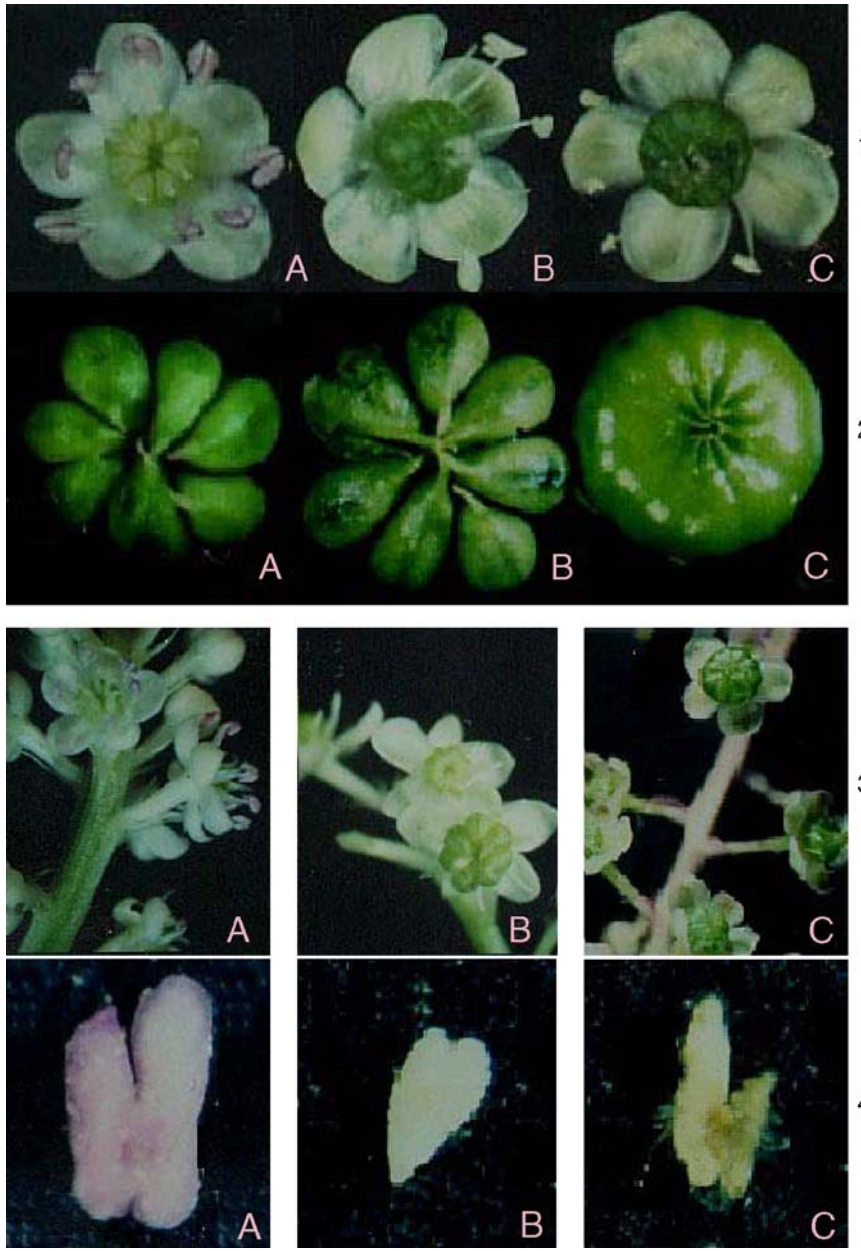

FIG. 3. Morphological comparison of flowers (1), fruits (2), stems and flowers (3), and anthers (4) on *P. esculenta* (A), *P. insularis* (B), and *P. americana* (C).

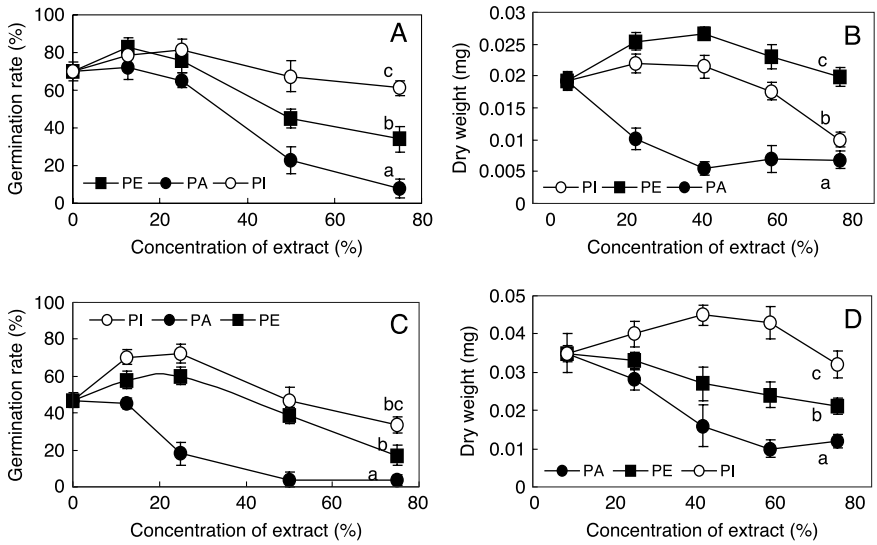

FIG. 4. Effect of the leaf extracts of *Phytolaccaceae* (PA: *P. americana*, PE: *P. esculenta*, PI: *P. insularis*) on seed germination and dry weight of *S. oleraceus* (A and B) and *L. indica* (C and D). Means (±SE, $N = 3$) with the same letter are not significantly different (Duncan's multiple range test, $P < 0.001$).

native species, *P. insularis*, had none (Figure 2). Therefore chlorogenic acid appears to be a candidate phenolic compound that could be used to differentiate between *P. esculenta* and *P. insularis*. Chlorogenic acid is derived from phenylalanine or tyrosine through the shikimic acid pathway and is more widespread in higher plants (Neish, 1964; Rice, 1984). In analysis of phenolic compounds from several plants, the unknown compounds in Figure 2 with Rt = 3.8, 5.5, and 14.5 min were identified as ρ-hydroquinone, gentistic acid, and scopoletin, respectively (Kim et al., 2000; Lee et al., 1997).

P. americana accumulates high concentrations of elements primarily in the leaves, whereas *P. esculenta* and *P. insularis* had lower concentrations (Table 1). Sakihama and Yamasaki (2002) reported that Al, Zn, Mn, and Cd stimulated phenoxyl radical-induced lipid peroxidation. The element concentrations in *P. americana*, especially Al and Mn, were higher than in *P. esculenta* or *P. insularis* (Table 1). *Ruta graveolens* treated with a mixture of Ni, Cu, Co, Cr, Cd, and Pb exhibited both qualitative and quantitative differences in phenolic concentration (Zobel et al., 1999), and phytoremediation plants in field accumulated high concentrations of metals in their foliage (Baker and Brooks, 1989; Raskin et al., 1997). Therefore, the accumulation of elements in the leaves of *P. americana* may be related to the high total phenolic concentration

that was observed in the leaves and may account for the species distribution. *P. americana* is widely distributed in polluted areas throughout South Korea, and its distribution has been correlated with absorption of heavy metals from polluted soil (Park, 1995a; Park et al., 1999).

Lee (1985) and Park (1995b) reported that the only difference between *P. esculenta* and *P. insularis* was the color of the anther. We found that the anther color of *P. esculenta* was pink and *P. insularis* was white and yellow (Figure 3A and B). *P. americana* showed different fruit, flower, stem, and anther color (Figure 3C). Recently *P. americana* has been found around Dodong on Ullung Island, which is visited by many people. This suggests that *P. americana* has invaded Ullung Island, possibly as a result of its high total leaf phenolic concentration and its ability to adapt to this environment. This invasion may threaten the distribution of *P. insularis*, the native species present on the island.

Allelopathic effects typically are the result of an interaction of a mixture of compounds rather than due to a single compound (Veronneau et al., 1997; Inderjit, 2003). Under field conditions, additive or synergistic effects among allelochemicals become more influential, especially at low concentrations (Dalton, 1989; Einhellig, 1995). Thus, we investigated the seed germination and dry weight of *S. oleraceus* and *L. indica* to test physiological effects of leaf extracts. The seed germination and dry weights of *S. oleraceus* and *L. indica* were dramatically inhibited by increasing concentration of *P. americana* extracts. The phytotoxic effects of *P. esculenta* and *P. insularis* were significantly different, although they had similar levels of total phenolic compounds (Figures 1, 2, and 4). We also investigated self-limiting phytotoxic compounds in *P. americana* to confirm if *P. americana* had self-constraining effects on its own seed germination and seedling growth. Seed germination of *P. americana* was little affected by extracts of *P. americana* (data not shown). Consequently, the extract of *P. americana* may decrease the invasiveness of neighbor species more than those of *P. insularis* and *P. esculenta* ensuring survival of their offspring. The phenolic compounds found in leaf extracts have been reported to reduce seed germination, seedling growth, and dry weight (Einhellig, 1995; Inderjit, 2003) as shown in the present study (Figure 4). Since 1993, *P. americana* has become a threat to native species in South Korea (Han et al., 1998; Park et al., 1999; Kim et al., 2000). The overall pattern of seed germination inhibition by leaf extracts containing phenolic compounds corresponds with the findings of previous studies (Pellissier, 1993; Lydon et al., 1997). Species containing abundant phenolic compounds strongly suppressed seed germination of other species. In our study, seedling growth and dry weight was slightly stimulated by 12.5–25% concentration of *P. insularis* and *P. esculenta* extract, but not with *P. americana* extract. Similarly, seed germination is stimulated by low concentrations of phenolic compounds (Heisey, 1990; Inderjit and Dakshini, 1998). Our results showed that *P. americana* also has abundant phenolic com-

pounds that inhibit seed germination and seedling biomass. We suggest that *P. americana* is a successful invader of existing plant communities and may be able to expand its habitats by employing foliar allelochemicals. We also predict that *P. americana* is more adaptable than *P. insularis* and *P. esculenta* to environmental changes through the production of self-defense allelochemicals. Therefore, we need to protect *P. insularis* and *P. esculenta* from *P. americana* to maintain species diversity in South Korea.

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ALLELOPATHIC EFFECTS OF VOLATILE MONOTERPENOIDS PRODUCED BY *Salvia leucophylla*: INHIBITION OF CELL PROLIFERATION AND DNA SYNTHESIS IN THE ROOT APICAL MERISTEM OF *Brassica campestris* SEEDLINGS

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Abstract—*Salvia leucophylla*, a shrub observed in coastal south California, produces several volatile monoterpenoids (camphor, 1,8-cineole, β-pinene, α-pinene, and camphene) that potentially act as allelochemicals. The effects of these were examined using *Brassica campestris* as the test plant. Camphor, 1,8-cineole, and β-pinene inhibited germination of *B. campestris* seeds at high concentrations, whereas α-pinene and camphene did not. Root growth was inhibited by all five monoterpenoids in a dose-dependent manner, but hypocotyl growth was largely unaffected. The monoterpenoids did not alter the sizes of matured cells in either hypocotyls or roots, indicating that cell expansion is relatively insensitive to these compounds. They did not decrease the mitotic index in the shoot apical region, but specifically lowered mitotic index in the root apical meristem. Moreover, morphological and biochemical analyses on the incorporation of 5-bromo-2′-deoxyuridine into DNA demonstrated that the monoterpenoids inhibit both cell-nuclear and organelle DNA synthesis in the root apical meristem. These results suggest that the monoterpenoids produced by *S. leucophylla* could interfere with the growth of other

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plants in its vicinity through inhibition of cell proliferation in the root apical meristem.

Key Words—Allelopathy, *Brassica campestris*, BrdU, camphor, 1,8-cineole, α-pinene, β-pinene, camphene, DNA synthesis, *Salvia leucophylla*, terpenoid, volatile growth inhibitor.

INTRODUCTION

The term "allelopathy" was first used by Molisch (1937) to define the phenomenon that one plant species (donor plant) releases chemicals into the environment and thereby affects the growth and development of other plants. In most cases, however, it remains unclear to what extent allelopathy plays a role in determining the superiority of a plant in natural plant communities because other interfering mechanisms (such as resource competition) may also operate simultaneously.

One of the best known and well-studied examples of allelopathy is the "*Salvia* phenomenon" (Müller et al., 1964). *Salvia leucophylla* is a shrub observed in coastal south California with its Mediterranean climate. It forms soft chaparral vegetation adjacent to areas of annual grassland, showing a characteristic vegetation patterning: annual grasses and forbs are excluded from the interior of the shrub thickets, and the thickets are frequently surrounded by areas of bare soil without grasses, extending 1–2 m beyond the crowns of the shrubs. The third to sixth meters beyond the shrub bear dense, but stunted herbage, and this inhibited vegetation gradually merges with normal grassland 6–10 m beyond the shrubs. The possible involvement of various factors other than allelopathy (for example, activity of small animals, edaphic factors, shading, nutritional conditions, salinity stress, and soil moisture) in this characteristic vegetation patterning was carefully examined and excluded (Müller, 1970, but also see Halligan, 1975).

Because the distance between shrub and inhibited herbs was beyond the lateral spread of the shrub system or crown, the involvement of volatile growth inhibitor(s) was suspected. Five volatile monoterpenoids (camphor, 1,8-cineole, β-pinene, α-pinene, and camphene) were found in *Salvia* leaves (Figure 1), and two of them (camphor and 1,8-cineole) were detected in the air around *Salvia* thickets (Müller, 1965). When added in pure reagent form, these volatile chemicals exhibit inhibitory effects on the seedling growth of test plants; camphor and cineole were most toxic (Müller and Müller, 1964). Müller (1970) also pointed out the importance of the soil as a reservoir of monoterpenoids because soil exposed to vapors from macerated *Salvia* leaves became toxic, and three monoterpenoids (camphor, cineole, and camphene) were detected in soil around *Salvia* shrubs. These results indicate that *S. leucophylla* produces and

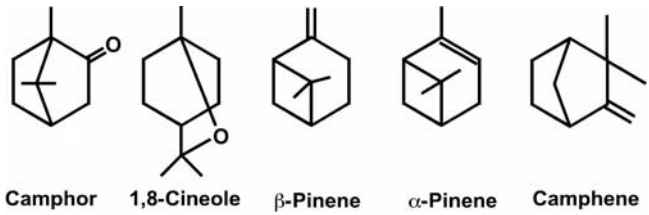

FIG. 1. Structures of monoterpenoids used in this study.

releases monoterpenoids, which adsorb to the soil in its vicinity and inhibit the growth of other plants, resulting in the characteristic vegetation patterning. However, the mode and mechanisms are not fully understood.

Koitabashi et al. (1997) examined the effects of 1,8-cineole on *Brassica campestris*, which was chosen as a test plant because of its rapid seed germination and uniform seedling growth. 1,8-Cineole inhibited growth of *B. campestris* seedlings in a dose-dependent manner, and its inhibitory effects were more severe on root than on hypocotyl growth. Treatment with 1,8-cineole decreased the mitotic index in the root apical meristem. Moreover, indirect immunofluorescence microscopy using an antibody raised against BrdU (a thymidine analog) revealed that treatment with 1,8-cineole decreased cell-nuclear and organelle DNA synthesis activities in root apical meristem. These results suggest that 1,8-cineole may interfere with the growth of other plant species by inhibiting DNA synthesis in the root apical meristem.

In the present study, the effects of five monoterpenoids produced by *S. leucophylla* on the germination and seedling growth of *B. campestris* were examined. The results demonstrated that each of them could act as a growth inhibitor, showing preferential inhibition of root growth. Quantitative analyses of the effects on cell expansion and cell proliferation revealed that they preferentially inhibit cell proliferation in the root apical meristem.

METHODS AND MATERIALS

Plant Material, Chemicals, and Treatment with Monoterpenoids. B. campestris L. var. *lacinifolia* (Bailey) Kitam. Seeds were purchased from Sakata Co., Ltd. (Yokohama, Japan). Seeds were soaked with 8 ml of deionized water on two layers of filter paper wad (Whatman No. 3, diam 55 mm) in transparent, airtight containers (4 cm diam $\times$ 6 cm high, 230 ml, 20 seeds per container) and incubated at 25°C under continuous illumination (80 μmol m^{-2} sec^{-1}). Monoterpenoids (camphor, 1,8-cineole, β-pinene, α-pinene, and camphene) were purchased from Aldrich (Wisconsin, USA). Camphor and

camphene, which are solid at room temperature, were dissolved in dimethylsulfoxide (DMSO) to give final concentrations of 1 M. The monoterpenoids were spotted onto a piece of filter paper (3.5 × 6 cm) hanging from the cap of the container and allowed to volatilize into the airspace within the container (Koitabashi et al., 1997). Concentrations in the airspace within the container were calculated assuming that the spotted compounds volatilize completely without any loss due to adsorption or leakage.

Measurement of Seedling and Cell Sizes. The lengths of hypocotyls and primary roots were measured with calipers. Only individual seeds that successfully germinated were used. For determination of cell size, root tips (5 mm long) and hypocotyls (full length) were excised from 4-day-old seedlings, fixed in an ethanol/acetic acid (9:1) solution overnight, followed by a washing in 90, 70, 50, and 30% ethanol, respectively, and were cleared with a chloral hydrate/glycerol/water solution (8:1:2, w/v/v) as described by Yadegari et al. (1994). Cells were observed under a microscope (BX-60, Olympus, Tokyo, Japan) equipped with Nomarski optics. Cortex cells at the root-hair-forming region of the primary roots and the epidermal cells at the middle region of the hypocotyls were chosen for determination of matured cell sizes. Photographs were taken using Neopan F black and white film (Fuji Film, Tokyo, Japan).

Frozen Sectioning and DAPI-Fluorescence Microscopy. Samples (root tips from 2-day-old seedlings or hypocotyls from 3-day-old seedlings) were fixed with formalin/acetic acid/50% ethanol (FAA, 1:1:18, v/v/v) for at least 12 hr. After fixation, samples were rinsed with 0.1 M sodium phosphate buffer (pH 7.0), dialyzed against 0.1 M sodium phosphate buffer containing increasing concentrations of sucrose (10, 20, and 30%, respectively), immersed in compound for frozen sectioning (O.T.C. Compound, Sakura Finetech, USA), and quickly frozen with liquid nitrogen. Frozen sections (7 μm thick) were prepared using a cryostat (OTE Cryostat, Bright, UK) at −26°C, mounted on gelatin-coated slides, and air-dried as previously described (Tamotsu et al., 1994). Sections on the slides were washed twice (10 min each) with PBS (100 mM sodium phosphate, pH 7.2, 150 mM NaCl), stained with staining solution [1 μg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI), 12.5% glycerol, 10 mM Tris–HCl, pH 7.6, 0.25 mM EDTA, 3.5 mM β-mercaptoethanol, 0.6 mM spermidine, 0.5% *n*-propylgalate], and the percentages of the cells in mitotic phase were counted using a fluorescence microscope (BX-60, Olympus).

BrdU Labeling and Indirect Immunofluorescence Microscopy with Anti-BrdU Antibody. Labeling of root tip cells with BrdU and indirect immunofluorescence microscopy with anti-BrdU antibody were performed essentially according to Koitabashi et al. (1997). *B. campestris* seedlings were labeled for 12 hr (from 36 to 48 hr after sowing) in the dark with 10 μM 5-bromo-2′-deoxyuridine (BrdU, a thymidine analog) supplemented with 1 μM 5-fluorodeoxyuridine (FldU) to improve labeling efficiency. The root tips (3 mm

long) were excised, fixed with 4% paraformaldehyde, dissolved in sodium cacodylate buffer (pH 7.4), and embedded in Technovit 7100 resin (Kulzer, Wehrheim, Germany) as described by Kuroiwa et al. (1990). Thin sections (1 μm thick) were cut with a glass knife on an ultramicrotome (MT-1, Sorvall, USA), expanded on water drops on a coverslip, and attached onto the coverslip by drying. The samples on the coverslip were first treated with anti-BrdU monoclonal antibody (Becton Dickinson Immunocytometry Systems, California, USA) and then with fluorescein isothiocyanate (FITC)-conjugated second antibody (goat anti-mouse IgGs, Tago, California, USA), and finally counterstained with DAPI, as described previously (Suzuki et al., 1992). The double-stained sections were examined under a fluorescence microscope (BX-60, Olympus). Localization of DNA was visualized as blue-white fluorescence of DAPI under UV excitation, whereas that of BrdU residue incorporated into newly synthesized DNA was visualized as yellowish-green fluorescence of FITC under blue-light excitation. Photographs were taken using color reversal films (Fuji chrome Provia 400, Fuji Film).

Immunodetection Analysis for Measuring DNA Synthesis Activities. To measure the total DNA synthesis activity in the root apical meristem, *B. campestris* seedlings were labeled for 12 hr (from 36 to 48 hr after sowing) in the dark with labeling solution (10 μM BrdU, 1 μM FldU), as described above. Approximately 40 root tips (1 mm long) were excised and collected in a 1.5-ml sampling tube, frozen with liquid nitrogen, ground into powder using a handmade microhomogenizer, dissolved in 125 μl of extraction buffer (50 mM Tris–HCl, pH 8.0, 100 mM EDTA, 300 mM NaCl, 4% SDS, 2% Sarkosyl), and incubated for 5 min at 65°C. Following phenol/chloroform extraction and chloroform extraction, the organic phases were successively re-extracted with 125 μl of HTE (50 mM Tris–HCl, pH 8.0, 20 mM EDTA), and the aqueous phases were combined (total 250 μl). After 5 μg of yeast tRNA was added to the combined aqueous phase, whole DNAs were precipitated with isopropanol and dissolved in 40 μl of TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). Concentrations of DNA were determined fluorimetrically using DyNA Quant (Amersham Biosciences, New Jersey, USA). The DNAs were dot-blotted (500 ng DNA/dot) onto nitrocellulose filters (Trans-blot, Bio-Rad, California, USA) using Dot-blot apparatus (Bio-Rad). The BrdU incorporated into DNA was detected with anti-BrdU monoclonal antibody (Becton Dickinson Immunocytometry Systems) using horseradish peroxidase (HRP)-conjugated second antibody (goat anti-mouse IgGs, Cappel, Ohio, USA) and a SuperSignal Substrate Western Blotting Kit (Pierce, Illinois, USA). To compensate for the minor differences in the DNA content of the samples, quantitative Southern blot hybridization was performed. Aliquots of the samples (approximately 20 ng DNA) were electrophoresed in an agarose gel, capillary-blotted onto nylon membrane (Biodine B, Pall, New York, USA), and hybridized with cloned

DNA fragment containing cytoplasmic 26S and 18S rRNA genes from rice (Sano and Sano, 1990) to detect and quantify the amount of cell-nuclear DNA. Detection of the hybridization signals was performed using Alkphos Direct (Amersham Biosciences). In both immunodetection and Southern hybridization experiments, standard curves were generated by blotting a dilution series of a sample. The signal intensities on the X-ray films (X-OMAT AR, Kodak) were measured with a densitometer (Densitograph AE, ATTO, Tokyo, Japan).

RESULTS

Effects of Volatile Monoterpenoids on Growth of B. campestris Seedlings. In the absence of volatile monoterpenoids, seed germination and seedling growth

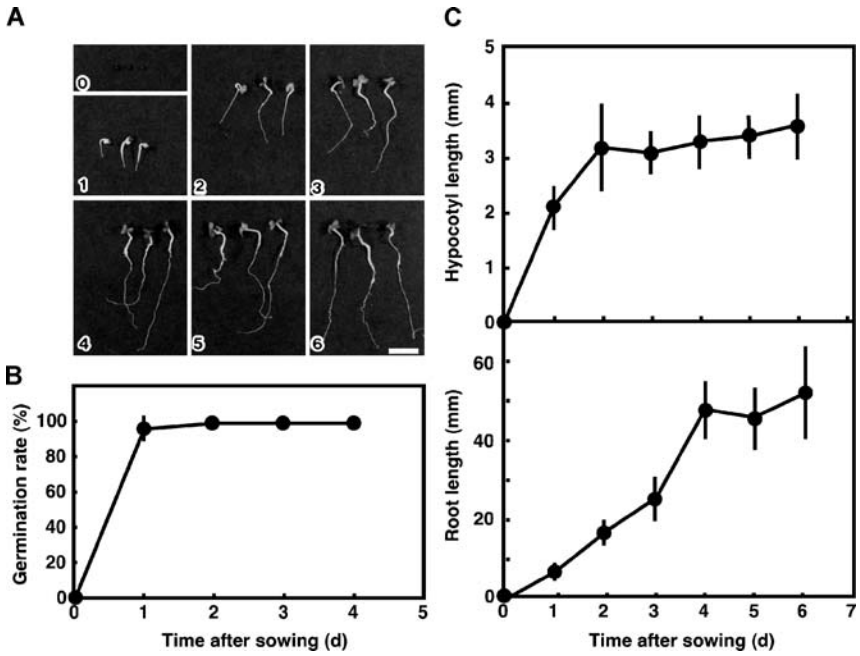

FIG. 2. Germination and seedling growth of *B. campestris* in the absence of monoterpenoids. (A) Photographs of the seeds or seedlings. Numbers represent the days after sowing. Scale bar = 5 mm. (B) Germination rate. (C) Hypocotyl and root lengths. In (B) and (C), average values from nine experiments (180 individuals) are shown. Vertical bars represent standard deviations.

of *B. campestris* proceeded rapidly and synchronously under our experimental conditions (Figure 2). Almost 100% germination was achieved within only 2 days after sowing. Hypocotyl length reached a maximum value (approximately 3.5 mm) on day 2, whereas maximum root length (approximately 50 mm) was achieved on day 4.

The effects of various concentrations of the five volatile monoterpenoids on seed germination were examined on day 4 (Figure 3A). Camphor and β-pinene slightly inhibited seed germination at 400 μM. At 1300 μM, in addition, 1,8-cineole inhibited seed germination. In contrast, α-pinene and camphene did not inhibit seed germination at any concentrations tested (up to 1300 μM), although they slightly delayed the progression of seed germination at high concentrations (data not shown).

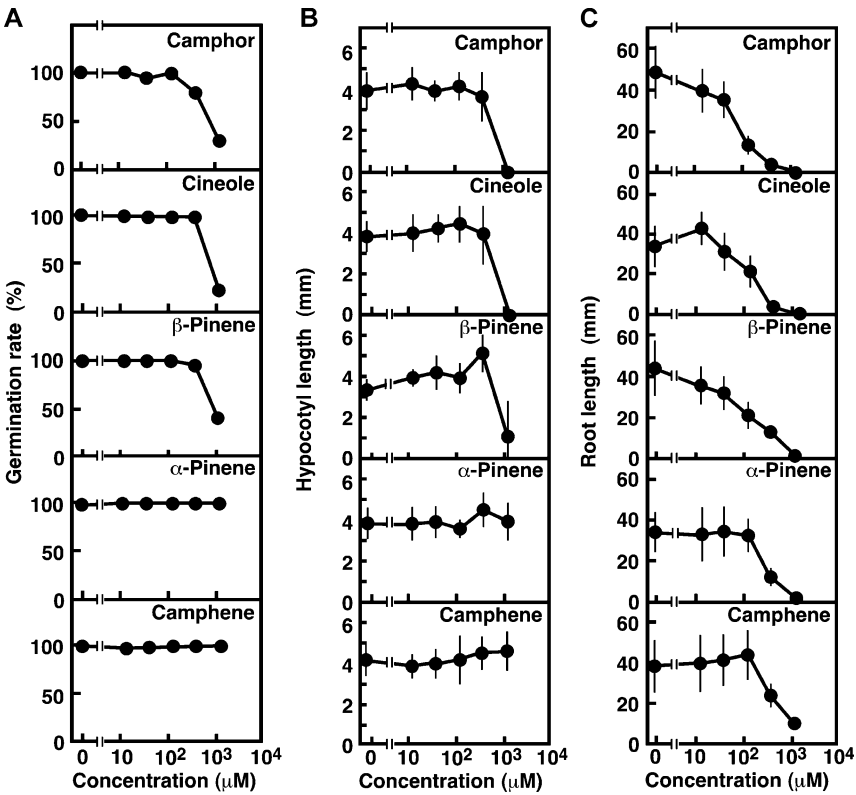

FIG. 3. Effects of the various concentrations of monoterpenoids. (A) Germination rate, (B) hypocotyls length, (C) root length examined on day 4. Each datum represents average values from 20 individuals. Vertical bars represent standard deviations.

The hypocotyl and root lengths of *B. campestris* seedlings that were grown for 4 days in the presence of various concentrations of monoterpenoids are shown in Figure 3B and C, respectively. Hypocotyl growth was relatively unaffected. No inhibition was observed at 400 μM or less for any of the monoterpenoids. At 1300 μM, camphor, 1,8-cineole, and β-pinene inhibited hypocotyl growth severely or partially, but α-pinene and camphene did not at any concentrations tested (up to 1300 μM). Root growth was more sensitive and was inhibited by all five monoterpenoids in a dose-dependent manner. Camphor, 1,8-cineole, and β-pinene shortened the root length even when they were added at low concentrations (130 μM or less). α-Pinene and camphene did not inhibit root growth at low concentrations (up to 130 μM), but did at higher concentrations (400 or 1300 μM). These results show that (1) all five monoterpenoids inhibit root growth more severely than hypocotyl growth or seed germination and (2) the strength of the monoterpenoids as growth inhibitors differs. The concentrations necessary to inhibit root growth to 50% of the control value (IC_{50}) were estimated to be approximately 90, 140, 150, 270, and 570 μM for camphor, 1,8-cineole, β-pinene, α-pinene, and camphene, respectively.

Effects of Volatile Monoterpenoids on Proliferation and Elongation of Shoot and Root Cells. For convenience, and to examine the mechanism of preferential inhibition of root growth by the monoterpenoids, we performed experiments at the concentrations where the root lengths of the 4-day-old seedlings were reduced to 25% of the control value (approximately 200, 300, 500, 750, and 1250 μM for camphor, 1,8-cineole, β-pinene, α-pinene, and camphene, respectively). At these concentrations, monoterpenoids did not affect hypocotyl growth (Figure 4A, also see Figure 3B and C).

The effects of monoterpenoids on the size of matured cells in hypocotyls and roots are shown in Figure 4B. Epidermal cells in the middle region of the hypocotyls and the cortex cells at the root-hair-forming regions of the primary roots were selected for cell size measurements as representatives of matured cells. None of the monoterpenoids decreased the size (length or width) of the matured cells in hypocotyls and roots, indicating that they did not inhibit cell expansion.

The effects of monoterpenoids on the mitotic indices in shoot apex (including both shoot apical meristem and young leaf primordia) and root apical meristem (within 400 μm from the tip of root proper) are shown in Figure 5. The mitotic indices of the shoot apex were scored 3 days after sowing, when the hypocotyl growth had completed and foliage leaves had started developing. The mitotic indices of the root apical meristem were scored 2 days after sowing, when the mitotic index in the root apical meristem of untreated seedlings reached the maximum value (Koitabashi et al., 1997). Monoterpenoids did not inhibit cell proliferation in the shoot apex under our experimental conditions. However, they all decreased the mitotic indices in the root apical meristem to

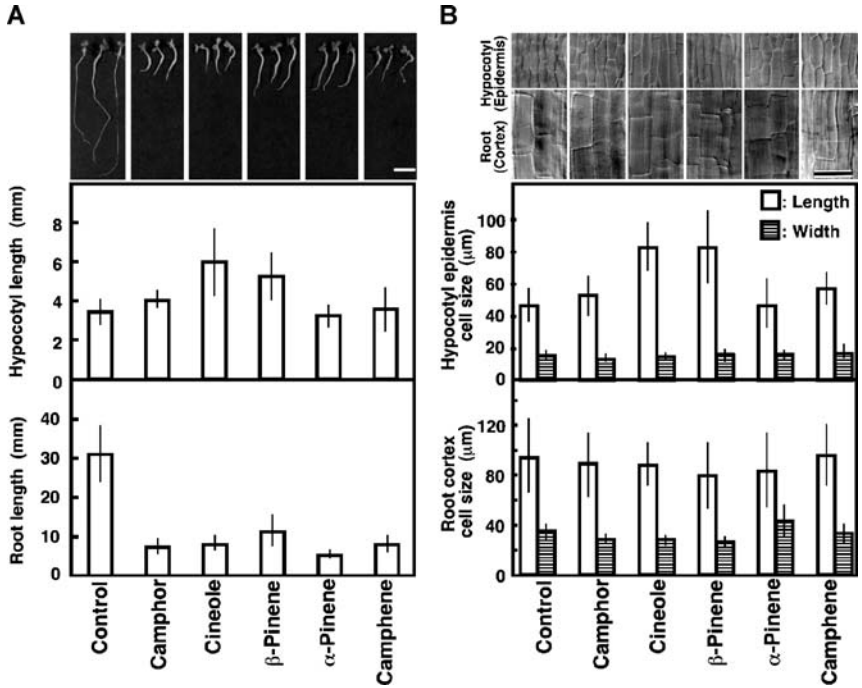

FIG. 4. Effects of monoterpenoids on the mature cell sizes in the hypocotyls and roots. The concentrations of monoterpenoids were adjusted so that root growth was reduced to approximately 25% of the control value. (A) Effects on the length of hypocotyls and roots. Photographs (top), hypocotyl length (middle), and root length (bottom) of the 4-day-old seedlings are shown. Each datum represents an average value from 20 individuals. Vertical bars represent standard deviation. Scale bar represents 5 mm. (B) Effects on the length (open box) and the width (hatched box) of matured cells in hypocotyl epidermis and root cortex. Nomarski images of mature cells (top), cell sizes of the matured hypocotyl epidermis (middle), and those of matured root cortex cells (bottom) are shown. Each datum represents average value from 40 cells. Vertical bars represent standard deviations. Scale bar represents 50 μm.

15–40% of the control value, demonstrating selective inhibition of cell proliferation in the root apical meristem.

Effects of Volatile Monoterpenoids on DNA Synthesis in Root Apical Meristem. In the root apical meristem of untreated seedlings, almost all cell nuclei and organelles were labeled clearly with BrdU (Figure 6a and b), reflecting their high DNA synthesis activities. In monoterpenoid-treated seedlings, by contrast, both labeling frequency and labeling intensity were lowered

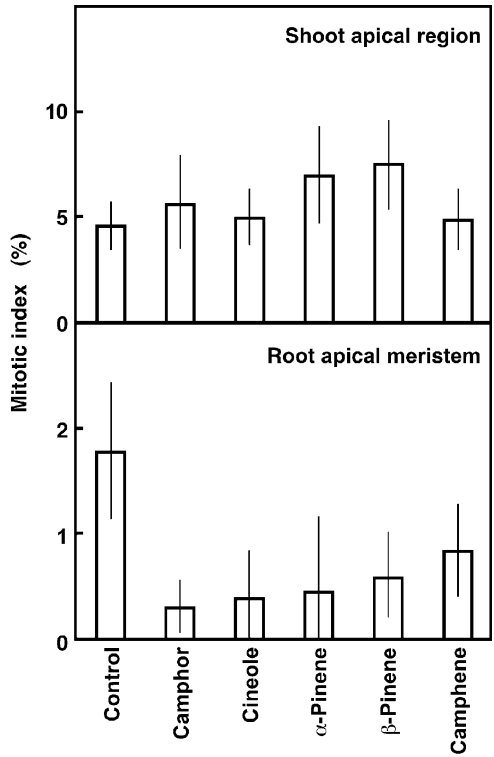

FIG. 5. Effects of the monoterpenoids on the mitotic index in shoot and root apical meristems. Top: Mitotic index of shoot apical region (including both shoot apical meristem and leaf primordia) of 3-day-old seedlings. Bottom: Mitotic index of root apical meristem (within 400 μm from the tip of root proper) of 2-day-old seedlings. Mitotic indices were counted on DAPI-stained frozen sections of the tissue under fluorescence microscopy. For convenience and clarity, only cells at meta- or anaphase were scored in the root apical meristem. Each datum represents average from ≧6 individuals (≧24 sections). Vertical bars represent standard deviations.

(Figure 6c–l), indicating that the monoterpenoids inhibited both cell-nuclear and organellar DNA synthesis within the root apical meristem.

The activity of DNA synthesis was determined quantitatively using an immunoblotting technique (Figure 7). The amount of BrdU residue incorporated into DNA (regardless of cell-nuclear DNA or organelle DNA) per unit cell-nuclear DNA was determined and was regarded as the activity of DNA synthesis per cell. The results demonstrate that treatment decreased the DNA synthesis activity in the root apical meristem to 7–40% of the control.

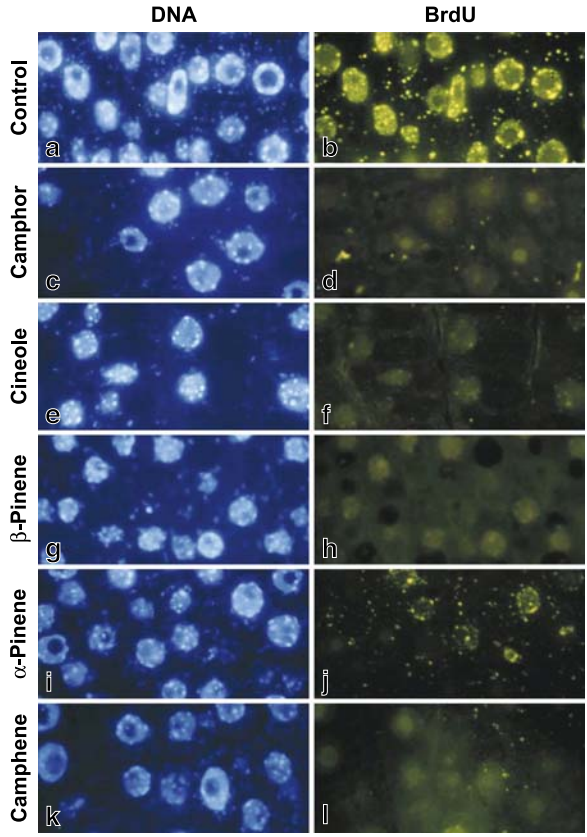

FIG. 6. Effects of monoterpenoids on DNA synthesis in the root apical meristem examined by immunofluorescence microscopy using anti-BrdU antibody. Technovit sections of the root apical meristem of *B. campestris* grown in the absence of terpenoids (a, b) or in the presence of camphor (c, d), 1,8-cineole (e, f), β-pinene (g, h), α-pinene (i, j), and camphene (k, l). *B. campestris* seedlings (1.5-day-old) were labeled with BrdU for 12 hr, embedded in Technovit 7100 resin, cut into thin sections, and examined by DAPI-fluorescence microscopy (a, c, e, g, i, and k) and by indirect immunofluorescence microscopy using anti-BrdU antibody (b, d, f, h, j, and l). Large, ovoid structures are cell nuclei, whereas small dots in the cytoplasm represent organelle nuclei. Dark areas and brighter spots of DAPI fluorescence within the cell nuclei are nucleoli and heterochromatin, respectively.

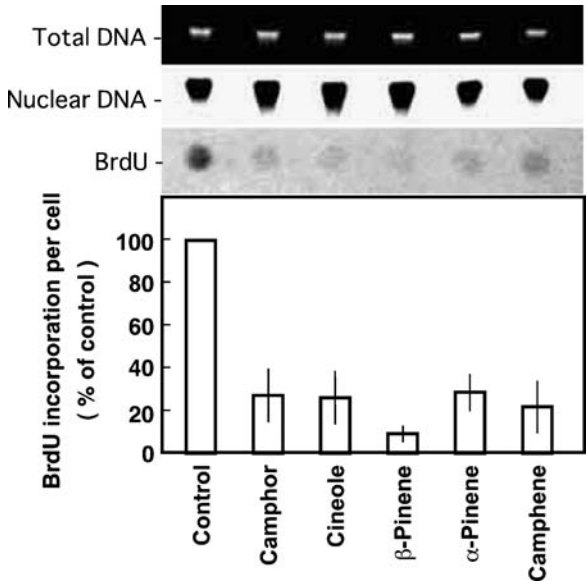

FIG. 7. Effects of monoterpenoids on DNA synthesis in root tips examined by immunodetection of the BrdU-labeled DNA on a nitrocellulose filter. *B. campestris* seedlings (1.5-day-old) were labeled with BrdU for 12 hr. Total DNA was extracted from root tips (1 mm long) and subjected to quantitative Southern hybridization analysis and immunodetection of BrdU residues incorporated into DNA. Shown are as follows: total DNA, EtBr-stained gel showing total DNA extracted from the root tips; nuclear DNA, hybridization signal of cell-nuclear rDNA to standardize the amount of cell-nuclear DNA in the sample; and BrdU, a result of immunodetection. Bottom: Relative amount of BrdU incorporated into unit amount of cell-nuclear DNA. Average values from three experiments (approximately 40 seedlings were used in each experiment) are shown. Vertical bars represent standard deviations.

DISCUSSION

Monoterpenoids as Growth Inhibitors. All five volatile monoterpenoids produced by *S. leucophylla* have the potential to inhibit the growth of *B. campestris* roots in a dose-dependent manner. *B. campestris* was chosen as the test plant because it germinates easily and uniformly and grows relatively quickly (Figure 2); these characters are important for bioassays (Dayan et al., 2000). The order of the strength of the five monoterpenoids as root growth inhibitors based on the IC_{50} values was estimated as camphor > 1,8-cineole > β-pinene > α-pinene > camphene. This order is in agreement with the report by Müller (1970)

that cineole and camphor were most toxic, among the monoterpenoids present in the *Salvia* leaves, to the growth of cucumber seedlings. Vaughn and Spencer (1993, 1996) reported that several monoterpenoids with oxygen-containing functional groups were especially inhibitory to germination of crop and weed seeds. As camphor and cineole have oxygen-containing functional groups while other monoterpenoids present in *S. leucophylla* leaves do not (Figure 1), our results support the argument concerning the relationship between monoterpenoid structure and function.

Mechanism for Root-Specific Growth Inhibition. All monoterpenoids inhibited root growth more severely than hypocotyl growth. One possible explanation is the differences in the actual concentrations of the monoterpenoids around hypocotyls and roots. Under field conditions, volatile monoterpenoids released from *S. leucophylla* are adsorbed to soil and then exhibit inhibitory effects onto other plants (Müller, 1970). Under our experimental conditions, similarly, monoterpenoids can be adsorbed onto a filter paper wad and accumulate to high concentrations, whereas concentrations in the airspace might become lower accordingly. However, we observed that the hypocotyl growth was largely unaffected by monoterpenoids even if they were forcibly associated with small pieces of papers (on which volatilized monoterpenoids should be adsorbed) during seedling growth (unpublished results), suggesting that the different sensitivities of hypocotyl and root growth could not be fully explained by differences in actual concentrations of monoterpenoids around them.

A more plausible explanation is differences in the growth modes of the two organs. Hypocotyl growth is largely dependent on elongation of each cell, which has already occurred in the embryo within the seed (Obroucheva, 1999), whereas root growth requires both proliferation and elongation of cells. The monoterpenoids did not inhibit cell expansion in either root or hypocotyls (Figure 4B), whereas they did inhibit cell proliferation and DNA synthesis in the root apical meristem (Figures 5–7). The observation that monoterpenoids did not inhibit root-hair formation, which depends on cell elongation, supports the opinion that cell expansion is relatively resistant to monoterpenoids (data not shown). Thus we conclude that the root-specific growth inhibition is observed because (1) cell expansion is relatively insensitive, whereas cell proliferation is sensitive, to monoterpenoids and (2) hypocotyl growth depends largely on cell expansion, whereas root growth requires not only cell expansion, but also cell proliferation.

Mechanism for Root-Specific Inhibition of Cell Proliferation. Although root-specific growth inhibition is largely explained by differences in the growth modes of roots and hypocotyls, the reason why cell proliferation in the shoot apical region was not affected by monoterpenoids (Figure 5) should be considered carefully. A decrease in the actual concentrations of monoterpenoids around the shoot apical region, resulting from adsorption, might be responsible.

To examine this possibility, measurement of actual concentrations of monoterpenoids around shoot and root apices is desired. Alternatively, if shoot and root apical meristems are exposed to the same concentrations of monoterpenoids (for example, by growing the seedlings in midair or in liquid culture media), this possibility could be examined easily. Another possibility is that permeability to monoterpenoids may be different between the root and shoot apex. In plants producing essential oils that are mainly composed of various monoterpenoids, production and accumulation of monoterpenoids are restricted to highly specialized structures (Mahmoud and Croteau, 2002). For example, peppermint (*Mentha* × *piperita* L.) stores monoterpenoids in the subcuticular space of the peltate glandular trichomes (Turner et al., 2000a,b), and volatilization of monoterpenoids from the storage sites occurs only at a low rate (Gershenzon et al., 2000). This indicates that the cuticle that covers the surface of aerial parts of a plant can provide a tight barrier against monoterpenoids. Alternatively, in the shoot apical meristem, the metabolic pathway that should be inhibited by terpenoids may be absent or modified to be more resistant to the compounds.

Mechanism for Inhibition of Cell Proliferation. Koitabashi et al. (1997) by using BrdU-immunofluorescence microscopy reported that treatment with 1,8-cineole lowered DNA synthesis activity in the root apical meristem of *B. campestris* seedlings. This procedure is advantageous in that DNA synthesis in cell nuclei and in organelles can be examined separately, but is disadvantageous in that quantitative estimation of the DNA synthesis activity is difficult. In the present study, therefore, a quantitative immunoblotting technique was applied to estimation of the DNA synthesis activity in the root tips. The results demonstrated that the DNA synthesis activity in the root tips of the monoterpenoid-treated seedlings was down to 7–40% of the control value (Figure 7). This is in agreement with the degrees of inhibition of the frequency of the cell division in root apical meristem (Figure 5) and root growth (Figure 4A). This suggests that monoterpenoids inhibit DNA synthesis in the root apical meristem, directly or indirectly, thereby exerting inhibitory effects on cell proliferation in the root apical meristem and, thus, on root growth.

Koitabashi et al. (1997) argued that 1,8-cineole might inhibit DNA synthesis indirectly because DNA replication in cell nuclei and organelles is inhibited simultaneously despite the fact that the nature of DNA synthesis machinery is rather different in nuclei and organelles. Indeed, there are several reports that monoterpenoids inhibit several animal enzyme activities that are not related to DNA synthesis. For example, 1,8-cineole inhibits pentoxyresorufin-*O*-dependentilase, a type of liver monooxygenase (de-Oliveira et al., 1999), and acetylcholinesterase (Savelev et al., 2003). These reports support the opinion that monoterpenoids inhibit some cellular metabolic pathway unrelated to DNA synthesis and ultimately stop DNA synthesis.

Recently, Romagni et al. (2000a,b) reported that 1,4-cineole (an analog of 1,8-cineole) inhibits root growth of test plants, and asparagine synthetase was identified as the primary target site of 1,4-cineole. The apparent similarities in the structures and symptoms suggest that 1,8-cineole may function in a similar way. However, 1,8-cineole did not inhibit asparagine synthetase activity *in vitro* (Romagni et al., 2000b), and root growth inhibition induced by 1,8-cineole could not be reversed by exogenously added asparagine (our unpublished results). Therefore, these two cineoles appear to have different modes of action (Romagni et al., 2000a) despite their similarities. The primary target site of 1,8-cineole and other monoterpenoids produced by *S. leucophylla* is still unclear.

Besides the possibility that inhibition of DNA synthesis is an indirect effect of monoterpenoids, it is also possible that monoterpenoids inhibit DNA synthesis directly. We found that *in vitro* DNA synthesis in the organelle nuclei isolated from BY-2 cultured tobacco cells (Sakai et al., 1999) was inhibited by high concentrations of monoterpenoids (unpublished results). DNA polymerases of plastids and mitochondria in higher plants resemble each other, and both appear similar to bacterial DNA polymerase I (Sakai et al., 1999; Sakai, 2001), suggesting that simultaneous inhibition of organelle DNA synthesis is possible. Moreover, because organelle DNA synthesis before cell nuclear replication is essential for subsequent cell propagation (Suzuki et al., 1996), inhibition of organelle DNA synthesis may result in the cessation of cell-nuclear replication and cell proliferation. The report that camphor induces unfolding of bacterial nucleoids (Harrington and Trun, 1997) also suggests that the target site may be around prokaryotic genetic systems. The primary target site of the volatile monoterpenoids should be examined in the future, taking these various possibilities into consideration.

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BIOTRANSFORMATION OF 2-BENZOXAZOLINONE TO 2-AMINO-(3*H*)-PHENOXAZIN-3-ONE AND 2-ACETYLAMINO-(3*H*)-PHENOXAZIN-3-ONE IN SOIL

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Abstract—An alternative to the use of synthetic pesticides is to exploit the natural defense chemicals produced by cereals. An important class of allelochemicals is cyclic hydroxamic acids and related benzoxazolinones. A prolonged degradation experiment of the allelochemical compound from rye 2-benzoxazolinone (BOA) was carried out for up to 90 d at 15°C at three different concentration levels, 3, 3000, and 30,000 nmol BOA g soil^{-1}, respectively, in a sandy loam soil. Two main degradation products, 2-amino-(3*H*)-phenoxazin-3-one (APO) and 2-acetylamino-(3*H*)-phenoxazin-3-one (AAPO), were identified and quantified by LC-ESI-MS-MS. The half-life of BOA increased with higher levels of BOA added to the soil. Half-lives of BOA, APO, and AAPO were determined by fitting a single first-order model to the degradation data. Half-life of BOA was determined to be 0.6 d in the 3 nmol BOA g soil^{-1} treatment. Half-lives of BOA, APO, and AAPO were 3.1, 2.7, and 2.1 d, respectively, in the 3000 nmol BOA g soil^{-1} treatment. In the 30,000 nmol BOA g soil^{-1} treatment, the half-lives were 31 d for BOA and 45 d for APO. The microbial community structure was not affected by addition of BOA to the soil as investigated by analysis of signature fatty acids. The results suggest that the exploitability of BOA for crop protection is dependent on the existing concentration of BOA in the soil and the timing of incorporation of hydroxamic acid synthesizing crops into the soil.

Key Words—*Secale cereale*, allelopathy, allelochemicals, hydroxamic acids, DIBOA, FAMEs.

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INTRODUCTION

Cyclic hydroxamic acids (benzoxazinones) and related benzoxazolinones are potential substitutes for synthetic pesticides in crop protection due to their allelopathic activity (Putnam and DeFrank, 1983; Barnes and Putnam, 1986). Several investigations have shown an inhibiting effect on the growth of weeds and reduced infestation by insects and pathogens on cereals producing cyclic hydroxamic acids (Bell and Nalewaja, 1968; Argandoña et al., 1980, 1981; Zúñiga et al., 1983; Pérez, 1990; Pérez and Ormeño-Nuñez, 1991; Niemeyer and Pérez, 1995; Wilkes et al., 1999).

Studies of the degradation kinetics for the benzoxazolinones and their metabolites provide necessary information for estimating the exploitability of these compounds in crop protection, as the weed-reducing effects are dependent on the half-lives in soil. The metabolite 2-amino-(3*H*)-phenoxazin-3-one (APO) has been reported to be more phytotoxic than 2-benzoxazolinone (BOA) (Gagliardo and Chilton, 1992). Soil temperature may influence degradation kinetics. In addition, exploitation of the benzoxazolinones and their metabolites in crop protection requires an estimation of the risk of leaching to aquatic environments, as the risk is likewise dependent on the half-lives of these compounds in soil.

Cyclic hydroxamic acids with the 1,4-benzoxazin-3-one skeleton are secondary plant metabolites found in several grasses (*Gramineae*), including the major agricultural crops rye (*Secale cereale*), wheat (*Triticum aestivum*), and maize (*Zea mays*) (Niemeyer, 1988b). In rye, several allelochemicals have been identified (Chou and Patrick, 1976) with the hydroxamic acid 2,4-dihydroxy-1,4-benxozaxin-3-one (DIBOA) being highest in concentration (Barnes et al., 1987). 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) is the most abundant allelochemical in wheat, whereas both DIMBOA and 2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one (DIM_2BOA) have been found in maize (Cambier et al., 1999). Rye contains minor amounts of the lactam 2-hydroxy-1,4-benzoxazin-3-one (HBOA) (Hofman and Hofmanová, 1969). Hydroxamic acids are chemically unstable in aqueous solutions and are rapidly decomposed to related benzoxazolinones (Bredenberg et al., 1962; Atkinson et al., 1991). Macías et al. (2005) showed an average half-life of 43 hr of DIBOA at concentrations of 1 and 5 mg g $soil^{-1}$ in nonsterilized water-flooded soils. DIM_2BOA hydrolyzes to 6,7-dimethoxy-benzoxazolin-2-one (M_2BOA), DIMBOA to 6-methoxy-benzoxazolin-2-one (MBOA), and DIBOA to BOA (Figure 1).

BOA and HBOA have several metabolites in common, including 2-amino-(3*H*)-phenoxazin-3-one (APO), 2-acetylamino-(3*H*)-phenoxazin-3-one (AAPO) (Figure 1), and *N*-(2-hydroxyphenyl)-malonamic acid (HPMA) (Zikmundová et al., 2002a). Three major classes of degradation products have been identified

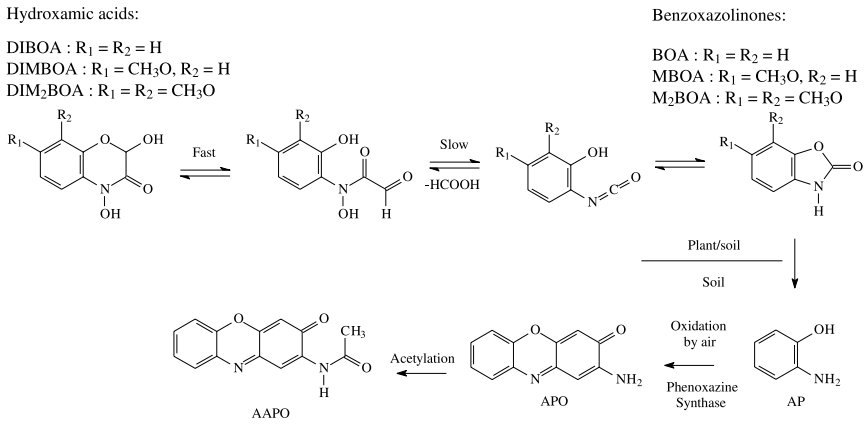


FIG. 1. Chemical transformation of cyclic hydroxamic acids to benzoxazolinones in plants or soils. In soil environments, the benzoxazolinones are degraded to AP. AP may be oxidized abiotically to APO by O_2 or due to catalysis by the enzyme phenoxazine synthase. APO may be further enzymatically acetylated to AAPO (Gents et al., 2005). Further transformation of APO and AAPO can occur as well. Modified after Sicker et al. (2000) and Zikmundová et al. (2002b).

from degradation of BOA and the lactam HBOA. These include aminophenoxazinones (Gagliardo and Chilton, 1992; Friebe et al., 1996; Zikmundová et al., 2002a; Fomsgaard et al., 2004), acetamides (Zikmundová et al., 2002b), and malonamic acids (Friebe et al., 1998; Yue et al., 1998; Vilich et al., 1999; Zikmundová et al., 2002a).

Several of these degradation products such as APO, AAPO, and HPMA have been found to be produced by different species of fungi grown in liquid media (Zikmundová et al., 2002a,b). Degradation of BOA has been reported to be conducted by both bacteria (Chase et al., 1991b; Friebe et al., 1996) and fungi (Friebe et al., 1998; Yue et al., 1998; Vilich et al., 1999; Zikmundová et al., 2002a,b). However, few degradation experiments of benzoxazolinones with identification and quantification of transformation products have been carried out in soil (Nair et al., 1990; Chase et al., 1991b; Gagliardo and Chilton, 1992; Kumar et al., 1993).

Studies of microorganisms in soil pose complex technical problems due to the heterogeneity of soils. Identification of microorganisms by classical methods with cultivation, isolation, and identification of microorganisms in Petri plates is time consuming. Furthermore, the selected medium may favor the growth of certain groups (Bakken, 1985), and the separation of the microorganisms from soil particles may be inconsistent (Schallenberg et al., 1989). An alternative method to estimate the community structure responsible for the microbial

processes taking place in environmental samples is by identification of biomarkers such as signature fatty acids. Biomarkers that are restricted to certain groups of organisms are used to define community structure. The narrow composition of specific signature fatty acids for subsets of the microbial community has been shown (Lechevalier, 1977). Any changes in the signature fatty acid patterns would thus indicate that the microbial community has been affected (Bååth et al., 1998).

The objective of this study was to determine the half-life of BOA at three different concentration levels. The chosen temperature condition at 15°C corresponds to a realistic soil temperature in the northern part of Europe in summertime. Other objectives were to identify degradation products from BOA and to identify changes in the microbial community structure due to addition of BOA by using the signature fatty acid pattern technique.

METHODS AND MATERIALS

Experimental Design. A degradation experiment of BOA was performed in soil by measuring the formation and degradation of the metabolites APO and AAPO. The experiment was performed with two main variables: concentration of BOA and time. The four initial concentrations were 0 (control), 3, 3000, and 30,000 nmol BOA g soil^{-1}. A total of 60 samples for each concentration level of BOA were incubated. At preselected time intervals, the incubation was terminated for sets of six replicates from d 0 to d 15 and for sets of three replicates from d 20 to d 90 for each concentration level. A recovery experiment of BOA, APO, and AAPO was carried out with the same sandy loam soil as used for the degradation experiment.

Soil Data. A sandy loam (Typic Agrudalf, classified according to USDA Soil Taxonomy) (Greve et al., 1998) soil was collected on the 8th of April 2003 from the upper 0–30 cm from a cultivated field near the Research Centre Flakkebjerg, Denmark. The previous cultivated crop in the field was spring-sown barley in 2001, 2002, and 2003. The soil was gently crushed and passed through a 2.00-mm-mesh-size sieve and stored at 4°C at field moisture concentration until start of the experiment. Characteristics of the sandy loam soil are as follows: clay (<2 μm) 18%, silt (2–20 μm) 15%, fine sand (20–200 μm) 40%, coarse sand (200–2000 μm) 24.2%, carbon 1.64%, and pH 6.8 (measured in water).

Solutions and Chemicals. A working standard solution of 40 g l^{-1} BOA (Acros Organics 98%) was prepared by dissolving BOA in MeOH (Ratburn Chemicals Ltd., Walkerburn, Scotland). APO and AAPO were dissolved in dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) at concentrations of 1.5 and 1.1 g l^{-1}, respectively. Gifts of APO were obtained from Professor

Chilton, NC State University, USA, and Professor Igarashi, Toyama Prefectural University, Japan. Later, APO (pure by elemental analysis, TLC, IR, and ^{1}H and ^{13}C NMR spectroscopy; Mp 256–258°C) and AAPO [pure by elemental analysis, TLC, IR, and ^{1}H and ^{13}C NMR spectroscopy; Mp (corr) 298–299°C] were synthesized in another project in our laboratory (Gents et al., 2005). Working standard solutions of BOA, APO, and AAPO for the degradation and the recovery experiments were obtained by serial dilution in MeOH of the stock standard solutions. HPLC-grade solvents hexane and methyl-*tert*-butyl ether were purchased from Ratburn Chemicals Ltd. and acetic acid from Riedel-de-Haën (Seelze, Germany).

Degradation Experiment. Five grams of chemically inert Ottawa quartz sand (particle size 20–30 mesh, Fisher Chemicals) was added to 100-ml Erlenmeyer flasks, and four different concentrations of BOA were added: 0 (control), 3, 3000, and 30,000 nmol BOA g soil^{-1}, respectively. The applied initial concentrations of 3, 3000, and 30,000 nmol BOA g soil^{-1} correspond to 0.4, 400, and 4000 μg BOA g soil^{-1}, respectively. MeOH from the BOA solutions was evaporated under nitrogen. Wet soil, 28.0 g, corresponding to 25.0 g of dry soil was added to the Erlenmeyer flasks and mixed. The soil was moistened evenly by addition of 2.2 ml of milliQ autoclaved water to obtain a water concentration corresponding to field capacity at 17.3%. The Erlenmeyer flasks were closed, and the holes in the stoppers were filled with wads of cotton wool allowing circulation of air. All samples were incubated in the dark at 15°C.

Sampling. The incubation was discontinued for six replicates of each treatment after 0, 1, 3, 5, 7, 10, and 15 d and for three replicates after 20, 30, 40, 50, 60, and 90 d. Each of these samples was stored at −20°C until freeze-drying. Later, the samples were freeze-dried for a period of 12–24 hr in random order. After freeze-drying, the soil was gently crushed and homogenized in the Erlenmeyer flasks and frozen again at −20°C.

Extraction and Chemical Analysis of BOA and Metabolites. The freeze-dried soil materials were transferred to PVC-free polyethylene bags and homogenized. Two times 3.0 g of mixed sand and soil from each of the homogenized samples were weighed out for fatty acid analyses and stored at −20°C in sealed polyethylene bags. The rest of each sample was stored in sealed PVC-free polyethylene bags at −20°C.

Extraction of the compounds from the soil was performed using an Accelerated Solvent Extraction 200 system (ASE) from Dionex. Nineteen grams of soil were added to the 33-ml extraction cell, and a thin layer of Ottawa sand was added to the top of the cell. The eluent was MeOH containing 0.5 vol.% glacial acetic acid. Setup for the ASE 200 was as follows: preheat for 5 min, heat for 5 min, static for 5 min, flush 80%, purge for 60 sec, cycles 2, pressure 1500 psi, and temperature 80°C. Extracts were collected in vials that

were filled to the same volume by addition of MeOH, corresponding to 50-ml solution. Extracts were stored at −20°C. During analysis, extracts were diluted to provide a signal close to the signal from external standards of BOA, APO, and AAPO. A comparison between a standard curve prepared in MeOH and a standard curve prepared in a soil extract showed that no matrix effect influenced the determination of the three compounds.

BOA and the degradation products were analyzed with Applied Biosystems MDS Sciex API 2000 liquid chromatography–tandem mass spectrometry (LC-MS-MS) system in turbo electrospray ionization (ESI) positive multiple reaction monitoring (MRM) mode. The identities of the metabolites were verified by comparison of the fragmentation patterns and retention times with synthetic reference compounds. BOA was detected at *m*/*z* 136/80, APO at *m*/*z* 213/185, and AAPO at *m*/*z* 255/213. The chromatography of the extracts was performed with gradient elution at a flow rate of 200 μl min^{-1} at 30°C. The column was a Hypersil BDS C18 (4.0 × 250 mm, 5 μm). Eluent-A consisted of 10% MeOH and 90% milliQ filtered water (v/v) with 20 mM glacial acetic acid. Eluent-B consisted of MeOH with 20 mM glacial acetic acid. The eluent program was as follows: 1 min 90% A followed by a linear gradient for 7 min to 30% A, which was kept for 7 min followed by a linear gradient in 5 min to 10% A, then back to 90% A in 1 min and 7-min restabilization with 90% A; the total runtime was 28 min.

The detection limits of the analytical method were determined according to Miller and Miller (1993). Two sets of the calibration solutions were prepared, and the linear parts of the calibration curves were used for regression analysis, resulting in detection limits of BOA at 0.2 nmol g $soil^{-1}$, APO at 0.04 nmol g $soil^{-1}$, and AAPO at 0.04 nmol g $soil^{-1}$, respectively.

Recovery Experiment. A recovery experiment was carried out as described for the degradation experiment except that the amount of wet soil was 11.2 g. Samples were not incubated but were placed at −20°C for 24 hr prior to extraction. Spiking concentrations of BOA were 3, 3000, and 30,000 nmol g $soil^{-1}$, whereas recovery experiments with APO were performed at concentrations of 3 and 700 nmol g $soil^{-1}$ and at concentrations of AAPO at 3 and 600 nmol g $soil^{-1}$, respectively. The applied concentrations of APO and AAPO correspond to 0.4 and 150 μg g $soil^{-1}$. In the spiking experiments with APO and AAPO at concentrations of 700 and 600 nmol g $soil^{-1}$, respectively, DMSO could not be evaporated so it was mixed into the soil. Recovery for BOA, APO, and AAPO was 73, 60, and 66%, respectively, in the 3 nmol g $soil^{-1}$ experiment, 75 and 83% for APO and AAPO, respectively, in the 700 and the 600 nmol g $soil^{-1}$ experiment, 75% for BOA in the 3000 nmol g $soil^{-1}$ experiment, and 92% for BOA in the 30,000 nmol g $soil^{-1}$ experiment (Table 1). The recovery estimates have not been used to correct the data from the degradation experiment.

TABLE 1. PERCENTAGE OF RECOVERY OF BOA, APO, AND AAPO FROM SPIKED FLAKKEBJERG SANDY LOAM SOIL

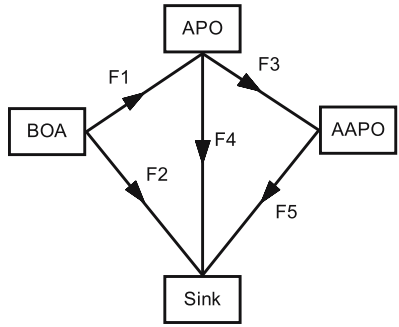
	Concentration of compound added to the soil				
Compound	3 nmol g soil^{-1} (%)	600 nmol g soil^{-1} (%)	700 nmol g soil^{-1} (%)	3000 nmol g soil^{-1} (%)	30,000 nmol g soil^{-1} (%)
BOA	72.9 ± 6.4			74.6 ± 11.2	91.7 ± 20.4
APO	60.4 ± 2.6		74.6 ± 8.3		
AAPO	66.0 ± 2.9	83.4 ± 8.1			

Means and standard deviation ($N = 7$). The molar masses of the compounds are BOA: 135; APO: 212, and AAPO: 254. 3000 nmol BOA corresponds to 400 μg BOA.

Kinetic Modeling. Degradation rates were obtained by modeling data for BOA, APO, and AAPO with ModelMaker 4 (AP Benson). A single first-order model was used, $dM\ dt^{-1} = -kM$ (Eq. 1), where M is the total amount of a substrate present at time t and k is the rate constant. In the flow transformation kinetic diagram (Figure 2), the transformation of BOA (Eq. 2), APO (Eq. 3), and AAPO (Eq. 4) is defined. Formation fraction is the fraction of a substance that is transformed from a precursor into a metabolite (e.g., ff_{APO} is the fraction of BOA transformed into APO) (Boesten et al., 2004). In case of first-order reactions, the formation fraction is equal to the proportion between the two rate constants for the transformation of BOA to APO and BOA to "sink," respectively (Boesten et al., 2004). The term "sink" (Eq. 5, Figure 2) is defined as a pool of nonspecified degradation products, including CO_2 and bound residues. The formation of 1 mol of APO requires 2 mol of BOA.

Extraction and Analysis of Fatty Acids. The extraction of fatty acids from whole cells was performed as a four-step procedure consisting of saponification, methylation, extraction, and wash according to Larsen et al. (2000). The method was followed except for a few modifications in the saponification process. One gram of the mixed sand and soil was saponified in 1.0 ml, 3.75 M NaOH in 50% MeOH and 50% water (v/v) in a screw cap test tube and vortexed for 5–10 sec. The tube was heated at 100°C in a water bath for 1 min, and the lids were tightened again. After an additional minute, the test tube was vortexed for 5–10 sec and returned to the water bath for 28 min followed by cooling on ice. Larsen et al. (2000) described the gas chromatography conditions for analysis of the extracted fatty acid methyl esters. The relative amount of each fatty acid was measured in proportion to the internal standard fatty acid 19:0.

The selected signature fatty acids for gram-positive bacteria were: 15:0I, 15:0A, 16:0I, 17:0I, and 17:0A; gram-negative bacteria: Cy17:0 and Cy19:0ω8; total bacteria: 15:0I, 15:0A, 16:0I, 17:0I, 17:0A, Cy17:0, Cy19:0ω8, 14:0,



Rate equations

$$dBOA \cdot dt^{-1} = -F1-F2 = -k_{BOA} \cdot BOA \cdot ff_{APO} - k_{BOA} \cdot BOA \cdot (1-ff_{APO}) \quad \text{(Eq. 2)}$$

$$dAPO \cdot dt^{-1} = F1-F3-F4 = k_{BOA} \cdot BOA \cdot ff_{APO} - k_{APO} \cdot APO \cdot ff_{AAPO} - k_{APO} \cdot APO \cdot (1-ff_{AAPO}) \quad \text{(Eq. 3)}$$

$$dAAPO \cdot dt^{-1} = F3-F5 = k_{APO} \cdot APO \cdot ff_{AAPO} - k_{AAPO} \cdot AAPO \quad \text{(Eq. 4)}$$

$$dSink \cdot dt^{-1} = F2+F4+F5 = k_{BOA} \cdot BOA \cdot (1-ff_{APO}) + k_{APO} \cdot APO \cdot (1-ff_{AAPO}) + k_{AAPO} \cdot AAPO \quad \text{(Eq. 5)}$$

where

BOA = Total amount of BOA present at time t
APO = Total amount of APO present at time t
AAPO = Total amount of AAPO present at time t
k_{BOA} = Rate constant for degradation of BOA
k_{APO} = Rate constant for degradation of APO
k_{AAPO} = Rate constant for degradation of AAPO
ff_{APO} = Formation fraction of APO
ff_{AAPO} = Formation fraction of AAPO

FIG. 2. Illustration of the flow transformation kinetic diagram between BOA and the metabolites APO, AAPO, and "sink" used in the modeling of the kinetics with definitions for the different rate equations.

10Me16:0, and 17:0; saprotrophic fungi 18:2ω6,9 and 18:3ω6,9,12; arbuscular mycorrhizal fungi: 16:1ω5; and protozoa: 20:4ω6,9,12,15. Nomenclature of the fatty acids is described by Vestal and White (1989).

Levels of significance of main treatments and their interactions were calculated by analyses of variance after testing for variance homogeneity with

Bartlett's test ($P > 0.05$). The statistical analyses were performed with StatGraphics Plus 4.0 (Statistical Graphics Corp.) for Windows.

RESULTS

In the 3 nmol BOA g soil^{-1} treatment, a rapid degradation of BOA was observed within the first 24 hr ($DT_{50} < 1$ d). In contrast to the 3000 and 30,000 nmol BOA g soil^{-1} treatments, no APO or AAPO was observed.

In the 3000 nmol BOA g soil^{-1} treatment, degradation of BOA started within the first 24 hr and was not complete after 30 d, as a background level was reached after 30 d at a concentration of 64 nmol BOA g soil^{-1} (Figure 3). The metabolite APO was already detected 24 hr after start of incubation, and the maximum level of 353 nmol APO g soil^{-1} was reached after 5 d, decreasing toward d 60 to 5.9 nmol APO g soil^{-1}. APO was further acetylated to AAPO. AAPO was detected after 24 hr, and the maximum concentration of 2.4 nmol AAPO g soil^{-1} was observed after 7 d, decreasing toward d 60 to 0.15 nmol APO g soil^{-1}. Half-lives of BOA, APO, and AAPO were estimated as 3.1, 2.7, and 2.1 d, respectively, from first-order fits to the degradation data (Table 2). The transformation of BOA into APO was 23%, and approximately 1% of APO was further acetylated into AAPO.

In the 30,000 nmol BOA g soil^{-1} treatment, the degradation time of BOA increased significantly compared to the 3000 nmol BOA g soil^{-1} treatment (Table 2). Degradation of BOA was incomplete after 90 d when a concentration of 158 nmol BOA g soil^{-1} was measured (Figure 3). APO again was identified within the first 24 hr and reached a maximum of 3950 nmol APO g soil^{-1} after 50 d and a concentration of 1583 nmol APO g soil^{-1} at d 90. At this concentration level of BOA, AAPO was not detected before d 7, and its maximum concentration of 7.6 nmol AAPO g soil^{-1} at d 60 was reached after the maximum concentration of APO had appeared. At d 90, 4.0 nmol AAPO g soil^{-1} was measured. In the 30,000 nmol BOA g soil^{-1} treatment, the estimated half-lives for BOA and APO were 31 and 45 d, respectively (Table 2). Formation fraction of APO was 21%. The decomposition rate for AAPO could not be determined because the maximum concentration was not seen until d 60.

The signature fatty acids for gram-positive bacteria (15:0I, 15:0A, 16:0I, 17:0I, and 17:0A) constituted about 12% of the total fatty acids in the soil samples (Figure 4). The relative value of signature fatty acids for total bacteria, gram-negative bacteria, saprotrophic fungi, arbuscular mycorrhizal fungi, and protozoa varied in proportion to gram-positive bacteria, depending on the existence of these groups of microorganisms in the soil. In general, no significant differences were measured as a function of time. Overall, no

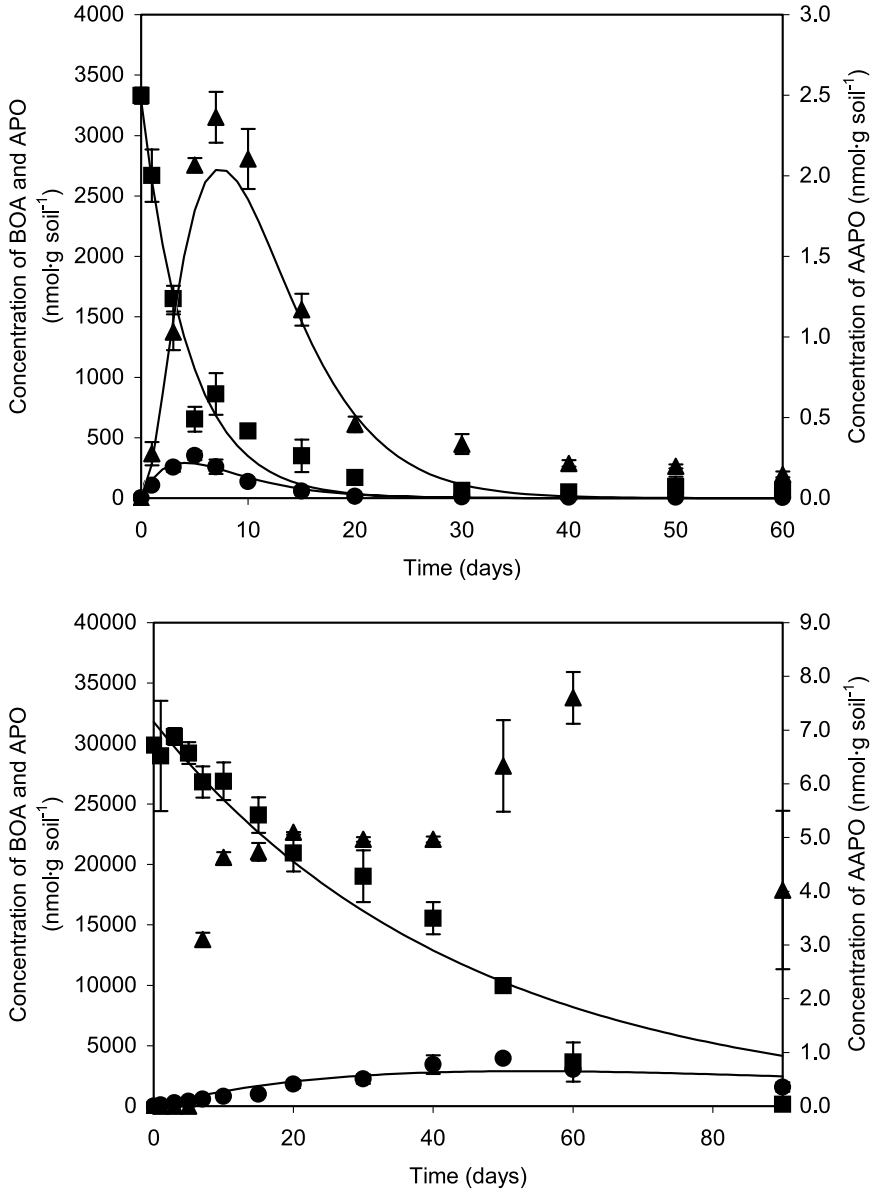


FIG. 3. Degradation kinetics of BOA in the 3000 nmol BOA g soil^{-1} (top) and 30,000 nmol BOA g soil^{-1} (bottom) treatments. BOA (■), APO (●), and AAPO (▲). Lines represent results from the single first order model (Eq. 1). Each point represents the mean of three replicates, and bars indicate standard deviation of means.

TABLE 2. DECOMPOSITION RATE CONSTANTS (k) FOR BOA, APO AND AAPO, FORMATION FRACTION (ff) OF APO AND AAPO, AND r^2 VALUES FOR THE THREE DEGRADATION EXPERIMENTS[a]

	Optimized value	Error	$t_{1/2}$[b]
3 nmol BOA g soil^{-1}			
k_{BOA}[c]	1.14 d^{-1}	0.28 d^{-1}	0.6 d
3000 nmol BOA g soil^{-1}			
k_{BOA}	0.23 d^{-1}	0.008 d^{-1}	3.1 d
k_{APO}	0.26 d^{-1}	0.09 d^{-1}	2.7 d
k_{AAPO}	0.34 d^{-1}	20.2 d^{-1}	2.1 d
ff_{APO}	0.23	0.07	
ff_{AAPO}	0.011	0.54	
r^2	0.98		
30,000 nmol BOA g soil^{-1}			
k_{BOA}	0.023 d^{-1}	0.001 d^{-1}	31 d
k_{APO}	0.015 d^{-1}	0.01 d^{-1}	45 d
ff_{APO}	0.21	0.08	
r^2	0.97		

[a] $N = 3$.
[b] Time required for 50% degradation of the compound.
[c] Data from d 0 and d 1 were used for modeling.

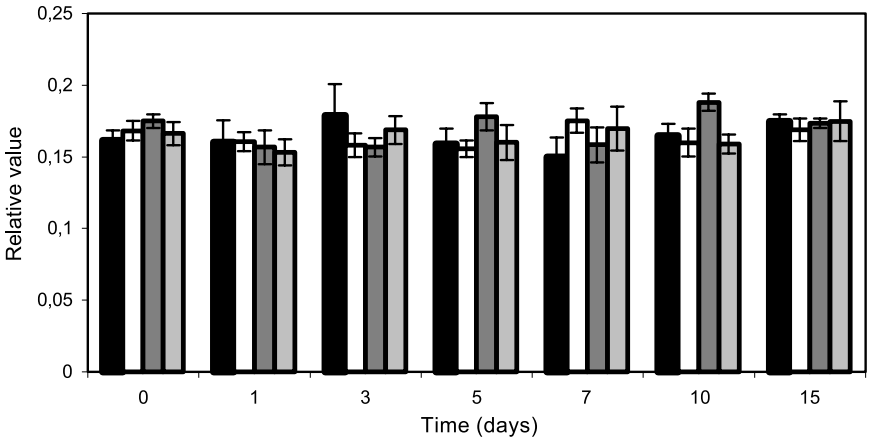


FIG. 4. Time course of changes in relative amount of signature fatty acids for gram-positive bacteria. Bars represent the total amount of the five signature fatty acids 15:0I, 15:0A, 16:0I, 17:0I, and 17:0A. The relative amount of each fatty acid was measured in proportion to the internal standard fatty acid 19:0. Each column represents the mean of five or six replicates, and bars indicate standard error of means. Control (black), 3 nmol BOA g soil^{-1} (white), 3000 nmol BOA g soil^{-1} (dark gray), and 30,000 nmol BOA g soil^{-1} (light gray).

TABLE 3. MULTIFACTOR ANALYSIS FOR EFFECTS OF BOA ON SIGNATURE FATTY ACIDS PATTERNS[a,b,c]

Factor	Gram-positive bacteria	Gram-negative bacteria[d]	Total bacteria	Saprotrophic fungi[d]	VAM fungi	Protozoa[d]
P values						
Time (A)	0.49	0.11	0.43	0.65	0.59	0.13
Concentration (B)	0.67	0.25	0.52	0.43	0.49	0.63
A × B	0.61	0.89	0.48	0.33	0.85	0.42

[a] $N = 5$ or 6.
[b] Significance ($P < 0.05$).
[c] Two factors: time (A), concentration (B), and the interaction (A × B).
[d] Data are log-transformed.

statistically significant differences were observed for the six different groups of microorganisms. Multifactor analysis of variance showed no significant effects ($P < 0.05$) of time or concentration of BOA for any of the six groups of microorganisms (Table 3).

DISCUSSION

This is the first report of quantification of APO and AAPO and the decomposition kinetics for BOA, APO, and AAPO in soil. In accordance with our observations, where unreacted BOA in the 3000 and 30,000 nmol BOA g soil^{-1} treatments was measured even after 90 d, several other investigations have also shown an incomplete transformation of BOA within 10–12 d (Nair et al., 1990; Chase et al., 1991a,b; Gagliardo and Chilton, 1992). Gagliardo and Chilton (1992) found unreacted BOA after 10 d of incubation in a sandy loam soil at an initial concentration of 37,000 nmol BOA g soil^{-1}. Similarly, these authors found that BOA was transformed into APO (Gagliardo and Chilton, 1992). In contrast to our work, a complete transformation of BOA after 3–4 d in a liquid media has been reported, which may indicate a faster transformation of BOA in liquid media compared to soil environments (Friebe et al., 1996). A further transformation of APO to AAPO was similarly reported in liquid media (Friebe et al., 1996; Zikmundová et al., 2002a). The slower degradation of BOA observed in our work might be due to the incubation temperature (15°C), which is lower than the temperatures (23–28°C) used in many degradation studies (Nair et al., 1990; Gagliardo and Chilton, 1992; Friebe et al., 1998; Yue et al., 1998; Vilich et al., 1999; Zikmundová et al., 2002a). Increasing the temperature may affect degradation kinetics, as a higher temperature may accelerate the growth of the microorganisms and thereby the enzyme-catalyzed processes, resulting in a shorter half-life of BOA.

In the kinetic modeling of BOA degradation in soil and ensuing formation and degradation of APO and AAPO, the single first-order model was chosen because this model described the first and dominating part of the curves correctly. Models with a low number of parameters should be used when possible (Boesten et al., 2004), even if the changes in the observed half-lives indicate that we are not dealing with true first-order processes. The decomposition rate and formation fraction of AAPO in the 3000 nmol BOA g soil^{-1} treatment showed a large error due to the low amounts of AAPO formed, making modeling of the data difficult. However, modeling the degradation of AAPO from its maximum concentration, which is the most obvious alternative, resulted in the same half-life. In the 30,000 nmol BOA g soil^{-1} treatment, the decomposition rate of AAPO could not be determined because the maximum concentration was seen at d 60 and only one data point later than d 60 was obtained.

This is the first study of the microbial community structure by whole cell fatty acid (WCFA) signatures after addition of an allelochemical compound to the soil. Measurements of changes in the signature fatty acid patterns showed no significant differences among the treatments. The maximum initial concentration level of 30,000 nmol BOA g soil^{-1} is high compared to a recently measured natural concentration of BOA in soil. Gents et al. (2005) found a maximum concentration of BOA in the soil of 1 nmol BOA g soil^{-1} 7 days after incorporation of rye into the soil. Several investigations have shown differences in the levels of hydroxamic acids and benzoxazolinones in cereals (Niemeyer, 1988a; Burgos et al., 1999). Niemeyer (1988a) found that wheat species differed by a factor of 75 in the concentration of DIMBOA (0.21 mmol kg^{-1} fresh weight to 16 mmol kg^{-1} fresh weight), and Burgos et al. (1999) found that rye species differed by a factor of 10 in the concentration of DIBOA and BOA (137 μg g^{-1} dry weight to 1469 μg g^{-1} dry weight). These considerable differences in the levels of hydroxamic acids in cereals make it reasonable to expect higher concentrations in soil than the 1 nmol BOA g soil^{-1} found by Gents et al. (2005). Higher local concentrations in soil may occur under natural circumstances in the rhizosphere around cultivars with high contents of hydroxamic acids and the ability to exude hydroxamic acids from roots. Even a uniform distribution in the top soil (10 cm) of the 50 nmol BOA estimated in rye residues by Yenish et al. (1995) would lead to a mean concentration of 33 nmol BOA g soil^{-1}. However, when the distribution is not even, concentrations of BOA high enough for initiating the formation of APO and AAPO could easily be imagined. In addition, the concentrations of hydroxamic acids in cereals have been investigated more intensively in wheat species compared to rye species, which makes it realistic to believe that the rye species with the highest concentrations of hydroxamic acids have not yet been identified. Moreover, natural plant breeding or even gene modification may increase the

concentrations of hydroxamic acids in cultivars. Therefore it is relevant to perform degradation studies at higher initial concentrations than 3 nmol BOA g soil^{-1}.

Even at the highest concentration level used in the present study, no observable changes in the microbial community structure could be detected. The soil used had an organic matter (OM) concentration of 2.8%, corresponding to 28 mg OM g soil^{-1}. By addition of 30,000 nmol BOA g soil^{-1}, additionally 14% extra OM was supplied. In the degradation experiments with 3 and 3000 nmol BOA g soil^{-1}, detection of a change in the microbial community structure by WCFA was less likely. The results indicate that BOA, APO, and AAPO have little influence on the amount of biomass for the different groups of microorganisms investigated. The WCFA method does not exclude the possibility that changes have taken place within the different groups, although statistics of the signature fatty acids by principal component analysis showed no such shift. Both bacteria (Chase et al., 1991b; Friebe et al., 1996) and fungi (Friebe et al., 1998; Yue et al., 1998; Vilich et al., 1999; Zikmundová et al., 2002a,b) have been shown to transform BOA, showing that several different groups of microorganisms pose this ability. A natural level of BOA in the soil used might have favored the presence of microorganisms that are adapted to use BOA as a carbon source. In addition, the growth of the microbial population may have been limited by available nutrients (e.g., nitrogen), and the formed APO and AAPO might have lowered the microbial activity at the highest BOA concentration used. Anzai et al. (1960) demonstrated that APO and AP (Questiomycin A and Questiomycin B) had considerable antibiotic effects.

At a concentration of 3 nmol BOA g soil^{-1}, we found a half-life of BOA of 0.6 d, which is in agreement with Gents et al. (unpublished data) who reported a half-life of BOA of less than 0.5 d at the same initial concentration of BOA. The short half-lives of less than 5 d for BOA, APO, and AAPO in soil at concentrations of less than 3000 nmol BOA g soil^{-1} may provide weed control for only a short period once these compounds are released into the soil environment. Therefore production of high hydroxamic acid synthesizing crops by natural plant breeding or even gene-modified cultivars with improved capacities for producing these compounds may provide a prolonged weed control. The fast decomposition rate of BOA is in accordance with the relative high water solubility of 8960 mg l^{-1}, making the compound highly available for degradation by microorganisms, which is also indicated by the low partition coefficient of 14.45 (octanol–water) (Hansch et al., 1995), resulting in a low sorption to soil organic matter. Sakaliene et al. (unpublished data) have found a K_d value for BOA of 3–5 l kg^{-1} in different soil types. A low sorption of BOA in soil increases the risk of leaching of BOA to aquatic environments and groundwater. However, the fast degradation rate reported in this study reduces this risk

substantially. As the concentration of BOA in soil increased, the formation of APO was favored. APO and AAPO have a stronger sorption in the soil compared to BOA (Sakaliene et al., unpublished data), making these compounds less bioavailable and with lower leaching potentials. In spite of a substantial increase in the half-lives of BOA, APO, and AAPO when performing degradation studies at a concentration level of 30,000 nmol BOA g soil^{-1}, the half-lives do not exceed values that would lead to a ban of their use, if we were dealing with conventional pesticides. Combining the key knowledge on the decomposition rate of BOA and its main metabolites with results of ongoing studies concerning the target effects of the compound on weeds (Kudsk et al., 2004; Mathiassen et al., 2004) and studies on nontarget effects on soil organisms (Čoja, 2004; Idinger, 2004) will provide a basis for evaluation whether breeding programs or genetic modification should be initiated for producing rye varieties with substantially higher concentrations of hydroxamic acids as an enhancement of their defense properties.

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RAPID COMMUNICATION

FLORAL ODOR VARIATION IN TWO HETEROSTYLOUS SPECIES OF *Primula*

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Abstract—Floral traits such as odor, color, and morphology are important pollinator attractants. Variation in floral traits may influence floral constancy, the tendency of pollinating insects to visit flowers of only one type. We investigated for the first time variation in odor between floral morphs in heterostylous species. We analyzed inter- and intraspecific odor variation in the "pin" and "thrum" floral morphs of sympatric *Primula elatior* and *P. farinosa* (Primulaceae). Floral volatiles were sampled with headspace sorption. Quantitative analysis and chemical identification were performed by gas chromatography coupled to mass spectrometry. The species produced different floral bouquets. *P. elatior* emitted mostly limonene with small amounts of α-pinene, myrcene, and sabinene. *P. farinosa* produced benzaldehyde, 4-oxoisophorone (2,6,6-trimethyl-2-cyclohexene-1,4-dione), benzyl alcohol, and benzyl acetate. These interspecific differences may play a role in promoting floral constancy and maintaining species integrity. Conversely, no differences were detected between the scents of pin and thrum morphs within each species. Heterostyly relies on pollinators visiting both floral morphs. There may be stabilizing selection against divergences in traits that may cause pollinators to develop floral constancy to only one of the floral morphs.

Key Words—Floral odor, heterostyly, *Primula*, pollination, headspace sorption, gas chromatography-mass spectrometry.

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INTRODUCTION

Floral traits such as odor, color, and shape are important pollinator attractants and have evolved with the behavior, morphology, and physiology of pollinating animals (Chittka et al., 1999). An intriguing aspect of pollinator behavior is floral constancy, the tendency of an insect to visit flowers of only one type while ignoring other rewarding flowers. Taxa exhibiting floral constancy include honeybees (*Apis mellifera*), bumblebees (*Bombus* spp.) and solitary bees (*Trichocolletes* spp.), moths and butterflies (Lepidoptera), beetles (Coleoptera), and flies (Diptera; Chittka et al., 1999 and references therein). Pollinators use floral traits to discriminate among different flowers. Thus, there may be strong selection upon floral odors, colors, and shapes that encourage constancy if they increase pollen transfer efficiency and/or reproductive success.

Many plant species have variable floral traits and distinct morphs within a population. Heterostyly is a genetic polymorphism in which plant populations comprise two (distyly) or three (tristyly) mating types that differ in the reciprocally arranged female and male reproductive organs (Barrett, 2002). Distylous species have two floral morphs, 'pin' (long style and low anthers) and 'thrum' (short style and high anthers) (Barrett, 2002). Heterostyly occurs in 155 angiosperm genera, including *Primula* (Primulaceae). There are approximately 425 species of *Primula*, 91% of which are distylous (Richards, 2003).

In heterostylous species, pollen is efficiently transferred from the anthers of one plant to the stigma of another plant by its precise placement on the body of the pollinator (Barrett, 2002). Furthermore, self-fertilization is generally prevented by a self-incompatibility system that inhibits germination of pollen from the same floral morph. Successful pollen exchange requires a pollinator to visit both pin and thrum floral morphs during a foraging bout. Therefore, in heterostylous species, fertilization success relies on pollinators failing to distinguish between pin and thrum morphs of a species. Consequently, within a population, pin and thrum morphs are unlikely to differ in traits that may influence floral constancy. Between species, however, significant differences in floral traits are likely to be selected for to reduce pollen wastage and hybridization. Here, we test for low intraspecific and high interspecific variation in floral traits by considering variation in floral odor between floral morphs, and between species, in sympatric populations of heterostylous *Primula elatior* (L.) Hill and *P. farinosa* L. (Primulaceae).

METHODS AND MATERIALS

Floral odor samples were collected from 30 plants of *P. elatior* (15 pin, 15 thrum), 39 plants of *P. farinosa* (23 pin, 16 thrum), and 10 from ambient air

at Klausenpass (1950 m), Switzerland, during July 2004. The odors were collected by headspace sorption by isolating the inflorescence in a polyethylene bag. Air was drawn from the bag and through a filter attached to a pump at ~100 ml/min, for 4 hr. Filters consisted of 5 mg of Porapak Q absorbent material sealed in glass capillaries. Before each use, filters were cleaned with 200 μl of dichloromethane. Filters were eluted with 50 μl of a 9:1 solution of hexane and acetone.

Gas Chromatography. Before analysis, 100 ng of octadecane (Fluka, purity 99.8%) were added as an internal standard to all samples. A 1.5-μl portion of each odor sample was injected splitless into a gas chromatograph (GC) (Agilent 6890N) at 40°C (3 min), followed by opening the split valve and programming to 150°C at a rate of 2.5°C/min, and then 250°C at a rate of 10°C/min. The GC was equipped with an HP-Innowax column [30 m × 0.32 mm (diam) × 0.25 μm (film thickness)] and a flame ionization detector (FID); helium was the carrier gas. Compounds were identified by analyzing samples on a GC coupled to mass spectrometry (MS) and comparing spectra and retention times with reference compounds.

Statistical Analyses. Statistics were calculated by using relative amounts of floral odor compounds. As data were not normally distributed and resisted transformation, nonparametric Mann–Whitney *U*-tests were used to compare the relative amounts of each compound between pin and thrum plants. There was no difference in the compounds produced by pin and thrum plants within each species (Mann–Whitney *U*-tests, all P values > 0.5), so the pin and thrum data were pooled for each species. Between-species comparisons were then conducted, also with Mann–Whitney *U*-tests.

RESULTS AND DISCUSSION

In our study populations, *P. elatior* and *P. farinosa* had relatively simple bouquets composed of common floral odors (Table 1, cf. Gregg, 1983; Knudsen et al., 1993; Knudsen, 2002). The two species produced qualitatively different bouquets. *P. elatior* emitted primarily limonene with small amounts of three other monoterpenes: myrcene, α-pinene, and sabinene (Table 1). *P. farinosa* produced benzaldehyde, 4-oxoisophorone (2,6,6-trimethyl-2-cyclohexene-1,4-dione), benzyl alcohol, and benzyl acetate (Table 1).

Some plant individuals overlapped in scent between the species: five of the 30 *P. elatior* samples contained some benzaldehyde, and two of the 23 *P. farinosa* samples contained a small amount of limonene (Table 1). This overlap may be a part of the natural variation in each species rather than hybridization, since *P. elatior* and *P. farinosa* are classified in different sections

TABLE 1. MEAN (± SEM) RELATIVE AMOUNTS OF VOLATILE COMPOUNDS IDENTIFIED FROM 'PIN' AND 'THRUM' FLORAL MORPHS OF *Primula elatior* AND *Primula farinosa* (PRIMULACEAE)

	Primula elatior		*Primula farinosa*		
Volatile compounds	Pin (n = 15)	Thrum (n = 15)	Pin (n = 16)	Thrum (n = 23)	U (P)
α-Pinene	0.75 ± 0.22	0.74 ± 0.22	0	0	320 (<0.001)
Sabinene	2.71 ± 0.44	2.05 ± 0.45	0	0	180 (<0.001)
Myrcene	2.01 ± 0.50	1.99 ± 0.50	0	0	280 (<0.001)
Limonene	93.9 ± 1.13	94.1 ± 1.34	0.10 ± 0.10	0.31 ± 0.30	0.0 (<0.001)
Benzaldehyde	0.63 ± 0.44	1.10 ± 0.72	34.5 ± 2.46	36.4 ± 2.75	0.0 (<0.001)
4-Oxoisophorone	0	0	27.5 ± 2.28	26.6 ± 1.77	0.0 (<0.001)
Benzyl alcohol	0	0	29.5 ± 2.66	28.4 ± 2.31	15 (<0.001)
Benzyl acetate	0	0	8.41 ± 1.08	8.33 ± 1.00	60 (<0.001)

n = Number of plants sampled. Statistics in final column compare the two species using data pooled from pin and thrum plants. Test statistic U and P values are derived from Mann–Whitney U-tests.

(*Primula* and *Aleuritia*, respectively), and no hybrids between these two species have been reported (Richards, 2003). The quantitative differences in the odor bouquets between the species were also highly significant for all compounds (Table 1).

The different odors emitted by *P. elatior* and *P. farinosa* may attract different pollinators. Limonene and the other monoterpenes emitted by *P. elatior* are produced by many plants with diverse pollinating taxa including bees, Lepidoptera, beetles, and fly species (e.g., Knudsen et al., 1993; Knudsen, 2002 and references therein). The few published observations of visitors to *P. elatior* suggest pollinators include species of bumblebees, Lepidoptera, beetles, and thrips (Schou, 1983). The compounds emitted by *P. farinosa* are often found in Lepidoptera-pollinated flowers (Andersson et al., 2002). Butterflies and day-flying moths reportedly visit *P. farinosa*, but Diptera (Syrphidae, Bombyliidae), bees (*Osmia* spp.), and bumblebees have also been observed (Hambler and Dixon, 2003). Some insect taxa may be attracted to both *Primula* species.

The insects pollinating *Primula* spp. at our study site have not yet been identified, nor has the specific role of floral odor in pollinator attraction been investigated in any *Primula* species. However, odor is a principal pollinator attractant in a wide range of flowering plants and is a major factor promoting floral constancy (Knudsen et al., 1993; Chittka et al., 1999 and references therein). Both *P. elatior* and *P. farinosa* are largely self-sterile and rely heavily on pollinators for fertilization (Richards, 2003).

Even if some pollinator species are shared, cross-pollination between the two *Primula* species seems improbable. Individual insects are unlikely to visit

both *Primula* species during a foraging bout, since the striking quantitative and qualitative differences in the floral bouquets should promote floral constancy to only one of the species. Even minor qualitative differences in floral scent can affect pollinator behavior. For example, trained moths can distinguish between flowers differing by a single volatile compound (Cunningham et al., 2004). Thus, scent differences could contribute to the maintenance of species integrity, and/or provide a selective advantage, e.g., by reducing pollen wastage or clogging of the stigmas with heterospecific pollen.

Intriguingly, we found no intraspecific variation in scent between the floral morphs in either *Primula* species. Even the relative amounts of each compound produced by the pin and thrum morphs were remarkably similar (Mann–Whitney *U*-tests: *P. elatior* all P values > 0.74, *P. farinosa* all P values > 0.58). Similarly, low intraspecific scent variation was detected in two other species: *Cycnoches densiflorum* (Orchidaceae; Gregg, 1983) and *Geonoma macrostachys* (Arecaceae; Gregg, 1983; Knudsen, 2002). As in heterostylous primulas, both *C. densiflorum* and *G. macrostachys* require pollinators to visit two floral morphs for successful fertilization. Thus, in these four species, stabilizing selection might operate against any divergence in scent that would cause pollinators to develop floral constancy to only one of the morphs.

Chittka et al. (1999) argue that floral constancy is rarely strong enough to isolate floral morphs or maintain species integrity. If this is so, some variation in traits between morphs or species may have little impact on pollinator behavior. However, if there are additional factors that limit which individual plants can exchange pollen for successful reproduction (for example, self-incompatibility in heterostylous primulas), then low scent variability that promotes constancy to both floral morphs may provide a significant reproductive advantage.

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RAPID COMMUNICATION

(3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-PENTACOSAPENTAENE, A KEY PHEROMONE COMPONENT OF THE FIR CONEWORM MOTH, *Dioryctria abietivorella*

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Abstract—The sex pheromone of the fir coneworm moth consists of a blend of (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-pentacosapentaene and (9*Z*,11*E*)-tetradecadienyl acetate. Analogous blends of polyunsaturated, long-chain hydrocarbons with much shorter chain aldehydes or alcohols recently have been discovered in three other moth species in the superfamily Pyraloidea. These combinations of components from two distinct structural classes may represent an important and widespread new pheromone blend motif within the Lepidoptera.

Key Words—Sex pheromone, *Dioryctria abietivorella*, (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-pentacosapentaene, (9*Z*,11*E*)-tetradecadienyl acetate, Pyralidae, Crambidae

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INTRODUCTION

Almost all of the female-produced pheromone blends from the more than 500 species in the lepidopteran suborder Ditrysia for which pheromones have been identified are composed of components with the same or similar chain lengths, structures, and volatilities (Ando et al., 2004; Witzgall et al., 2004). Furthermore, for a particular species, the blend components usually belong to only one of two general structural classes. The first class consists of C_{10} to C_{18} primary alcohols and their corresponding acetates and aldehydes, whereas the second consists of C_{17} to C_{23} polyunsaturated hydrocarbons and epoxides (Ando et al., 2004). Exceptions to this pattern are rare; the pheromone of the tomato fruit borer, *Neoleucinodes elegantalis* (Crambidae: Pyraustinae), a blend of (*E*)-11-hexadecenol and (3*Z*,6*Z*,9*Z*)-tricosatriene, is one such example (Cabrera et al., 2001).

In spring of 2004, we found that the pheromone blends of two pyralid moths, the navel orangeworm (NOW), *Amyelois transitella* (subfamily Phycitinae), and the meal moth, *Pyralis farinalis* (subfamily Pyralinae), contain components of dissimilar structural types. The major component of the NOW blend was identified as (11*Z*,13*Z*)-hexadecadienal (Coffelt et al., 1979), but this single component was only weakly attractive. Using wind tunnel bioassay-directed fractionations and coupled gas chromatography–electroantennography (GC-EAG), we discovered that (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-tricosapentaene and (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-pentacosapentaene are components of the NOW blend, whereas the meal moth blend contains (11*Z*,13*Z*)-hexadecadienal and (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-tricosapentaene (J.G. Millar, L.P.S. Kuenen, and J.S. McElfresh, unpublished data). Although the pentaenes elicit weak EAG responses, they are critical components of the attractants. The presence of the pentaenes in NOW has been independently verified (Leal et al., 2005).

The fir coneworm, *Dioryctria abietivorella* (Pyralidae: Phycitinae), causes substantial damage in coniferous seed orchards in western North America. The major component of its pheromone [(9*Z*,11*E*)-tetradecadienyl acetate, (9*Z*,11*E*)-14:Ac] had been known since 1985 (G. Grant, unpublished data), but, as with NOW, the single component was minimally attractive in field trials. Here, we report that (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-pentacosapentaene is a crucial component of the pheromone blend of the fir coneworm.

METHODS AND MATERIALS

Douglas fir cones infested with fir coneworms, collected near Chico, CA, were held in individual containers under ambient laboratory conditions. Emerging moths were segregated by sex and placed in a light box, 16:8 L:D, in an

environmentally controlled room. Pheromone glands of virgin females were clipped off ca. 6 hr after lights off and extracted in pentane. Extracts were analyzed by coupled GC-EAG on DB-5 and DB-WAX columns as previously described (McElfresh and Millar, 1999). An aliquot of a composite extract from 212 females was analyzed by GC-MS (HP-6890 GC interfaced to an HP 5973 mass selective detector, electron impact ionization, 70 eV), using splitless injections with a DB-5MS column programmed from 50 to 250°C at 10°C/min. A second aliquot was treated with 10 μl of a solution of 4-methyl-1,2,4-triazoline-3,5-dione (MTAD, 2 mg/ml in CH_2Cl_2), and the adduct was analyzed by GC-MS (injector 300°C; program 100°C/1 min, 10°/min at 300°C, hold 20 min).

(3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-Pentacosapentaene was synthesized from (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-icosapentaenoic acid ethyl ester (Tokyo Kasei Kogo Co., Tokyo, Japan) by reduction with $LiAlH_4$, tosylation of the resulting alcohol, and alkylation with pentyl magnesium bromide with CuI catalysis (Jain et al., 1983). Isomerically pure (9*Z*,11*E*)-14:Ac was prepared by a new synthesis (J.A. Moreira and J.G. Millar, unpublished data). (*Z*)-9-14:Ac was obtained from Bedoukian Research (Danbury, CT, USA).

Field trials were conducted in a seed orchard near Vernon, BC, Canada, during August–September, 2004. Test compounds were loaded onto 11-mm gray rubber septa (The West Co., Lionville, PA, USA) in 100 μl hexane, with 5 mg/ml each of butylated hydroxytoluene (BHT) and Sumisorb 300 added to the hexane solutions as stabilizers. Doses tested are shown in Table 1. Baited sticky traps were hung from branches (~1.5 m above ground), and spaced ~25 m apart.

TABLE 1. TRAP CATCHES (MEAN ± SE) OF MALE *D. abietivorella* IN PHEROMONE-BAITED TRAPS IN A SEED ORCHARD NEAR VERNON, BRITISH COLUMBIA[a]

Lure blend			
(9*Z*,11*E*)-14:Ac (μg)	Pentaene[b] (μg)	(*Z*)-9-14:Ac (μg)	Mean trap catch
100	0	0	0
100	100	0	2.8 ± 0.9 c
100	500	0	51.4 ± 6.4 a
100	0	3.3	0
100	100	3.3	2.8 ± 1.1 c
100	500	3.3	27.6 ± 7.8 b
Blank			0

[a] Trial conducted from August 12–September 14, 2004, with five replicates counted weekly. Trap catch data analyzed by ANOVA followed by Student–Newman–Keuls (SNK) test. Means followed by different letters are different at $\alpha \leq 0.05$. Zero values were not included in the ANOVA or SNK analyses.

[b] Pentaene = (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-pentacosapentaene.

RESULTS AND DISCUSSION

GC-EAD analyses indicated strong and consistent antennal responses to two compounds in pheromone gland extracts (Figure 1, peaks 1 and 2). The major component was identified as (9*Z*,11*E*)-14:Ac (peak 2) by comparisons of retention times on DB-5 and DB-WAX GC columns, mass spectra, and EAG responses with those of authentic standards. The position of the diene was verified by diagnostic ions (*m/z* 194, base peak, *m/z* 336, 16%) in the mass spectrum of the MTAD adduct. The earlier eluting peak 1 was present in insufficient amounts to obtain a mass spectrum. However, its retention indices on DB-5 and DB-WAX columns matched those of (*Z*)-9-14:Ac, and comparisons with tabulated retention index values of all possible C14 monoene acetates confirmed the position and geometry of the double bond (Marques et al., 2000). However, blends of these two compounds attracted virtually no moths in field trials. In early 2004, our discovery of pentaene compounds in NOW extracts prompted us to carefully examine our composite coneworm extract (212 females) for similar compounds, resulting in the discovery of (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-pentacosapentaene (Figure 1, peak 3) in an approximately 1:1 ratio with (9*Z*,11*E*)-14:Ac. The identification was confirmed by exact

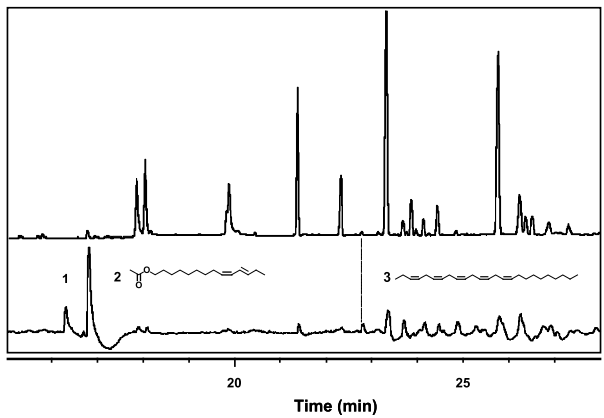

FIG. 1. Gas chromatogram (top trace) and corresponding electroantennogram trace from the antenna of a male *D. abietivorella* moth challenged with an aliquot of pheromone gland extract from female moths. Peak 1, (*Z*)-9-14Ac; peak 2, (9*Z*,11*E*)-14:Ac; and peak 3, (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-pentacosapentaene. Other EAD responses were to straight- and branched-chain cuticular hydrocarbons. Conditions: DB-5 column (30 m × 0.25 mm ID, 0.25 μm film; 70°C/1 min, 10°/min–275/20 min. Approximately four female equivalents injected splitless.

matches of retention times with those of an authentic standard on polar and nonpolar columns and by its diagnostic mass spectrum characterized by even-mass fragments that unequivocally placed all the double bonds (Underhill et al., 1983, and references therein). In particular, cleavage between C_8 and C_9 with hydrogen transfer from C_5 gave a strong $C_2H_5(CH{=}CH)_3H]^+$ ion (*m*/*z* 108, 38% of base peak), whereas cleavage between C_{10} and C_{11} with hydrogen transfer from C_{14} produced the analogous $C_9H_{19}(CH{=}CH)_3H]^+$ ion (*m*/*z* 206, 9% of base peak) from cleavage and rearrangement from the other end of the chain. Because of the small amounts present, even in a composite sample from 212 moths, it has not been possible to verify that all the double bonds have the *Z* configuration, for example, by GC-FTIR. Nevertheless, the exact retention time and mass spectral matches, coupled with the strong biological activity (see below) provide strong evidence that the natural compound is the all-*Z* isomer.

(3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-Pentacosapentaene elicited unexpectedly weak responses from male moth antennae in GC-EAD analyses (Figure 1, peak 3). However, field trials demonstrated that this compound is a key component of the pheromone blend (Table 1). Although (9*Z*,11*E*)-14:Ac (Table 1) and (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-pentacosapentaene (second trial, data not shown) were not active as single components, a 5:1 ratio of the pentaene:acetate was attractive to male moths. When added to the 5:1 blend, (*Z*)-9-14:Ac decreased attraction at the rate tested, despite being present in pheromone gland extracts (Table 1).

These recent examples of pheromone blends from four related species in the superfamily Pyraloidea, consisting of one or more polyunsaturated long-chain hydrocarbons in combination with much shorter-chain aldehydes, alcohols, and acetates, suggest that these combinations represent a new and possibly widespread motif in lepidopteran pheromone chemistry. These examples also suggest a possible reason (i.e., missing long-chain components) why the pheromone blends of some other moths (e.g., the sugar cane borer, *Diatraea saccharalis* (Crambidae)) have proven so elusive.

These blends have other remarkable features. First, their dissimilar components almost certainly arise from two independent pheromone biosynthesis pathways, with the shorter chain components probably being synthesized in the pheromone gland and the longer chain hydrocarbons possibly being synthesized in oenocyte cells and transported to the gland through the hemolymph (see Jurenka, 2004). Second, it is unclear how calling female moths successfully emit optimal blends of compounds with such substantial differences in vapor pressure. Third, it is surprising, but not unprecedented (e.g., Cabrera et al., 2001), that the polyunsaturated hydrocarbon components elicit relatively weak responses from male antennae, despite their being crucial to eliciting behavioral responses. Answers to these and other questions may be forthcoming from ongoing explorations of these novel pheromone blends.

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SOIL MICROVARIATIONS AS A SOURCE OF VARIABILITY IN THE WILD: THE CASE OF SECONDARY METABOLISM IN *Origanum dayi* POST

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Abstract—The volatile components of *Origanum dayi* Post were analyzed in 10 wild populations grown in a limited area. ANOVA tests showed no significant differences among the compositions of plants that grew in different locations, which suggests that differences in composition are of genetic origin and do not spring from environmental variation. However, the use of new statistical methods (such as use of the correlation coefficient, *r*, as a parametric value) revealed that, despite their reduced range of variation, most of the 22 soil properties (SPs) measured correlated with the composition of the volatile components. This analysis also indicated that the main volatile components were modified in parallel in response to SP variations. It is concluded that variations in soil properties affected the composition of volatile components in *O. dayi*, and that the main influencing factors were soil microelement contents and texture. This study highlights the need for highly sensitive statistical tools to determine the actual influence of environmental factors in natural environments, especially when their range of variation is small.

Key Words—Canalization, plasticity, secondary metabolism, essential oil, *Origanum dayi*, analysis of variability, ANOVA, nongenotypic effects, soil microvariation effects.

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INTRODUCTION

Variations in soil composition and structure may influence the composition of essential oils in plants. For example, soil fertility was shown to affect the composition of the essential oils in *Mentha arvensis* (Singh et al., 1989), *Origanum syriacum* (Omer, 2000), *Daucus carota* (Schaller and Schnitzler, 2000), and many other species (e.g., Adler et al., 1989; Piccaglia et al., 1989; Thomas and Cracker, 1991; Dragar and Menary, 1995; Khan et al., 1999). Variations in micronutrient contents and soil sodicity also affect the essential oil composition (Ram et al., 1999; Singh and Misra, 2000).

However, in all these cases, the observed changes in essential oil content or composition resulted from wide variations of a single soil parameter that were imposed to evaluate its influence. However, such a mode of variation does not necessarily match the conditions encountered in natural habitats. In the wild, many soil properties vary simultaneously over narrow ranges, even within an apparently homogeneous area. In such a case, it is not clear whether low amplitude variations of soil properties affect plant secondary metabolism. The present study used highly sensitive statistical tools to evaluate the effects of soil microvariations on the composition of plant volatile components, specifically in *Origanum dayi* Post (Lamiaceae). This endemic subshrub, a copious producer of volatile components (Dudai et al., 2003), grows wild in the Northern Negev and Judean Desert in Israel (Feinbrun-Dothan, 1987). Most of the individuals belonging to this species grow in a small area in the southeastern Judean Mountains (a north–south strip, 15 km long and ~5 km wide), at the boundary between the Mediterranean and the desert land flora. This area is relatively uniform in altitude, between 700 and 900 m above sea level (asl), with annual mean rainfall ranging from 250 to 350 mm. It was chosen for testing the influence of microvariations in soil properties and structure on the accumulation of the volatile components in plants.

METHODS AND MATERIALS

Plant and Soil Sampling. Ten sites were selected. In each, the vegetative shoot was harvested from each of six individuals that were randomly sampled within a 15-m radius. Sampling was conducted between 6:00 and 8:00 A.M. on July 18, 2002, when most of the upper parts of the plant stem were still vegetative.

One soil sample from 0.05- to 0.2-m depth was taken in the middle of each sampling site. Soil samples were dried in a ventilated oven at 70°C and ground by hand. The gravel (fragments up to 7.5-cm diam) content in the soil samples was determined by dry sieving. Crushed soil was sieved through a 2-mm sieve

TABLE 1. SOIL PROPERTIES IN VARIOUS SAMPLES AND THEIR AVERAGES (AV) AND COEFFICIENTS OF VARIATION (CV%)

	Mechanical composition											Macronutrients					Micronutrients content			
No.	Clay (%)	Silt (%)	Sand (%)	Gravels (%)	$CaCO_3$ (%)	OM (%)	pH	EC (dS m^{-1})	Cl^- (mmol/l)	Na^+ (mmol/l)	SAR	$N–NO_3$ (mg/kg soil)	$P–PO_4$ (mg/kg soil)	K^+ (mmol/l)	PAR	ΔF	Fe (mg/kg soil)	Mn (mg/kg soil)	Zn (mg/kg soil)	Cu (mg/kg soil)
1	30.2	35.1	34.6	7.6	17.3	1.3	7.7	0.9	2.4	1.5	0.7	0	4.1	0.22	0.1	3365	11.1	16.1	0.8	1.6
2	30.2	19.1	50.6	13.3	25.4	1.6	7.6	1.3	2.6	2.7	1.1	11.7	15.8	0.5	0.2	3010	19.7	23.5	1.8	3.0
3	14.2	15.1	70.6	69.3	62.1	1.7	7.6	1.2	2.0	1.1	0.5	29.8	11.1	0.45	0.2	3051	4.1	9.8	5.4	0.9
4	26.2	39.1	34.6	8.6	12.1	2.4	7.7	1.2	2.5	2.4	1.0	35.7	10.2	0.31	0.1	3267	18.7	28.1	1.7	2.0
5	34.2	31.1	34.6	11.9	20.2	1.4	7.9	0.4	1.3	0.8	0.6	0	38.7	0.09	0.1	3697	14.8	17.0	0.9	2.5
6	30.2	35.1	34.6	6.6	13.7	1.6	8	0.7	1.3	1.5	0.9	5.2	9.7	0.25	0.1	3211	15.4	16.9	1.0	2.0
7	22.2	35.1	42.6	5.6	18.1	4.1	7.2	1.4	2.7	1.3	0.5	0.9	47.5	0.69	0.2	2880	22.1	30.1	3.1	3.4
8	22.2	39.1	38.6	10.4	14.9	2.5	7.9	0.7	1.7	1.4	0.8	20.6	14	0.23	0.1	3283	15.7	19.3	1.2	2.1
9	26.2	31.1	42.6	16.1	24.6	2.4	7.9	0.7	1.4	1.5	0.8	1.7	26.6	0.53	0.3	2788	11.6	15.1	1.2	2.4
10	42.2	27.1	30.6	51	66.9	0.7	7.5	0.7	2.4	1.4	0.8	4.3	31.5	0.63	0.4	2638	5.8	6.6	24.2	14.4
Av	27.8	30.7	41.4	20.0	27.5	2.0	7.7	0.9	2.0	1.6	0.8	11.0	20.9	0.4	0.18	3119	13.9	18.3	4.1	3.4
CV	27.3	26.4	28.5	108.8	72.6	47.6	3.1	36.2	27.7	36	26.0	120.0	68.7	50.8	54.4	9.4	41.9	40.5	174.1	114.2

and stored pending further analysis. Values of soil properties (SPs) at the 10 sampling sites and their coefficients of variation (CV) are presented in Table 1. Calculation of the coefficient of variation (CV) of each SP revealed that nitrogen, phosphorus, zinc, manganese, gravel, and $CaCO_3$ were the most variable (Table 1).

Chemical and Physical Analysis. Essential oil was extracted from a 2-cm segment of the upper part of a nonflowering shoot of each plant sample. A mixture of methyl *tert*-butyl ether (MTBE) and 10 μg/ml of isobutyl benzene (as internal standard) were combined with the shoot segment at 5 ml MTBE per gram (fresh weight) of shoot, according to Lewinsohn et al. (1993). This was shaken gently for 2 hr at room temperature. The resulting extract was purified by passage through a Pasteur pipette containing anhydrous Na_2SO_4 and silica gel 60 (230–400 mesh, Merck). This purification was to dry the sample and to remove polar substances of high molecular weight that would interfere with the GC-MS analyses (Dudai et al., 2001).

Methyl *tert*-butyl ether extracts were analyzed on an HP-GCD apparatus equipped with an Rtx-5SIL MS (30 m × 0.25 mm i.d. × 0.25 μm) fused-silica capillary column (Restek). The carrier gas was helium flowing at 0.8 ml/min. Samples were injected in both split and splitless injection modes. The injector temperature was 250°C, and the transfer line temperature was 280°C. Column conditions were 50°C for 1 min, 50–260°C at 5°C/min, and 10 min at 260°C. The mass range was acquired by working in the EI mode (70 eV), over an *m*/*z* range of 41–350. Compounds were identified by comparing their relative retention indices and spectra with those of authentic standards or with those reported in the literature (Adams, 2001) and by using the NIST 98 and QuadLib 1607 GC-MS libraries.

For all soil samples, the mechanical composition was determined with a hydrometer (Day, 1956), the organic matter (OM) content by the Walkley–Black method (Allison, 1965), and the $CaCO_3$ content by a volumetric method (Allison and Moodie, 1965). The pH, electrical conductivity (EC), sodium adsorption ratio (SAR), and potassium adsorption (PAR) values and the Cl^-, Na^+, Ca + Mg, and K^+ concentrations were determined in saturated soil paste. The SAR and PAR were calculated according to Equations (1) and (2), respectively.

$$\mathrm{SAR} = \frac{(\mathrm{Na})}{(\mathrm{Ca} + \mathrm{Mg})^{0.5}} \tag{1}$$

$$\mathrm{PAR} = \frac{(\mathrm{K})}{(\mathrm{Ca} + \mathrm{Mg})^{0.5}} \tag{2}$$

where the cations concentration are expressed in mmol/l.

$N–NO_3$ concentration in the soil samples was determined by extraction with 1 M KCl (Keeney and Nelson, 1982) and the available $P–PO_4$ concentration by extraction with 0.5 M $NaHCO_3$ (Olsen and Sommers, 1982). The available micronutrients, Fe, Zn, Mn, and Cu, in the soil samples were determined by extraction with diethylenetriaminepentaacetic acid (Lopert and Inskeep, 1996).

Statistical Analyses. The statistical tools used in this study were based on comparison of r values (the correlation coefficients for linear correlation) considered as parameters. However, since the r coefficients are not normally distributed, their comparison is only possible after a z-transformation, which converts them into parameters with near normal distributions (Sokal and Rohlf, 1981), according to Equation (3):

$$z = 0.5 \times \ln\left[(1 + r)/(1 - r)\right] \tag{3}$$

As previously shown by Amzallag (1999a,b, 2000), such a conversion enables the r coefficients to be used as parameters that quantify the level of linkage between a pair of variables.

For each soil property (SP), an influence index was calculated as follows:

$$\ln\,(\mathrm{SP}) = 1/(m-1)\sum z\left(\mathrm{SP}, Y_j\right) \tag{4}$$

where Y_j are the $m-1$ components other than SP considered to be representative of the essential oil (m = 21; see Results and Discussion).

For a volatile component Y, the coefficient of variation, $\mathrm{CV_L}(Y)$, in a location L is defined in Equation (5)

$$\mathrm{CV_L}(Y) = 100 \times \mathrm{SD_L}(Y)/\mathrm{AVG_L}(Y) \tag{5}$$

where SD and AVG are the standard deviation and the mean value, respectively, of the parameter Y for the population considered (L). Canalization of a parameter quantifies the level of uniformity of expression of a character among plants exposed to the same soil conditions (the same location). For a volatile component Y, the canalization $[C(Y)]$ is determined according to Debat and David (2001) as the inverse value of the mean of the within-localities values of the CV of Y:

$$C(Y) = n \times 1\Big/\left[\sum \mathrm{SD_{L}}i(Y)/\mathrm{AVG_{L}}i(Y)\right] \tag{6}$$

where $L_1, \ldots, L_n$ are the n localities considered.

Plasticity quantifies the influence of environmental variations on the composition of the volatile components. For each component Y, the plasticity $[P(Y)]$ was calculated as the coefficient of variation between mean values of Y (each determined at one location):

$$P(Y) = 100 \times \mathrm{SD}[\mathrm{AVG_{L1}}(Y), \ldots, \mathrm{AVG_{L}}n(Y)]\Big/\tilde{Y}, \tag{7}$$

TABLE 2. RELATIVE COMPOSITION (%) AND CONTENT (%, w/w) OF VOLATILES OF *Origanum dayi* OBTAINED BY *Tert*-BUTYL METHYL ETHER (MTBE) EXTRACTION[a]

	Populations																			
	1		2		3		4		5		6		7		8		9		10	
Compounds	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD
α-Thujene	1.11	0.22	0.91	0.17	0.90	0.33	0.93	0.19	0.97	0.23	0.88	0.26	0.97	0.18	1.05	0.21	0.91	0.31	1.06	0.37
α-Pinene	3.06	0.18	2.86	0.30	2.89	0.26	2.77	0.22	2.93	0.25	2.96	0.27	2.71	0.26	2.64	0.30	2.63	0.35	2.93	0.33
Camphene	0.21	0.21	0.34	0.18	0.34	0.25	0.43	0.24	0.40	0.26	0.18	0.22	0.32	0.30	0.24	0.16	0.08	0.12	0.00	0.18
Sabinene	3.31	0.39	3.12	0.39	3.28	0.32	3.08	0.15	3.08	0.42	3.54	0.69	3.16	0.41	3.55	0.46	3.51	0.50	3.51	0.37
β-Pinene	4.44	0.38	4.25	0.62	4.44	0.35	4.15	0.57	4.43	0.33	4.85	0.43	3.99	0.39	4.07	0.56	4.36	0.57	4.51	0.72
Myrcene	1.82	0.24	1.64	0.39	3.38	1.37	3.26	1.54	2.36	1.35	2.44	0.98	1.66	0.36	2.18	1.35	2.42	1.15	2.59	1.60
α-Terpinene	0.32	0.11	0.42	0.17	0.33	0.18	0.34	0.11	0.35	0.10	0.33	0.06	0.33	0.08	0.37	0.14	0.33	0.07	0.35	0.11
Unknown RI 1020	0.26	0.05	0.20	0.18	0.25	0.09	0.30	0.05	0.34	0.13	0.23	0.05	0.29	0.09	0.25	0.03	0.21	0.10	0.22	0.05
p-Cymene	0.46	0.23	0.50	0.36	0.56	0.32	0.41	0.17	0.52	0.27	0.29	0.11	0.34	0.15	0.32	0.14	0.17	0.09	0.22	0.15
Limonene	0.62	0.15	0.69	0.39	0.76	0.22	0.63	0.28	0.47	0.12	0.46	0.16	0.58	0.16	0.75	0.46	0.99	1.50	0.69	0.22
1,8-Cineole	39.32	5.34	36.31	3.28	39.25	4.95	36.68	4.77	36.35	5.94	39.00	5.36	36.57	6.78	37.34	6.39	37.27	5.81	40.93	9.69
γ-Terpinene	1.59	0.41	1.56	0.45	1.51	0.54	1.44	0.33	1.60	0.34	1.20	0.17	1.36	0.36	1.32	0.22	1.14	0.22	1.52	0.27
(*E*)-Sabinene hydrate	7.74	2.49	8.53	2.42	8.22	4.75	7.99	2.93	7.99	4.73	6.61	2.59	12.15	8.11	8.70	3.97	8.47	5.54	9.38	7.15
Terpinolene	0.28	0.03	0.28	0.05	0.26	0.08	0.27	0.04	0.43	0.31	0.26	0.02	0.26	0.02	0.27	0.03	0.26	0.04	0.29	0.05
Linalool	0.57	0.19	0.53	0.37	0.22	0.06	0.67	0.57	0.49	0.34	0.61	0.26	0.73	1.00	0.81	0.76	0.99	1.01	0.40	0.20
(*Z*)-Sabinene hydrate	2.42	0.65	3.81	1.26	3.28	1.51	3.11	1.43	2.67	0.83	2.61	1.00	2.55	0.85	3.72	2.02	3.35	1.89	2.30	1.03
(*Z*)-*para*-menth-2-en-1-ol	0.09	0.04	0.13	0.06	0.10	0.09	0.12	0.04	0.12	0.05	0.09	0.02	0.10	0.04	0.11	0.03	0.09	0.03	0.09	0.04
δ-Terpineol	1.01	0.26	0.98	0.23	1.03	0.23	1.01	0.18	0.92	0.14	1.01	0.07	0.92	0.22	0.97	0.16	1.15	0.24	1.04	0.26
Borneol	0.48	0.44	0.94	0.43	0.88	0.59	1.30	0.80	1.13	0.61	0.70	0.38	0.87	0.74	0.74	0.37	0.30	0.26	0.32	0.24
Terpinen-4-ol	2.11	0.72	2.78	1.17	2.25	1.95	2.28	0.49	2.64	1.02	1.90	0.50	2.03	0.71	2.02	0.46	1.87	0.70	1.64	0.71
α-Terpineol	8.49	1.23	8.57	1.23	8.40	3.18	8.39	1.66	8.72	2.15	6.71	1.36	8.32	1.50	8.29	1.44	8.99	3.79	8.54	1.48
(*E*)-Sabinene hydrate acetate	9.47	2.78	5.31	2.12	5.91	3.27	6.39	2.59	6.80	2.20	6.59	4.75	6.53	2.31	6.59	2.79	5.31	2.91	6.24	1.91
2-Hydroxycineole	1.06	2.59	0.01	0.02	0.02	0.01	0.02	0.01	0.03	0.02	0.52	1.22	0.03	0.03	0.02	0.02	0.01	0.00	0.03	0.01
(*Z*)-Sabinene hydrate acetate	0.24	0.11	1.68	1.09	0.93	0.78	0.98	1.36	0.48	1.00	1.31	1.50	0.89	1.19	1.45	1.55	1.86	2.25	0.62	0.76

Linalyl acetate	1.31	0.60	3.60	1.56	1.42	0.85	1.69	0.54	2.15	1.40	2.66	1.05	1.90	1.59	1.94	1.34	3.07	2.06	1.67	0.94
Bornyl acetate	0.32	0.28	0.43	0.27	0.45	0.48	0.57	0.29	0.65	0.22	0.37	0.24	0.36	0.39	0.33	0.21	0.16	0.13	0.14	0.10
Terpinen-4-yl acetate + pinocarvyl acetate	0.29	0.09	0.21	0.08	0.20	0.12	0.32	0.13	0.17	0.07	0.14	0.02	0.19	0.06	0.23	0.06	0.17	0.04	0.21	0.15
Unknown RI 1312	0.12	0.04	0.15	0.05	0.17	0.11	0.17	0.06	0.17	0.08	0.11	0.03	0.13	0.04	0.14	0.04	0.12	0.04	0.11	0.05
α-Terpinyl acetate	0.06	0.04	0.06	0.03	0.06	0.04	0.06	0.03	0.06	0.02	0.05	0.02	0.05	0.04	0.04	0.01	0.06	0.03	0.04	0.02
Eugenol	0.19	0.12	0.26	0.18	0.35	0.10	0.54	0.45	0.33	0.09	0.26	0.08	0.21	0.05	0.24	0.04	0.19	0.08	0.31	0.17
Vanillin	0.05	0.01	0.06	0.01	0.05	0.01	0.05	0.02	0.06	0.02	0.06	0.02	0.04	0.01	0.05	0.02	0.06	0.01	0.05	0.03
β-Caryophyllene	1.54	0.17	1.82	0.72	1.67	0.82	2.11	0.67	2.31	1.16	2.93	1.05	2.29	0.72	2.51	0.75	2.42	0.60	2.47	0.60
α-Humulene	0.06	0.01	0.07	0.03	0.07	0.03	0.08	0.02	0.09	0.04	0.12	0.03	0.09	0.03	0.10	0.02	0.09	0.02	0.09	0.03
allo-Aromadendrene	0.13	0.12	0.06	0.10	0.25	0.13	0.24	0.06	0.19	0.06	0.20	0.05	0.31	0.12	0.20	0.07	0.12	0.01	0.33	0.14
Valencene	0.01	0.02	0.02	0.03	0.05	0.04	0.08	0.02	0.07	0.03	0.09	0.04	0.07	0.02	0.05	0.02	0.08	0.03	0.09	0.02
Unknown RI 1508	0.09	0.02	0.09	0.05	0.11	0.04	0.13	0.03	0.11	0.05	0.16	0.09	0.15	0.05	0.11	0.03	0.13	0.04	0.15	0.03
Unknown RI 1522	0.53	0.32	0.48	0.51	0.53	0.49	0.78	0.40	0.81	0.63	0.71	0.54	0.35	0.43	0.41	0.48	0.56	0.47	0.01	0.01
Unknown RI 1528	0.33	0.20	0.30	0.32	0.34	0.32	0.51	0.27	0.52	0.41	0.46	0.36	0.23	0.29	0.27	0.32	0.37	0.32	0.00	0.00
β-Caryophyllene oxide	0.53	0.19	0.78	0.18	0.74	0.20	0.71	0.30	0.63	0.21	0.63	0.14	0.83	0.09	0.80	0.30	0.44	0.25	0.59	0.19
α-Selinene	0.01	0.03	0.06	0.06	0.03	0.04	0.06	0.02	0.10	0.09	0.06	0.02	0.05	0.04	0.05	0.04	0.08	0.04	0.02	0.01
(*Z*)-Methyl jasmonate	0.04	0.01	0.07	0.03	0.03	0.02	0.04	0.02	0.06	0.03	0.04	0.02	0.06	0.01	0.05	0.02	0.05	0.02	0.04	0.01
Intermediol	1.04	0.18	1.22	0.56	1.24	0.45	1.39	0.42	1.14	0.46	1.52	0.77	1.76	0.59	1.34	0.46	1.56	0.46	1.92	0.28
(*Z*)-Methyl epijasmonate	0.20	0.04	0.33	0.12	0.18	0.05	0.17	0.07	0.22	0.10	0.17	0.07	0.21	0.06	0.19	0.09	0.17	0.02	0.11	0.03
Kunzeaol	0.16	0.12	0.17	0.19	0.18	0.17	0.30	0.16	0.30	0.24	0.27	0.22	0.14	0.17	0.15	0.18	0.22	0.19	0.00	0.00
Unknown RI 1738	0.00	0.00	0.00	0.00	0.02	0.05	0.04	0.06	0.09	0.18	0.28	0.57	0.08	0.17	0.04	0.05	0.03	0.03	0.02	0.03
Manoyl oxide	0.04	0.04	0.13	0.18	0.10	0.08	0.05	0.04	0.18	0.10	0.21	0.13	0.07	0.06	0.13	0.07	0.03	0.05	0.07	0.05
Unknown RI 2082	0.16	0.07	0.45	0.28	0.21	0.10	0.09	0.05	0.15	0.11	0.27	0.29	0.15	0.10	0.23	0.30	0.15	0.17	0.11	0.08
Unknown RI 2128	0.06	0.02	0.19	0.09	0.09	0.05	0.14	0.08	0.13	0.07	0.12	0.09	0.11	0.04	0.14	0.09	0.09	0.05	0.10	0.05
Unknown RI 2169	0.93	0.14	0.89	0.23	0.82	0.24	0.92	0.13	1.02	0.26	0.71	0.13	0.89	0.19	0.81	0.10	0.73	0.33	0.77	0.14
Unknown RI 2235	0.20	0.05	0.18	0.06	0.16	0.05	0.21	0.03	0.23	0.06	0.15	0.03	0.20	0.05	0.19	0.04	0.18	0.10	0.16	0.04
Unknown RI 2384	0.06	0.03	0.20	0.09	0.10	0.06	0.16	0.09	0.16	0.12	0.13	0.09	0.13	0.06	0.16	0.08	0.09	0.07	0.09	0.05
Unknown RI 2620	0.37	0.12	0.79	0.31	0.50	0.38	0.61	0.30	0.90	0.75	1.08	1.01	0.54	0.22	0.59	0.43	1.13	0.89	0.40	0.17
Extract, % (w/w)	28.04	4.93	22.48	7.91	26.61	2.37	25.90	6.11	29.22	5.89	27.03	2.18	28.54	5.21	27.46	7.54	31.83	7.93	34.09	4.45

[a]Mean and SD values of 52 main volatile components of *O. dayi* from the 10 locations. Quantity of each component was calculated as the percentage of the total volatile compounds. Mean and SD values were calculated on the basis of six samples harvested from each of the 10 locations.

where $\tilde{Y}$ is the overall mean value of Y, calculated by Equation (7)

$$\tilde{Y} = 1/n \times \sum \mathrm{AVG}_{\mathrm{L}i}(Y) \tag{8}$$

RESULTS AND DISCUSSION

Volatile Components of O. dayi. The volatile components of *O. dayi* were analyzed separately for each of the six individual plants sampled from each of 10 localities, and an average composition was determined for each location by pooling data from the six related individuals. About 52 volatile components were separated, of which the most abundant were 1,8-cineole, (*E*)-sabinene hydrate, α-terpineol, and (*E*)-sabinene-hydrate acetate (Table 2).

TABLE 3. CHARACTERISTICS OF THE 21 VOLATILE COMPONENTS SELECTED FOR FURTHER ANALYSIS[a]

Compounds	Average	Min	Max	*P* values
1,8-Cineole	37.901	23.445	52.713	0.9169
(*E*)-Sabinene hydrate	8.579	2.516	25.350	0.8434
α-Terpineol	8.342	4.840	14.421	0.8568
(*E*)-Sabinene hydrate acetate	6.514	0.392	13.547	0.4608
β-Pinene	4.348	3.150	5.336	0.2001
Sabinene	3.314	2.420	4.662	0.2619
(*Z*)-Sabinene hydrate	2.981	1.121	6.716	0.4368
α-Pinene	2.839	2.135	3.327	0.1302
Myrcene	2.374	1.076	5.720	0.1248
β-Caryophyllene	2.207	0.755	4.415	0.0812
Terpinen-4-ol	2.152	0.657	5.997	0.6047
Linalyl acetate	2.141	0.300	6.466	0.0544
γ-Terpinene	1.423	0.950	2.533	0.2463
Intermediol	1.412	0.451	2.812	0.0663
(*Z*)-Sabinene hydrate acetate	1.044	0.000	6.134	0.4445
δ-Terpineol	1.005	0.523	1.388	0.7900
α-Thujene	0.968	0.471	1.747	0.8182
Unknown RI 2169	0.848	0.414	1.276	0.1959
Unknown RI 2620	0.690	0.027	3.047	0.1894
β-Caryophyllene oxide	0.668	0.192	1.202	0.0465
Kunzeaol	0.190	0.000	0.588	0.1396
Sum	91.940			

[a] The *Y* components are ordered according to their quantitative importance. Mean, maximal, and minimal values were calculated (as % of the total components) on the basis of the whole population of 60 samples. The *P* value was calculated for difference among the 10 defined populations (ANOVA).

Only 21 of the 52 compounds were subjected to further analysis: they included all the compounds present at more than 1%, but also some of those with the longest retention times (Table 3). These 21 components (designated as Y) account for about 92% of the total volatile components that form the average composition of the 60 *O. dayi* samples analyzed; all of them varied considerably in their abundance, as indicated by the minimum and maximum values presented (Table 3).

In spite of this wide variation, between-population variation was similar to within-population variation (Table 3), which suggests that the samples taken from the 10 locations belong to the same population of *O. dayi*. Moreover, no significant difference ($P < 0.05$ one-way ANOVA test) could be found among the 10 populations for any individual *Y* component, except for caryophyllene oxide, which accounts for less than 1% of the total compounds (Table 3). These results suggest that variations in soil structure and composition (Table 1) had no real influence on the composition of Y, so that variations in the composition of *Y* could be attributed to genetic variation inherent to the sampling of a wild population.

Reinterpretation of the Observed Variability. If most of the observed variations in composition were of genetic origin, it would be expected to exhibit similar amplitudes in all locations, and no relationship would be expected between within-location variability (i.e., the variability of each volatile component, among the six samples analyzed in each location) and between-population variability (i.e., the variability among mean values calculated for each population). For each of the 21 components of *Y* (Table 3), the CV calculated for each one of the 10 populations (Table 2) was averaged, and this value (mean of CV values) was plotted as a function of the CV calculated between the 10 mean

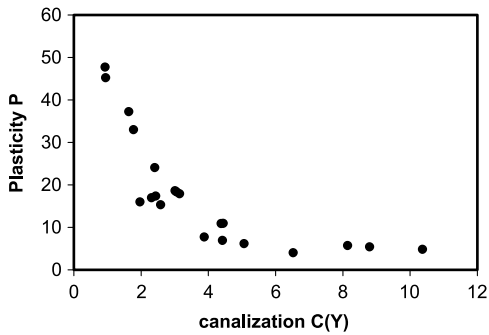

FIG. 1. Relationship between plasticity and canalization in representation of the 21 main components of the essential oil from *O. dayi*. For each volatile component *Y*, canalization and plasticity [respectively $C(Y)$ and $P(Y)$] were calculated as described in Methods and Materials.

TABLE 4. *r* VALUES FOR THE CORRELATION BETWEEN STRUCTURE AND PROPERTIES OF SOIL AND REPRESENTATION OF EACH VOLATILE COMPOUND[a]

A. Soil texture and composition

Compounds	Calc	Sand	Silt	Clay	Gravels	Org
α-Thujene	0.073	−0.480	0.350	0.370	−0.043	−0.230
α-Pinene	0.232	−0.093	−0.265	0.429	0.191	−0.705
Sabinene	0.193	−0.186	0.168	0.108	0.159	−0.209
β-Pinene	0.246	−0.112	−0.205	0.393	0.236	−0.692
Myrcene	0.419	0.275	−0.171	−0.245	0.588	−0.227
1,8-Cineole	0.662	−0.041	−0.205	0.284	0.610	−0.606
γ-Terpinene	0.319	0.096	−0.388	0.264	0.280	−0.463
(*E*)-Sabinene hydrate	0.128	0.073	0.023	−0.140	0.012	0.655
(*Z*)-Sabinene hydrate	−0.136	0.503	−0.263	−0.499	−0.011	0.180
δ-Terpineol	0.293	0.100	−0.152	0.006	0.301	−0.255
Terpinen-4-ol	−0.254	0.323	−0.348	−0.131	−0.182	−0.037
α-Terpineol	0.233	0.147	−0.233	0.019	0.183	0.016
(*E*)-Sabinene hydrate acetate	−0.278	−0.397	0.437	0.15	−0.285	−0.175
2-Hydroxycineole	−0.198	0.261	−0.104	−0.295	−0.16	0.296
Linalyl acetate	−0.270	0.049	−0.211	0.149	−0.340	0.043
β-Caryophyllene	−0.199	−0.508	0.462	0.295	−0.263	0.134
β-Caryophyllene oxide	−0.078	0.351	−0.092	−0.446	−0.011	0.477
Intermediol	0.323	−0.262	−0.132	0.265	0.167	0.247
Kunzeaol	−0.629	−0.018	0.261	−0.25	−0.458	0.168
Unknown RI 2169	−0.283	−0.082	0.066	0.056	−0.272	0.058
Unknown RI 2620	−0.394	−0.077	0.062	−0.033	−0.369	0.066
Influence index	0.296	0.221	0.226	0.239	0.258	0.314

B. Soil properties

Compounds	Delta F	PAR	SAR	Satur	pH	Conduct
α-Thujene	0.078	0.02	−0.077	0.312	−0.151	−0.265
α-Pinene	0.318	−0.25	−0.025	−0.193	0.029	−0.22
Sabinene	−0.379	0.362	0.097	−0.317	0.388	−0.496
β-Pinene	0.074	−0.023	0.086	−0.532	0.489	−0.568
Myrcene	0.013	−0.025	−0.065	−0.475	0.208	−0.023
1,8-Cineole	−0.350	0.380	−0.072	−0.441	−0.037	−0.261
γ-Terpinene	0.325	−0.233	−0.113	0.010	−0.333	0.112
(*E*)-Sabinene hydrate	−0.487	−0.503	−0.413	0.407	**−0.834**	0.505
(*Z*)-Sabinene hydrate	0.009	−0.133	0.326	−0.195	0.244	0.260

TABLE 4. CONTINUED

B. Soil properties						
Compounds	Delta F	PAR	SAR	Satur	pH	Conduct
δ-Terpineol	−0.523	0.495	0.332	−0.514	0.279	−0.129
Terpinen-4-ol	0.550	−0.565	0.075	0.038	0.040	0.226
α-Terpineol	0.134	0.235	−0.104	−0.068	−0.266	0.104
(*E*)-Sabinene hydrate acetate	0.489	−0.455	−0.178	0.303	0.033	−0.199
2-Hydroxycineole	−0.360	0.228	0.434	−0.152	0.282	0.135
Linalyl acetate	−0.185	0.152	0.505	−0.060	0.274	−0.036
β-Caryophyllene	−0.118	0.175	0.118	0.163	0.368	−0.493
β-Caryophyllene oxide	0.072	−0.192	−0.16	0.333	−0.447	0.597
Intermediol	**−0.767**	**0.814**	0.002	0.163	−0.406	0.072
Kunzeaol	**0.673**	**−0.705**	0.073	0.052	0.564	−0.124
Unknown RI 2169	0.640	−0.555	−0.197	0.405	−0.212	0.144
Unknown RI 2620	0.108	−0.102	0.232	−0.188	**0.629**	−0.386
Influence index	0.35	0.35	0.18	0.26	0.34	0.27

[a] The significant correlations ($P < 0.05$ for $|r| > 0.632$, $df = 8$) are indicated in bold characters. For each SP, the influence index was calculated as described in Methods and Materials.

values presented in Table 2 (CV of the mean values). A significant and positive relationship was observed ($r = 0.904$, $df = 19$, $P < 0.001$, not shown), which indicates that the variability observed cannot be considered simply as noise. Rather, the CV of the 10 mean values reflects the level of *plasticity* of each component of Y. The significant relationship observed (Figure 1) between plasticity and canalization (calculated as defined in Methods and Materials) suggests that the levels of variation within and between populations are not determined mainly by genetic variability. Therefore, more detailed analysis of any links between soil properties and essential oil composition is required.

Linkage between Soil Parameters and Volatile Components. For each soil property (SP_i) ($1 \leq i \leq 22$), a numerical value $SP_i(l_k)$ was designated for each location l_k ($1 \leq k \leq 10$) (Table 1). In parallel, for each volatile compound Y_j ($1 \leq j \leq 21$), a numerical value $Y_j(l_k)$ was designated for each location l_k (Table 2). Thus, for each pair (SP_i, Y_j), a correlation coefficient (termed r_{ij}) could be determined on the basis of variation of these variables among the 10 locations.

The *r* coefficients were calculated for the 462 possible (i, j) pairs presently defined (Tables 4 and 5). This analysis revealed an influence of many SPs on *Y* values. For example, the percentage of 1,8-cineole, the most widely represented volatile component in the essential oil of *O. dayi* (Table 3), appears to be correlated with the percentage of calcareous components in the soil (Table 4A),

TABLE 5. *r* VALUES FOR THE CORRELATION BETWEEN MICROELEMENT OR MICROELEMENT CONTENTS AND REPRESENTATION OF EACH VOLATILE COMPOUND[a]

A. Macroelement concentration

Compounds	Cl meq	Na	Ca + Mg	N	P	K
1,8-Cineole	0.270	−0.230	−0.290	−0.280	−0.002	−0.125
(*E*)-Sabinene hydrate	0.077	−0.112	−0.225	−0.238	−0.300	−0.332
α-Terpineol	−0.447	−0.278	−0.532	−0.202	−0.197	0.073
(*E*)-Sabinene hydrate acetate	−0.515	−0.247	−0.578	−0.286	−0.298	−0.278
β-Pinene	−0.208	0.109	−0.111	**0.696**	−0.287	−0.116
Sabinene	0.016	−0.297	−0.336	−0.090	−0.283	0.140
(*Z*)-Sabinene hydrate	0.460	0.031	0.090	0.063	−0.021	−0.172
α-Pinene	0.543	−0.113	0.561	−0.194	**0.736**	**0.714**
Myrcene	−0.074	0.461	0.225	0.522	−0.315	−0.081
β-Caryophyllene	−0.202	−0.141	−0.227	0.054	−0.325	0.269
Terpinen-4-ol	0.098	0.321	0.265	0.192	−0.06	−0.346
Linalyl acetate	0.256	0.006	0.074	−0.021	0.327	0.233
γ-Terpinene	0.126	−0.25	−0.181	−0.272	−0.279	−0.48
Intermediol	−0.246	0.434	0.123	0.174	−0.11	0.252
(*Z*)-Sabinene hydrate acetate	−0.239	0.457	−0.008	−0.254	0.083	0.148
δ-Terpineol	−0.546	−0.215	−0.459	−0.245	0.312	−0.02
α-Thujene	0.416	0.204	**0.633**	0.460	0.098	0.134
Unknown RI 2169	0.153	−0.074	0.058	−0.193	0.531	**0.720**
Unknown RI 2620	−0.479	0.077	−0.076	0.213	−0.197	**−0.626**
β-Caryophyllene oxide	0.303	0.017	0.190	0.010	0.189	−0.375
Kunzeaol	**−0.717**	0.032	−0.330	−0.252	0.088	−0.194
Influence index	0.330	0.203	0.285	0.248	0.257	0.306

B. Microelement contents

Compounds	Fe	Zn	Mn	Cu
1,8-Cineole	−0.221	0.354	−0.227	0.402
(*E*)-Sabinene hydrate	−0.356	0.158	−0.327	0.141
α-Terpineol	−0.440	0.300	−0.564	0.292
(*E*)-Sabinene hydrate acetate	−0.509	0.190	**−0.645**	0.144
β-Pinene	−0.518	0.208	−0.346	0.005
Sabinene	**−0.797**	**0.684**	**−0.788**	0.546
(*Z*)-Sabinene hydrate	−0.182	−0.243	−0.126	0.189
α-Pinene	0.298	0.257	0.370	0.296
Myrcene	0.199	−0.405	0.210	−0.427
β-Caryophyllene	−0.521	0.189	−0.470	0.130
Terpinen-4-ol	0.381	−0.497	0.377	−0.476
Linalyl acetate	−0.151	0.126	−0.050	0.142
γ-Terpinene	−0.070	−0.144	−0.024	−0.135
Intermediol	0.281	−0.290	0.195	−0.233

TABLE 5. CONTINUED.

B. Microelement contents				
Compounds	Fe	Zn	Mn	Cu
(*Z*)-Sabinene hydrate acetate	0.405	−0.278	0.200	−0.121
δ-Terpineol	0.189	0.133	−0.017	0.264
α-Thujene	0.478	−0.125	−0.533	−0.171
Unknown RI 2169	−0.018	**0.646**	−0.059	**0.694**
Unknown RI 2620	0.379	**−0.766**	0.395	**−0.736**
β-Caryophyllene oxide	0.342	−0.293	0.415	−0.245
Kunzeaol	0.255	−0.443	0.075	−0.324
Influence index	0.364	0.353	0.339	0.319

[a] The significant correlations ($P < 0.05$ for $|r| > 0.632$, $df = 8$) are indicated in bold characters. For each SP, the influence index was calculated as described in Methods and Materials.

but also with the concentrations of microelements, especially iron and manganese, in the soil (Table 5B).

Not all the SPs exhibited the same influence on the volatile compounds. For example, Na content and SAR showed no significant correlation with any *Y* component. In contrast, the percentage of organic matter in the soil and the zinc and potassium contents were each significantly correlated with three *Y* parameters (Tables 4 and 5).

To estimate the precise influence of each SP on essential oil composition, the *r* coefficients were converted into *z* values by Equation (3). For each SP_i, the *influence index* [$In(SP_i)$] of its effect on essential oil composition was calculated by Equation (4), as described in Methods and Materials. Calculation of this index indicated that the soil factors that most influenced the essential oil composition are iron content, delta F, PAR, Zn content, and pH (Tables 4 and 5).

Nature of the Nonsignificant Correlations. Less than 10% of the correlations presented in Tables 4 and 5 were statistically significant ($P < 0.05$ for $r \geq 0.632$, $df = 8$). Indeed, such a result is not surprising because the analysis was applied to wild populations for which genetic and environmental variations (even microvariations in soil properties between surface and deep soil layers) are constitutive. In such a context, attention should be given not only to significant correlations, but also to the trends in variations of low *r* values. For this reason, we have considered the *r* values (after their *z*-transformation) as parameters that quantify the linkage between a soil property and a volatile component. Before performing such an analysis, it is necessary to verify whether variations in *z* values really reflect the strength of the relationship between a soil parameter and a volatile component.

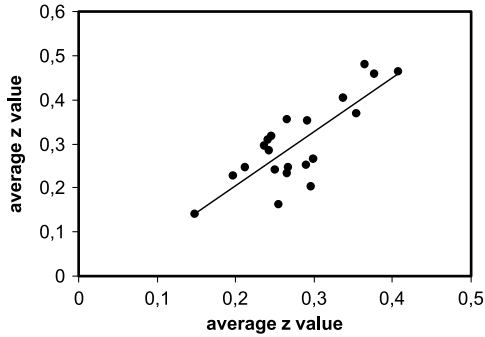

FIG. 2. Relationship between influence of structural and composition parameters of soil on representation of the 21 main components of the essential oil of *O. dayi*. The influence index (determined as the average value of *z*-transformed absolute *r* values) was calculated separately for structural parameters (from Table 4) and for composition parameters (Table 5). A highly significant correlation ($r = 0.805$, $df = 19$, $P < 0.001$) is observed.

TABLE 6. CLASSIFICATION OF SOIL PARAMETERS ACCORDING TO THEIR EFFECT ON BETWEEN-POPULATION VARIABILITY IN COMPOSITION OF VOLATILE COMPONENTS IN *O. dayi*

	Significant correlation	Nonsignificant correlation
Soil structure	Calcareous (0.570)	Sand (−0.216)
	Clay (0.601)	Silt (−0.227)
	Gravel (0.517)	
	Org. (−0.761)	
Soil properties	Satur (−0.431)	Delta F (−0.04)
	Conduct. (−0.456)	PAR (0.204)
		SAR (−0.161)
		PH (0.055)
Macroelements	Na (−0.556)	Cl (−0.007)
	Ca + Mg (−0.491)	N (−0.335)
		P (−0.339)
		K (−0.108)
Microelements	Fe (−0.669)	
	Zn (0.487)	
	Mn (−0.672)	
	Cu (0.440)	

For each soil parameter SP, the *r* correlation between CV of the *X* component and its $z_{sp}(X)$ value was calculated. The *r* values are indicated in parentheses. Significant correlations ($P > 0.05$, $df = 19$) are observed for $|r| > 0.43$.

For each *Y* component, all the *z*-transformed absolute values of the *r* coefficients for relationships with the SPs listed in Table 4 were averaged. A similar calculation was performed for the SPs listed in Table 5. Thus the corresponding average *z*-values calculated from Tables 4 and 5, respectively, were plotted against one another, and a highly significant correlation was observed ($r = 0.805$, $df = 19$, $P < 0.001$, Figure 2). This suggests that components with low average *z* values may be considered to be stable, and those with high *z* values to be highly dependent, for their production, on the various soil characteristics.

Before drawing conclusions about fluctuations in *z* values, it is also necessary to verify whether variations in *r* coefficients reflect a real influence on the composition of the essential oil in *O. dayi*. For example, if the highest r_{ij} values coincided with the most widely variable SPs, one might conclude that the strength of the relationship was biased by the range of SP variation. To test this point, the coefficient of variation of each SP was calculated from the data in Table 1 and plotted as a function of the corresponding influence index (Tables 5 and 6). The very low *r* value obtained for this correlation ($r = 0.13$, $df = 20$, $P > 0.05$ not shown) indicates that the differences in the amplitude of SP variations did not significantly influence the strength of the relationship with *Y* parameters.

Linkage Between z Values and Canalization. Networks are characterized by buffering properties with regard to perturbations (Amzallag, 2001). For this reason, and to test whether low *r* values may also be considered to reflect a phenomenon, we investigated the relationship between canalization of a Y_j character calculated by means of Equation (6) and the *z*-transformed r_{ij} value calculated for the relationship with an SP_i. We tested this link for the organic matter content in the soil (abbreviated as *org*), a soil property with wide variations in *r* values (Table 4). When the canalization of a volatile component

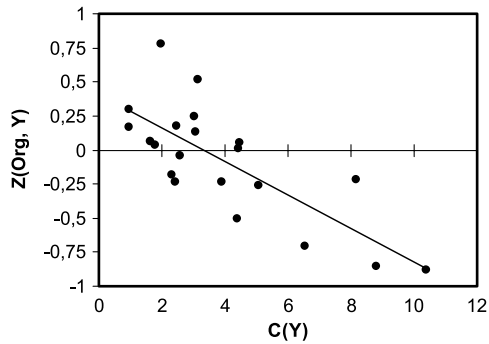

FIG. 3. Relationship between canalization of a volatile component and the influence of the percentage of organic matter in the soil on its variation. A highly significant correlation ($r = -0.761$, $df = 19$, $P < 0.001$) is observed.

TABLE 7. PARALLELS IN THE INFLUENCE OF MACROELEMENTS ON COMPOSITION OF THE VOLATILE COMPONENTS IN *O. dayi*[a]

SP values	Cl	Na	Ca + Mg	N	P	K
Cl		0.023	**0.690**	0.118	0.377	0.302
Na	0.517		**0.564**	**0.554**	**−0.630**	−0.007
Ca + Mg	**0.737**	0.436		**0.464**	**0.466**	0.319
N	0.196	0.396	0.347		−0.278	−0.108
P	0.024	−0.406	0.058	−0.502		**0.652**
K	0.534	0.175	0.542	−0.096	0.222	

[a]Upper half: r coefficient of correlation between $z(\mathrm{SP}, Y)$ of two SP variables; lower half: r between two SP variables. Upper half: The correlations (indicated in bold characters) are significant ($P < 0.05$) for $r \geq 0.433$ (upper half, $df = 19$) and for $r \geq 0.632$ (lower half, $df = 8$).

Y was plotted as a function of $z(org, Y)$, a highly significant negative correlation was found (Figure 3). This confirms that the z values may be considered as parameters *per se*, independently of the statistical significance of the r coefficients. Moreover, it reveals that the main components of the essential oil of *O. dayi* behave as a globally integrated system, which suggests that there is a network of relationships among the metabolic pathways.

A similar relationship was studied for the other soil properties. Of the 22 properties that were measured (Table 2), 11 showed a significant correlation between the CV and the $z_{sp}(X)$ value of the X component (Table 6). Therefore, we suggest that all the microelements and soil texture parameters directly affect the secondary metabolic network as a whole. Concerning the other 11 soil properties, they may also influence the secondary metabolic network, but their influence cannot be revealed by simple analyses of the r correlation coefficient.

Redundancy in Data. A parallel between the $z(\mathrm{SP}, Y)$ values of two soil properties may be a consequence of a correlation between their variations in the soil samples. To test this point, the relationships between the contents of the

TABLE 8. PARALLELS IN THE INFLUENCE OF MICROELEMENTS ON COMPOSITION OF VOLATILE COMPONENTS IN *O. dayi*[a]

	Fe	Zn	Mn	Cu
Fe		**−0.687**	**0.808**	**−0.585**
Zn	−0.546		**−0.656**	**0.951**
Mn	**0.941**	−0.569		**−0.549**
Cu	−0.350	**0.954**	−0.441	

[a]Upper half: r coefficient of correlation between $z(\mathrm{SP}, Y)$ of two SP variables; lower half: r between two SP variables. Upper half: The correlations (indicated in bold characters) are significant ($P < 0.05$) for $r \geq 0.433$ (upper half, $df = 19$) and for $r \geq 0.632$ (lower half, $df = 8$).

various macroelements in the soils were calculated. A significant correlation was observed only between the variations of Cl and Ca + Mg concentrations in the soil solution (lower half of Table 7). In contrast, seven of the 15 correlations between the z(SP, Y) values of two SP variables calculated for macroelements were significant (upper half of Table 7). This indicates that the parallels observed in the influence of the various macroelements on Y values were not trivial, but that they reflected their common influence on these metabolic pathways. A similar analysis was performed for the microelements, and real parallels were found between the influence of copper and zinc contents in soil and between those of iron and manganese contents (lower half of Table 8). For this reason, it is impossible to determine which of the microelements in each of these pairs (Cu or Zn, Fe or Mn) actually influences this secondary metabolism. However, nontrivial antagonistic interactions between the effects of Fe and Mn, and between those of Cu and Zn, were revealed by the significant negative correlations between their z values (upper half of Table 8). Again, the significant correlations observed between z values (upper half of Tables 7 and 8) confirm that they should be considered as parameters that quantify the influence of soil properties on the composition of the essential oil in *O. dayi*.

Conclusions and Implications. Analysis of variance (ANOVA) is generally used to test the importance of fluctuations of a variable on parameters of a system. The present study reveals a more complex situation. The fact that a one-way ANOVA test does not reveal significant differences does not always imply that the observed variability is noise that is inherent to the environmental and genetic heterogeneity (Figure 1). In the present case, many soil factors have a real influence on the abundance of certain volatile components in *O. dayi* (Tables 4–6, Figure 3). It is even difficult to point to a soil property (except perhaps the SAR) that is devoid of influence on the secondary metabolism in these natural conditions.

The inability of ANOVA tests to reveal such an influence may arise from one or more of many causes, which are not mutually exclusive: (1) opposing influence of various soil parameters prevents the emergence of a significant trend; (2) the range of variation of the SP is too small to generate significant differences large enough to be evaluated by tests based on comparison of mean values; (3) the influence of soil properties is partly buffered by the metabolic network; (4) the wide range of genetic variability inherent in the wild population hides any clear trend.

To overcome these problems, in laboratory experiments, the range of variation of SPs is generally increased artificially. However, it is not always possible to extrapolate the effects of amplified variations to the responses of plants exposed to natural conditions. For example, macronutrients are considered to be the main soil factors influencing production of the volatile components (Dudai, 2005). However, calculation of the indices of influence

(Tables 4 and 5) for all the soil properties indicated the K, P, and N contents to be secondary factors; they were ranked 10th, 16th, and 17th, respectively, in order of importance, among 22 factors. In the present study, we found that the most important soil factors in influencing composition of the essential oil in *O. dayi* were microelements (Table 6 and Figure 3), which are generally ignored in studies of the influence of soil nutrients on secondary metabolism.

Until now, *r* correlation coefficients have been used to test the significance of the linkage between two variables. This leads to an all-or-none segregation of the linkages into significant and nonsignificant ones: a segregation which classifies the *r* value *per se* as devoid of significance. The importance of variations in *r* values to the understanding of physiological processes has already been highlighted (Amzallag, 1999a,b, 2001), and the present study confirms that *r* values, after being *z*-transformed, may reflect the strength of the influence of a soil factor on the composition of the essential oil. As shown by the highly significant correlations observed (e.g., Figures 2, 3, and Tables 6–8), even low values of *r* are of importance in such a representation. In that sense, we should also consider *r* as a variable and not only as a parameter.

In many cases, the $z(\mathrm{SP}, Y)$ values calculated for two soil properties are interrelated (e.g., Tables 7 and 8), which indicates that variations in at least some of the soil properties (e.g., the iron, manganese, copper, and zinc contents) affect the secondary metabolism as a whole (Table 6). This "integrated effect" in the influence of some of the soil properties suggests that the processes that produce the main components of the essential oil in *O. dayi* are not controlled independently, but that they behave as a part of an integrated metabolic network. It also suggests that genetic variability is not necessarily reflected in the variability in the composition of the essential oil. A similar conclusion arose from a comparison between populations of *Pistacia lentiscus*, with respect to their genetic variability and variations in their essential oil composition (Barazani et al., 2003). It seems, therefore, that the chemical composition of the essential oil reflects discrete stable states of the metabolic network, rather than strict genetic control over the production of each volatile component.

The influence of environmental factors on the biosynthesis of essential oil in *O. dayi* is not the only highly networked process to be found in plants. Meristems and differentiating tissues are also organized in a complex regulation network of communication, through the plant growth regulators they produce (Amzallag, 1999a). The source–sink relationships also generate a complex network that involves both sugars and PGRs as information-carrying substances (Gibson, 2000), and even gene expression in plants is now understood to be regulated as a network of interactions (Finnegan, 2001). This suggests that the quantitative use of statistical parameters as variables should not be restricted to our understanding of the factors influencing production of essential oils of commercial value. Rather, these parameters may be useful tools in elaborating

our oversimplified representation of the relationships between organisms and their natural environments.

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EXPRESSION OF CONSTITUTIVE AND INDUCIBLE CHEMICAL DEFENSES IN NATIVE AND INVASIVE POPULATIONS OF *Alliaria petiolata*

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Abstract—The Evolution of Increased Competitive Ability (EICA) hypothesis posits that invasive plants in introduced habitats with reduced herbivore pressure will evolve reduced levels of costly resistance traits. In light of this hypothesis, we examined the constitutive and inducible expression of five chemical defense traits in *Alliaria petiolata* from four invasive North American and seven native European populations. When grown under common conditions, significant variation among populations within continents was found for trypsin inhibitors and peroxidase activity, and glucosinolates and trypsin inhibitors were significantly jasmonate-inducible across populations. Across populations, constitutive levels of glucosinolates and trypsin inhibitors were negatively correlated with their degree of induction, with three North American populations tending to have lower constitutive levels and higher inducibility of glucosinolates than the seven European populations. Alliarinoside and isovitexin 6″-*O*-β-D-glucopyranoside levels were both higher in North American plants than in European plants, but levels of these compounds were generally increased by jasmonate in European plants and decreased by the same treatment in North American plants. Aside from the tendency for invasive populations to have reduced constitutive glucosinolate levels coupled with increased inducibility, little support for the predictions of EICA was evident in the chemical defenses that we studied.

Key Words—*Alliaria petiolata*, defense proteins, evolution, glycosides, herbivory, induced defenses, invasive plants, jasmonic acid.

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INTRODUCTION

Explaining the apparent increased vigor of invasive plant species in novel habitats has received a great deal of attention recently. One hypothesis that has motivated a number of studies is the Evolution of Increased Competitive Ability (EICA) hypothesis (Blossey and Nötzold, 1995). Among a broader suite of predictions, the EICA hypothesis posits that invasive plant populations in introduced habitats with reduced herbivore pressure will evolve lower levels of costly herbivore resistance traits and higher fitness than populations from the native range, given enough time. The expression of resistance traits by plants is costly to growth and fitness (Cipollini et al., 2003a), and relaxation of herbivore pressure can quickly select for reduced expression of resistance traits in plants (Mauricio and Rausher, 1997). Increased growth rates due to reduced expression of unnecessary resistance traits may be one explanation for the success of some invasive species.

Comparisons of invasive and native plant populations in common garden studies have revealed mixed support for EICA thus far. In *Lythrum salicaria*, plants from one invasive North American population grew larger in a European garden and were less resistant to a root-feeding weevil than plants from one native European population (Blossey and Nötzold, 1995). However, performance of a leaf-feeding beetle did not vary. Willis et al. (1999) found that native *L. salicaria* populations were no more resistant to herbivores than invasive North American and Australian populations, despite having higher phenolic contents. In *Solidago gigantea*, invasive European populations incurred more ambient herbivore damage than native North American populations in a North American garden (Meyer et al., 2001). In *Solidago canadensis*, however, invasive European populations were less fit than plants from native North American populations in a North American garden and were equally responsive to simulated herbivory (van Kleunen and Schmid, 2003). Siemann and Rogers (2001) showed that invasive North American populations of *Sapium sebiferum* grew faster and had lower levels of tannin in a North American garden than native Chinese populations, but were equally susceptible to herbivory. In *Alliaria petiolata*, Bossdorf et al. (2004a) demonstrated higher feeding rates by a specialist weevil on invasive North American populations than on native European populations in the laboratory, but no variation in susceptibility to a generalist caterpillar or in tolerance to simulated herbivory. Bossdorf et al. (2004b) also showed that, while similar in the absence of competition, invasive populations were less fit than their native counterparts when in competition with them, challenging the notion of improved competitive ability in invasive populations.

Although the EICA hypothesis makes clear predictions about the evolution of resistance traits in plants, few tests of EICA have examined defense chemistry. In those that have (e.g., Willis et al., 1999; Siemann and Rogers, 2001), comparisons have been restricted to a single compound, which did not explain patterns of resistance among native and invasive populations. In addition, no

tests of EICA have examined variation in inducibility among populations or the relationship between constitutive and induced defense expression. We examined the degree to which the expression of five chemical defenses in *A. petiolata* conformed to predictions of the EICA hypothesis. *A. petiolata* is an aggressive invader of deciduous woodlands throughout North America (Nuzzo, 2000). In Europe, *A. petiolata* is consumed by as many as 70 different herbivores (including five monophagous specialists) and seven pathogens, while only 11 total herbivores (no monophagous specialists) and few pathogens have been reported to consume it in North America (Blossey et al., 2001). Some information on the defense chemistry of this plant is available (e.g., Haribal and Renwick, 2001; Renwick et al., 2001; Cipollini, 2002b), but no comparisons of defense expression in native and invasive populations have been reported.

We first examined the expression of glucosinolates, peroxidase, and trypsin inhibitor activity in several native and invasive populations of this plant. We selected these defenses because they are known to be expressed by *A. petiolata* (Cipollini, 2002b), they are associated with resistance to generalist insects and/or pathogens in other mustards (e.g., Traw et al., 2003; Cipollini et al., 2004), and they are associated with fitness costs in other mustards (Mauricio and Rausher, 1997; Cipollini, 2002a). Each of these defenses has also been shown to be inducible by wounding, insect feeding, and/or jasmonic acid (JA) application in *A. petiolata* or other mustards (Cipollini and Bergelson, 2000; Cipollini, 2002b; Cipollini et al., 2003b, 2004). Second, we examined the expression of alliarinoside and isovitexin-6″-*O*-β-D-glucopyranoside, two glycosides in *A. petiolata* that are known to deter feeding by the mustard specialist *Pieris napi oleraceae* (Renwick et al., 2001), but for which little other information is available. Since *A. petiolata* both lacks monophagous specialists and supports fewer generalist herbivores in North America (Blossey et al., 2001), we predicted that North American populations would show reduced constitutive expression of each chemical defense, assuming that the expression is costly (Cipollini et al., 2003a). According to defense theory, the maintenance or increased inducibility of defenses should accompany reduced constitutive defense expression in populations where this is observed, lest plants be rendered defenseless (Koricheva et al., 2004). As plant defenses vary in their costs, and herbivores are differentially responsive to them, examination of a broader suite of defenses and their modes of expression may help to explain the varying support for EICA that has been detected thus far.

METHODS AND MATERIALS

Study System. A. petiolata. [(M. Bieb) Cavara and Grande; Brassicaceae] is a self-fertile Eurasian biennial, first recorded on Long Island, NY, in the 1860s,

that is spreading rapidly in North America (Nuzzo, 2000). *A. petiolata* flourishes in moist woodlands with moderate exposure to light, but it can grow in a diversity of habitats (Byers and Quinn, 1998; Meekins and McCarthy, 2000). It exhibits remarkable morphological plasticity to local environmental conditions (Byers and Quinn, 1998), it can exude allelopathic chemicals (e.g., Roberts and Anderson, 2001, but see McCarthy and Hanson, 1998), and it produces numerous seeds (Cavers et al., 1979).

Experimental Design. We used seeds from four North American populations from Ohio and Pennsylvania, USA, and seven European populations from the UK and the Netherlands (Table 1). These populations represent only a portion of the native and introduced ranges of garlic mustard, but we have considered population a random factor in our statistical analyses to broaden the generality of our results. Within each population, several mature seeds were collected from at least 10 widely spaced individuals and pooled. Seeds of each population were stratified on moistened filter paper at 4°C until germination, and seedlings were grown in a greenhouse as in Cipollini (2002b). Briefly, germinated seeds were transplanted in Pro-Mix BX potting medium in 500-ml pots and grown under ambient light supplemented with fluorescent lights timed to be on for 13 hr each day. Greenhouse temperatures averaged 22 ± 5°C. Plants were watered with distilled water as needed and were not fertilized in addition to the starter nutrients present in the potting medium. After 35 days of growth, half of the plants in each population were treated with a foliar spray of jasmonic acid (JA) as in Cipollini (2002a). Jasmonic acid is a wound-related hormone that mediates the expression of several chemical defenses in plants (e.g., Cipollini

TABLE 1. LATITUDE AND LONGITUDE OF EUROPEAN AND NORTH AMERICAN POPULATIONS OF GARLIC MUSTARD USED IN THIS STUDY

ID	Location	Latitude	Longitude
Native			
Bil	Bilthoven, The Netherlands	52°8′N	5°12′E
Eld	Arnhem, The Netherlands	51°59′N	5°55′E
Mar	Austerlitz, The Netherlands	52°5′N	5°19′E
Ral	Renkum, The Netherlands	51°58′N	5°44′E
Cav	Caversham, UK	51°28.5′N	1°0′W
Lin	Lincoln, UK	53°13′N	0°32′W
Rea	Reading, UK	51°26.5′N	0°57′W
Invasive			
Idl	Ligonier, PA	40°14′N	79°14′W
Wat	Waterman, PA	40°37′N	79°9′W
CF	Cedarville, OH	39°44′N	83°48′W
WSU	Fairborn, OH	39°48′N	84°1′W

et al., 2004). Induction of defenses by exogenous application of this hormone reasonably mimics induction by herbivore damage (e.g., Thaler et al., 1996). Use of jasmonic acid allows the careful control of the level and timing of induction, and it removes the potentially confounding effects of differential population susceptibilities and herbivore-derived elicitors that would limit generalizations extending from the use of an actual herbivore for induction. In preliminary experiments, we found that exogenous application of this hormone induces trypsin inhibitors in *A. petiolata* in a manner similar to wounding (C. Hillstrom and D. Cipollini, unpublished data). Uninduced plants in each population received an aqueous control spray. Four days after treatment, samples from the fourth true leaves of all plants were harvested for chemical analysis. For each population, levels were assessed in 5–10 replicate plants in each treatment.

Chemical Analyses. Total glucosinolates were assessed in methanol extracts of leaves using the glucose release method as in Siemens and Mitchell-Olds (1998), as modified in Cipollini (2002b). Due to sampling error, the Cedar Falls population was not included in the glucosinolate analysis. Peroxidase activity was assessed in soluble protein extracts of leaves using a spectrophotometric assay as in Cipollini et al. (2004). Trypsin inhibitors were assessed in soluble protein extracts using a radial diffusion assay as in Cipollini and Bergelson (2000). Levels of alliarinoside and isovitexin 6″-*O*-β-D-glucopyranoside were assessed in water-soluble fractions of ethanol extracts of leaves by HPLC, as in Haribal and Renwick (2001), with the following modifications. Compounds were extracted from two to three plants from each population receiving each treatment in boiling ethanol for 10 min, then diluted with water before a solvent change to pure water. Glycosides were isolated using a Phenomenex Luna C18 (150 × 4.6 mm, 5 μm) column attached to a Waters 2690 HPLC system equipped with a photodiode array detector. A water/acetonitrile gradient from 5 to 30% acetonitrile over 10 min was used. We identified the two glycosides by their retention time and UV absorption spectra. Peak areas at 218 nm were used to quantify the expression of each compound. We verified the identity of these two compounds by electronspray ionization (ESI)/MS and MS/MS on an liquid chromatography quadrapole (LCQ) ion-trap mass spectrometer (ThermoQuest, USA).

Statistical Analysis. Mean chemical defense levels were analyzed with mixed-model nested analysis of variance (ANOVA) with continent, population nested within continent, and JA treatment as main effects, including all interactions. Population and its interaction with other factors were considered random factors, and continent and JA treatment fixed factors. The generalized linear model procedure (PROC GLM) using the random statement in SAS V. 8.02 (SAS Institute, Cary, NC, USA) was used to complete these analyses. Type III expected mean squares were used in all tests, which utilized Satterthwaite

F values. The approximate degrees of freedom in the denominator of each *F* statistic have been rounded to the nearest whole number. While most populations were still represented, the population factor was excluded in the analysis of alliarinoside and isovitexin 6″-*O*-β-D-glucopyranoside due to lack of replicate samples within some populations. Thus, only continent, JA and their interaction were included in the fixed effect ANOVA for these two defenses. Pearson correlations were performed among and between constitutive and induced levels of trypsin inhibitor, peroxidase, and glucosinolates using the treatment means for each population. In addition, Pearson correlations were performed between constitutive levels of each of these defenses and the percent difference between their constitutive and induced levels (their *degree* of inducibility), again by using population means. All data were transformed as needed to meet the assumptions of ANOVA. All analyses were conducted using SAS V. 8.02 (SAS Institute, Cary, NC).

RESULTS

Total glucosinolate levels were similar among continents overall and among populations within continents, but were significantly induced by JA treatment by about 26% overall (Table 2, Figure 1A). While no significant interaction was detected in the ANOVA, North American populations tended to have lower constitutive and higher induced levels than European populations.

TABLE 2. *F*-VALUES FROM MIXED-MODEL ANOVA EXAMINING THE EFFECT OF CONTINENTAL ORIGIN, POPULATION WITHIN CONTINENT, AND JASMONIC ACID (JA) TREATMENT ON CHEMICAL DEFENSE LEVELS IN FOURTH TRUE LEAVES OF GARLIC MUSTARD FROM NATIVE EUROPEAN AND INTRODUCED NORTH AMERICAN POPULATIONS *P*-VALUES ARE IN PARENTHESES

Source	TI	GS	POD
Continent	$F(1,11) = 0.03$ (0.867)	$F(1,9) = 0.01$ (0.908)	$F(1,11) = 2.02$ (0.182)
Population (Cont)	$F(9,9) = 4.50$ (<0.018)	$F(8,8) = 0.53$ (0.809)	$F(9,9) = 4.79$ (<0.014)
JA treatment	$F(1,23) = 88.73$ (<0.001)	$F(1,25) = 4.93$ (0.036)	$F(1,19) = 0.24$ (0.631)
Continent × JA	$F(1,24) = 0.37$ (0.550)	$F(1,27) = 2.73$ (0.110)	$F(1,20) = 1.19$ (0.288)
Pop (Cont) × JA	$F(9,183) = 1.10$ (0.364)	$F(8,168) = 1.27$ (0.263)	$F(9,183) = 1.41$ (0.187)

TI = trypsin inhibitor, GS = glucosinolates, POD = peroxidase.

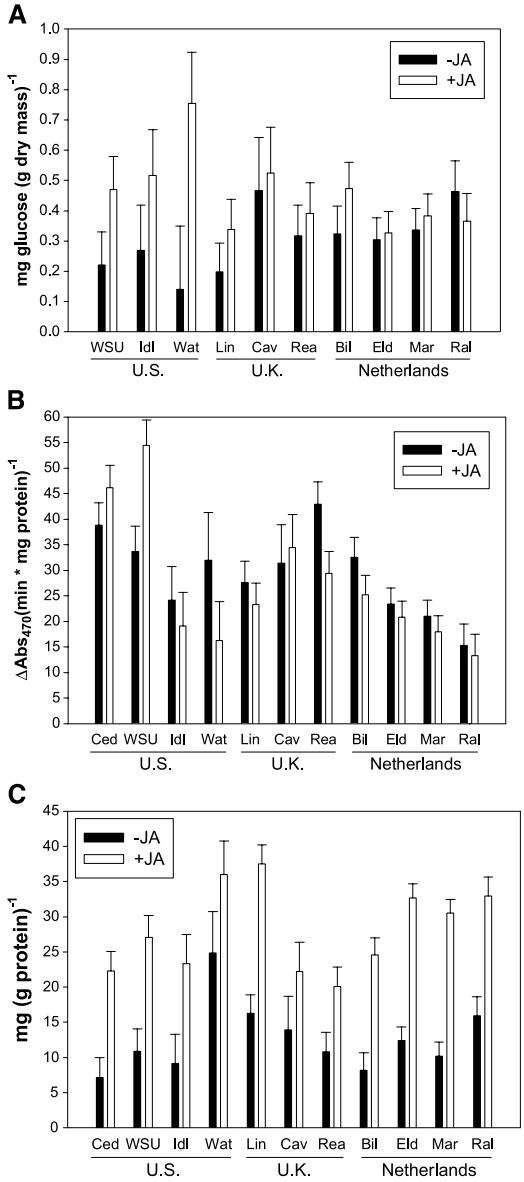

FIG. 1. Constitutive and jasmonic acid induced levels of chemical defenses in leaves of 39-day-old *Alliaria petiolata* from native European and introduced North American populations. (**A**) Glucosinolate content. (**B**) Peroxidase activity. (**C**) Trypsin inhibitor content. Bars represent the mean ± 1 SE, N = 5–10. See Table 1 for population abbreviations.

No variation among continents in peroxidase activity was found, but populations varied within continents by as much as 112% (Table 2, Figure 1B). Although not significant, an interesting pattern was present in the response of populations to JA treatment. Two North American populations displayed higher peroxidase activities after JA treatment, and two populations displayed lower

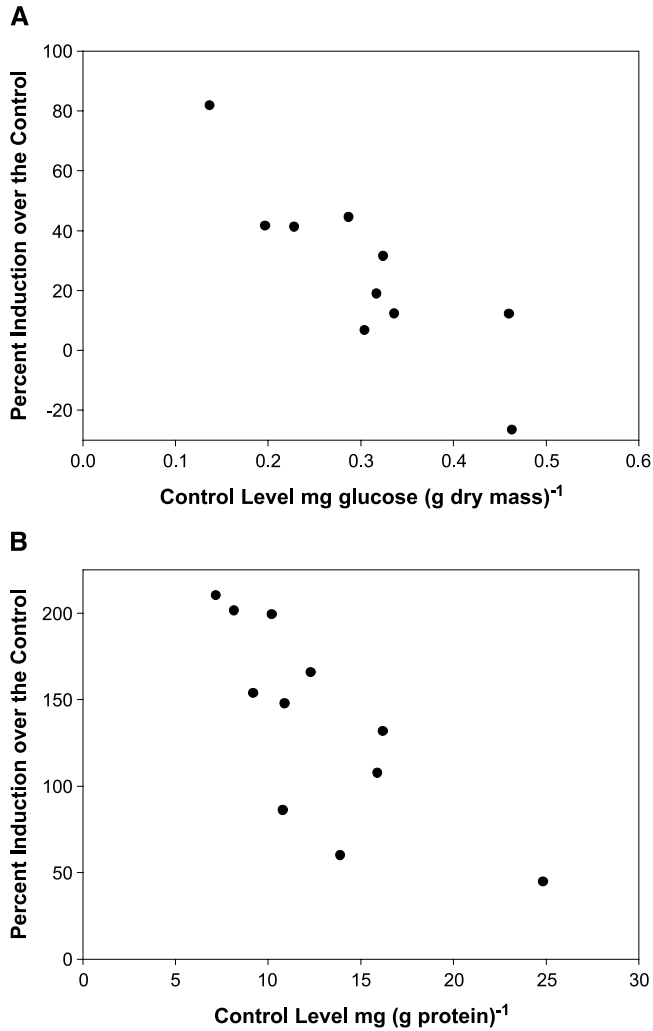

FIG. 2. Correlations between mean constitutive defense levels for each population and mean percent induction by jasmonic acid over control levels. (**A**) Glucosinolates. (**B**) Trypsin inhibitors. Each point represents the mean of 5–10 plants for one population.

peroxidase activities. Six of seven European populations displayed lower peroxidase levels after JA treatment.

No differences among continents were detected in trypsin inhibitor content overall, but populations varied within continents by as much as 300% (Table 2, Figure 1C). JA treatment significantly increased trypsin inhibitor expression by an average of 130% across populations.

Only two significant correlations among and between constitutive and induced *levels* of chemical defenses were detected. Constitutive and induced levels of peroxidase were positively correlated ($r = 0.637$, $P = 0.035$, $N = 11$), and constitutive and induced levels of trypsin inhibitor were positively correlated ($r = 0.697$, $P = 0.017$, $N = 11$). However, constitutive levels of both glucosinolates ($r = -0.867$, $P = 0.001$, $N = 10$; Figure 2A) and trypsin inhibitors ($r = -0.764$, $P = 0.006$, $N = 11$; Figure 2B) were negatively correlated with their degree of induction across all populations. In particular, constitutive glucosinolate levels were 36% lower on average in the three North American populations than in the seven European populations, but were four times more inducible on average.

Neither continent [$F(1,31) = 0.202$, $P = 0.657$] nor JA treatment [$F(1,31) = 0.009$, $P = 0.926$] affected alliarinoside levels independently. However, the response of alliarinoside to JA treatment varied by continent [$F(1,31) = 7.17$, $P = 0.013$]. Constitutive levels of alliarinoside were high in North American plants and decreased by JA, but were low in European plants and increased by JA (Table 3). Levels of isovitexin 6″- *O*-β-D-glucopyranoside varied by continent, being higher overall in North American plants than in European plants [$F(1,31) = 10.81$, $P = 0.003$, Table 3]. The effect of both JA treatment [$F(1,31) = 3.387$, $P = 0.076$] and the interaction of continent and JA treatment [$F(1,31) = 3.077$, $P = 0.09$] on levels of this compound approached significance. The response to JA treatment of this compound by continent was similar to that of alliarinoside (Table 3).

TABLE 3. MEAN (± 1SE) ALLIARINOSIDE AND ISOVITEXIN 6″-*O*-β-D-GLUCOPYRANOSIDE CONTENTS IN FOURTH TRUE LEAVES OF 39-DAY-OLD *Alliaria petiolata* PLANTS FROM NATIVE EUROPEAN AND INVASIVE NORTH AMERICAN POPULATIONS TREATED WITH JASMONIC ACID (JA) OR A CONTROL SOLUTION UNITS ARE IN AU_{218} PER GRAM LEAF EQUIVALENT ($\times 10^5$).

Continent	Treatment	Alliarinoside	Isovitexin glucopyranoside
Europe	Control	3.83 (2.24)	7.99 (1.02)
Europe	JA	10.3 (2.93)	8.44 (1.50)
North America	Control	12.9 (3.17)	17.5 (1.77)
North America	JA	4.81 (3.88)	10.2 (1.78)

DISCUSSION

We examined the degree to which the expression of five chemical defenses in young *A. petiolata* plants conformed to predictions of the EICA hypothesis in a subset of populations from the introduced and native range of this plant. Our population sampling was relatively restricted, so our results should be interpreted cautiously, but little evidence for the predictions of EICA was found in this study.

Although few continental differences were detected among the populations, significant variation among populations within continents was found in the expression of trypsin inhibitor and peroxidase, and both trypsin inhibitor and glucosinolates were jasmonate-inducible. Jasmonate regulation of these two defenses is consistent with findings in other mustard species including *Arabidopsis thaliana* (Cipollini et al., 2004). There were no negative correlations among or between constitutive or induced levels of any of these compounds, but constitutive levels of glucosinolates and trypsin inhibitor were negatively correlated with their degree of induction across all populations. This finding is in accordance with plant defense theory (e.g., Koricheva et al., 2004), suggesting that there are tradeoffs between constitutive expression and inducibility of these two defenses in this plant. In particular, the three North American populations included in the glucosinolate analysis generally displayed lower constitutive expression and increased inducibility of these chemicals relative to the European populations, a pattern that supports the predictions of EICA. Continental patterns in the expression of trypsin inhibitor and peroxidase activity do not support the predictions of EICA.

Expressing low constitutive levels and high inducibility of a defense, such as glucosinolates vs. high constitutive levels and low inducibility, may be a cost-saving strategy resulting from reduced selective pressure by herbivores (Koricheva et al., 2004). However, either strategy may result in the same benefits for resistance. If so, our results could explain the finding of no differences in susceptibility to the generalist caterpillar, *Spodoptera littoralis*, in the invasive and native populations of *A. petiolata* studied by Bossdorf et al. (2004a). Performance of a related caterpillar on *A. thaliana* is negatively related to glucosinolate and trypsin inhibitor content and is reduced by jasmonate (Cipollini et al., 2004). It is unclear how our results might explain the variation in susceptibility to the monophagous specialist weevil, *Ceutorhyncus scrobicollis*, among the populations studied by Bossdorf et al. (2004a), since it is unknown how it responds to the defenses that we measured. This herbivore is currently absent from the North American fauna and is being considered for release as a biocontrol agent (Blossey et al., 2001). Understanding its response to variation in host defense chemistry could inform biocontrol efforts.

Although variation among populations and correlations with other defenses could not be assessed due to a restricted sample size, North American plants of *A. petiolata* displayed higher constitutive levels of alliarinoside and isovitexin 6″-*O*-β-D-glucopyranoside than European plants, in stark contrast to the predictions of EICA. This finding suggests that invasive North American populations may be as well or better defended than native populations from herbivores that are sensitive to these compounds. Although *A. petiolata* has escaped many European herbivores (Blossey et al., 2001), herbivore pressure in North America may be sufficient to select for high levels of these defenses in invasive populations. Benefits to resistance against North American mustard specialist herbivores, such as *P. napi oleracea*, may have favored the maintenance of constitutively high levels of these glycosides. Slugs are one of the more important generalist herbivores of this plant in North America (Blossey et al., 2001; D. Cipollini, personal observation) and may also produce enough damage to select for the maintenance of certain chemical defenses. Why glycoside levels declined in response to jasmonate in North American populations, but generally increased in response to jasmonate in European populations is unknown.

Despite the focus on costs of defense as an impediment to competitive ability, expression of chemical defenses may be a critical part of the invasiveness of *A. petiolata*. Although monophagous specialists may be lacking, *A. petiolata* has retained a substantial degree of resistance to herbivores in its introduced range, as some North American mustard specialists and several generalist herbivores either perform poorly or cannot survive on it (Renwick et al., 2001; D. Cipollini, personal observations). Although presumably physiologically costly, expressing high levels of resistance by *A. petiolata* could benefit individual plants if high defense levels force herbivores to consume more palatable interspecific neighbors, favoring competitive release of *A. petiolata*. In addition to their interactions with herbivores, some chemical defenses may also have alternative benefits that could contribute to invasiveness and select for their maintenance in the invasive range. For example, glucosinolates have been associated with allelopathy in *A. petiolata* (e.g., Roberts and Anderson, 2001) and may confer competitive ability to this plant in species mixtures. Expression of allelopathic chemicals with multiple roles in resistance contributes to the invasiveness of plants such as *Centaurea maculosa* (Bais et al., 2002). In addition to defense against herbivores and pathogens, peroxidase has numerous roles in plant development and stress tolerance (Campa, 1991). Alternative benefits may be provided by the more recently discovered flavonoid glycosides, including allelopathy or protection from photooxidation, but they have been little studied to date.

In summary, analysis of five chemical defenses of *A. petiolata* revealed little support for the predictions of EICA, although population variability and jasmonate regulation was demonstrated for some defenses. Variation in the

constitutive and induced expression of chemical defenses with multiple roles in stress resistance may explain the idiosyncratic results that have accumulated in tests of EICA in this and other invasive plants, but most studies have failed to fully consider chemical defenses. The finding that some chemical defenses may be expressed as highly or more in invasive genotypes as in native genotypes suggests that some chemical defenses may contribute to invasiveness and that the general expectation that they will trade off with competitive ability in an ecological context may not always hold true.

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CHANGES IN THE COMPOSITION OF PHOSPHOLIPID FATTY ACIDS AND STEROLS OF MAIZE ROOT IN RESPONSE TO MONOTERPENES

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Abstract—Terpenes are thought to be important in plant–plant interactions because of their phytotoxic action on seed germination and growth. Herein, the effects of five volatile monoterpenes on root sterols and phospholipid fatty acid (PLFA) composition have been studied during maize seedling germination. The investigated monoterpenes (camphor, 1–8 cineole, geraniol, menthol, and thymol) were applied at their respective IC_{80} (concentration causing 80% inhibition). They quantitatively affected free sterols and PLFA composition, thus producing an increase in the percentage of unsaturated PLFAs, stigmasterol of the free sterol fraction, and saturated steryl ester fatty acids. Alcoholic and nonalcoholic monoterpenes appeared to have different modes of action. The former affected unsaturated fatty acid and stigmasterol to a greater extent, and accordingly they could interfere in seedling growth by changes in the membrane lipids.

Key Words—Allelopathy, fatty acid, monoterpenes, sterol, *Zea mays*, geraniol, 1,8-cineole, menthol, camphor, thymol, phytotoxicity, membrane.

INTRODUCTION

Monoterpenes are toxic towards vascular plants (Vaughn and Spencer, 1993, 1996; Scrivanti et al., 2003). An extensive study by Vaughn and Spencer (1993) showed that 18 volatile monoterpenes are inhibitory to the germination and growth of nine different plant species. They were generally phytotoxic to corn, wheat, and alfalfa. Notwithstanding, the mode and mechanism by which these

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compounds affect the growth of other plant species have been poorly examined at chemical levels (Abrahim et al., 2000; Romagni et al., 2000).

In recent years, the lipid composition of various plant tissues has been shown to influence a number of factors crucial to growth and development (Grandmougin-Ferjani et al., 1997; Borst et al., 2000; Hellgren and Sandelius, 2001; Harker et al., 2003). Phospholipids are more than just structural components of membranes; they can be cofactors for membrane enzymes, signal precursors, or signaling molecules themselves (Laxalt and Munnik, 2002). Plant sterols, like other sterols, are primary components of cellular membranes where they regulate fluidity and permeability. This structural role is often described as "bulk" function. Additionally, sterols can participate in the control of membrane-associated metabolic processes; their involvement in signal transduction events has been reported in mammalian cells (Hartmann, 1998). It is known that free and esterified sterols participate in the formation of biomembranes, and during plant development the sterol content is not static (Huang and Grunwald, 1988; Grandmougin et al., 1989; Izzo and Navari-Izzo, 1993; Moreau et al., 1998).

Since monoterpenes are lipophilic compounds, the question arises whether they act on plant growth by affecting lipid composition. This investigation was conducted to evaluate how monoterpenes affect the most abundant free and esterified sterols, i.e., sitosterol, stigmasterol, and campesterol (Dyas and Goad, 1993; Hartmann, 1998) and phospholipid fatty acid (PLFA) composition of roots during maize seedling growth.

METHODS AND MATERIALS

Chemicals. The monoterpenes used were 1,8-cineole, thymol, menthol, geraniol, and camphor. Previous studies indicated these were the most active compounds (Asplund, 1968; Vaughn and Spencer, 1993, 1996; Koitabashi et al., 1997) They were obtained from ICN Pharmaceuticals Co. (Costa Mesa, CA, USA) and were of high purity.

Plant Material. Maize seeds (*Zea mays* L.) were rolled in the upper 3 cm of a 20-cm-long paper towel scroll. Individual scrolls were moistened with 25 ml of distilled H_2O and placed upright in 3 l flasks. The scrolled seeds were germinated for 3 d at 27 ± 1°C in the dark. At the end of this period, the length of the roots was 6 ± 1 cm, and the seedlings were harvested and transferred to bioassay.

Bioassay. Three-d-old seedlings (0 hr of treatment) were placed in 3 l desiccator flasks on Whatman No. 1 filter paper wetted with 15 ml of distilled water, and a 5-ml glass beaker was placed in the center. A sample of 2 ml of liquid (1,8-cineole and geraniol) or 2 g of solid (thymol, menthol, and camphor)

monoterpenes (volatile source) was added to the beaker. No direct physical contact occurred between the compounds and the seedlings. These amounts saturated the headspace, reaching concentrations of 21.7, 2.0, 1.9, 2.5, and 7.4 mg l^{-1} of 1,8-cineole, thymol, geraniol, menthol, and camphor, respectively. These concentrations corresponded to the inhibitory concentration 80 (IC_{80}), defined as the concentration that inhibited 80% of root growth at 96 hr of treatment compared with the control (Zunino and Zygadlo, 2004). Flasks were placed in the dark at 27 ± 1°C and atmospheric pressure for 24, 48, and 96 hr. Then, the plants were harvested, the roots dissected, and the different experimental parameters were measured. The central beakers were left empty in the controls.

Analysis of Volatile Compounds. Volatiles from headspace of glass desiccators were trapped by using a 10-ml gastight syringe and were analyzed by gas chromatography (GC) and GC/mass spectrometry (MS). A gas chromatograph (Shimadzu R1A) equipped with a flame ionization detector was used. A split inlet (split ratio 200:1) was used to inject volatiles into a DB-5 capillary GC column (30 m × 0.25 mm i.d., and 0.25 μm film thickness), and ramped column temperature conditions (60°C for 3 min, increased to 240°C at 4°C min^{-1}) were used. Detector temperature was 280°C. The carrier gas was He, and it was applied at a constant flow of 0.9 ml min^{-1}. Individual peaks were identified by using a mass selective detector (Perkin-Elmer Q700 gas chromatograph–mass spectrometer) and co-injection with standards. GC-MS was performed under the same conditions. The ionization potential of MS was 70 eV. The quantity of monoterpenes in the headspace was determined by the external standard method (Vaughn and Spencer, 1993). Standard curves were generated by analysis of known concentrations of each compound dissolved in *n*-C_6H_{14}.

Lipid Extraction and Analysis. Lipids were extracted from the roots (20 ± 5 g fresh weight) with $CHCl_3$:methanol (87:13) in a Soxhlet apparatus for 12 hr. Extracts were dried on dry Na_2SO_4, taken to dryness under vacuum, and chromatographed on preparative silica gel thin-layer plates (silica gel 60 G F254) using hexane–ether (4:1 by volume) as the developing system. Sterols were visualized under UV light. Three fractions were eluted corresponding to steryl esters (SE), free sterol (FS), and phospholipids (PL). Cholesteryl myristate, sitosterol, and phosphatidylcholine were used as markers, respectively. The FS fraction was scraped off and eluted in dichloromethane, filtered to remove the residual silica, dried in a rotatory evaporator, and then purified by a bidirectional thin-layer chromatography (TLC; hexane–ether 4:1 and benzene–ether 4:1 as developing system) (Rohmer et al., 1972). SE and PL fractions were saponified with 1 N methanolic KOH to obtain the respective fatty acids. Fatty methyl esters from SE and PL were prepared by transesterification through treatment with 1 N H_2SO_4 in dry methanol for 30 min (Grosso et al., 1994) and analyzed by GC. Quantitative determinations of total PLFA were made with

heptadecanoic (C17:0) methyl esters as an internal reference. Analytical GC was performed on a Shimadzu GC-R1A gas chromatograph (FID) fitted with Supelcowax-10 capillary column (30 m × 0.25 mm i.d.). Column temperature was programmed from 180 to 240°C (4°C min^{-1}). Injector and detector temperature was 250°C, and N_2 was used as the carrier gas at a flow rate of 20 ml min^{-1}. The injection volume was 2 μl.

SE fraction sterols were recovered from unsaponifiable matter and together with FS were subjected to GC analysis. Qualitative and quantitative analysis were carried out using a Shimadzu GC-R1A gas chromatograph (FID) fitted with DB5 capillary column (30 m × 0.25 mm i.d.). Column temperature was programmed from 240°C to 290°C (4°C min^{-1}). Injector and detector temperature was 300°C, and N_2 was used as the carrier gas at a flow rate of 1 ml/min. The injection volume was 2 μl.

Sterol identities were confirmed by comparison with authentic compounds under identical conditions using a GC mass-selective detector.

Statistical Analyses. Experimental values are means and SDs of three independent experiments. A two-way analysis of variance (ANOVA) was used to evaluate treatment time (T) and monoterpenes (M) effects together with their interaction (T × M). Significance (at $P \leq 0.05$) was assessed using DGC multiple range test (Di Rienzo et al., 2002).

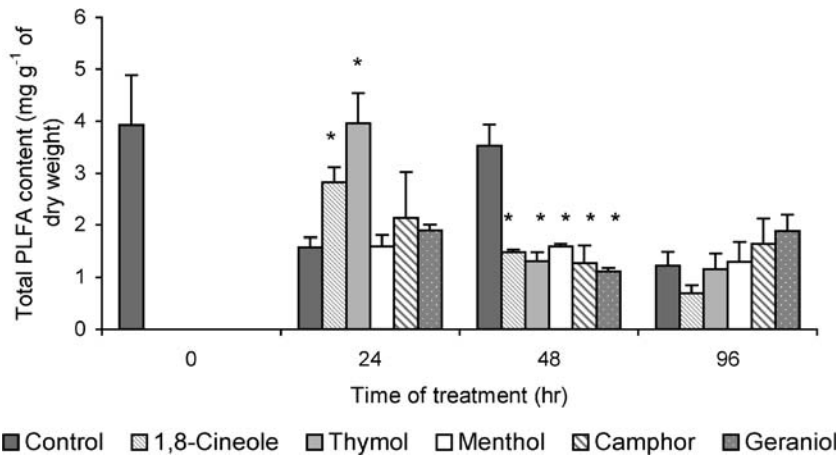

FIG. 1. Effect of monoterpenes on total PLFA content in maize roots. Values are means and SD. *Denotes significant differences between treatment and control for each time of treatment, according to DGC multiple range test at $P \leq 0.05$ ($n = 3$).

RESULTS

Effect of Monoterpenes on Phospholipid Fatty Acid Content. Each treatment and the control reduced the total PLFA content of roots during the period that goes from 0 to 96 hr (Figure 1). Control roots and roots treated with menthol, camphor, and geraniol showed a decrease of total PLFA at 24 hr, but soon after a recovery occurred in control roots at 48 hr (but not with treatments). Otherwise, roots treated with 1,8-cineole and thymol showed a decrease of the total PLFA from 48 hr. Nonetheless, the total PLFA of each treatment and control at 96 hr was lower than that observed at 0 hr (Figure 1).

In maize roots, the FAs 16:0, 16:1, 18:0, 18:1, 18:2, and 18:3 were the main contributing ones (Grandmougin et al., 1989; Bohn et al., 2001). The percentage of FA 18:2, 18:1, and 16:0 was higher than the other FAs, 16:1, 18:0, and 18:3 (Table 1). Saturated FAs did not show any significant changes

TABLE 1. EFFECT OF 1,8-CINEOLE, THYMOL, MENTHOL, GERANIOL, AND CAMPHOR ON PLFA COMPOSITION OF MAIZE (*Zea mays*) ROOTS

		PLFA (% of total)						
Monoterpenes	Time (hr)	16:0**	16:1*	18:0*	18:1*	18:2*	18:3**	Unsaturated/ saturated*
Control	0	27.63^{1}	1.43^{a}	1.46^{a}	9.17^{a}	56.41^{b}	3.90^{1}	2.5^{b}
	24	29.92^{b1}	1.08^{a}	1.77^{a}	9.47^{a}	53.82^{b}	4.12^{a1}	2.2^{b}
	48	28.39^{a1}	1.81^{a}	1.83^{a}	7.77^{a}	55.88^{b}	4.32^{a1}	2.3^{b}
	96	27.68^{a1}	2.60^{a}	7.57^{b}	18.40^{b}	39.27^{a}	4.49^{a1}	1.8^{a}
1,8 Cineole	24	24.80^{a1}	5.30^{b}	2.03^{a}	20.72^{b}	44.23^{a}	2.92^{a1}	2.8^{b}
	48	28.80^{a1}	4.90^{b}	3.55^{a}	13.03^{a}	45.85^{a}	3.87^{a1}	2.1^{b}
	96	31.25^{a1}	2.11^{a}	3.41^{a}	10.91^{a}	47.19^{a}	5.14^{a1}	1.6^{a}
Thymol	24	25.12^{a1}	1.07^{a}	2.60^{a}	15.80^{b}	51.7^{b}	3.70^{a1}	2.6^{b}
	48	28.19^{a1}	1.93^{a}	2.73^{a}	8.80^{a}	54.17^{b}	4.18^{a1}	2.2^{b}
	96	30.26^{a1}	1.04^{a}	2.76^{a}	6.73^{a}	54.06^{b}	5.16^{a1}	2.0^{b}
Menthol	24	30.14^{b1}	1.75^{a}	2.04^{a}	7.48^{a}	55.01^{b}	3.58^{a1}	2.1^{b}
	48	27.63^{a1}	1.86^{a}	1.87^{a}	8.30^{a}	56.22^{b}	4.12^{a1}	2.4^{b}
	96	29.70^{a1}	1.79^{a}	2.24^{a}	7.02^{a}	55.04^{b}	4.21^{a1}	2.1^{b}
Geraniol	24	25.06^{a1}	1.23^{a}	1.66^{a}	8.30^{a}	59.90^{b}	3.86^{a1}	2.7^{b}
	48	28.69^{a1}	1.24^{a}	2.18^{a}	7.53^{a}	56.60^{b}	3.77^{a1}	2.2^{b}
	96	29.19^{a1}	2.62^{a}	2.61^{a}	7.72^{a}	54.03^{b}	3.84^{a1}	2.2^{b}
Camphor	24	25.66^{a1}	2.28^{a}	3.77^{a}	11.95^{a}	52.19^{b}	4.15^{a1}	2.4^{b}
	48	28.42^{a1}	3.74^{b}	2.50^{a}	11.18^{a}	50.70^{b}	3.47^{a1}	2.2^{b}
	96	25.64^{a1}	4.40^{b}	3.28^{a}	14.51^{b}	46.99^{a}	5.18^{a1}	2.5^{b}

Results are the means of three independent experiments.

*ANOVA with interaction. Values having different letters are significantly different from each other according to DGC multiple range test at $P \leq 0.05$.

**ANOVA without interaction. For each monoterpene, values in columns followed by different numbers and for each time value in rows followed by different letters are significantly different according to DGC multiple range test at $P \leq 0.05$.

with treatments with the exception of FA 18:0 that increased from 48 to 96 hr in control roots. At 24 hr, roots treated with 1,8-cineole, thymol, geraniol, and camphor showed lower percentage of FA 16:0 than controls. For each monoterpene, the percentage of FA 18:0 was lower than controls at 96 hr. Among unsaturated FAs, there was a decrease from 0 to 96 hr in the percentage of 18:2 for controls, 1,8-cineole, and camphor. Compared with the untreated control, the composition of 18:2 at 96 hr was higher in each treatment, except in the 1,8-cineole and camphor treatments where their composition was similar to that of the controls (Table 1). A significant increase in the FA 18:1 was observed at 96 hr in the control and camphor treatments. This was not observed with the other treatments, which showed lower percentages of 18:1 than those at 96 hr. At 24 hr, 1,8-cineole and thymol treatments showed higher values of 18:1 than controls. FA 18:3 showed changes neither in time nor with treatments. Whereas FA 16:1 increased only with camphor during treatments (showing

TABLE 2. EFFECT OF 1,8-CINEOLE, THYMOL, MENTHOL, GERANIOL AND CAMPHOR ON SEFA COMPOSITION OF MAIZE (*Zea mays*) ROOTS

		SEFA (% of total)						
Monoterpenes	Time (hr)	16:0**	16:1*	18:0*	18:1**	18:2*	18:3**	Unsaturated/ saturated**
Control	0	19.96^{1}	3.01^{a}	5.48^{a}	21.83^{1}	49.71^{b}	Tr^{1}	3.4^{2}
	24	12.44^{a1}	1.35^{a}	2.25^{a}	29.27^{b1}	54.68^{b}	Tr^{a1}	5.8^{b2}
	48	14.88^{a1}	0.98^{a}	6.43^{a}	27.91^{a1}	49.80^{b}	Tr^{a1}	4.0^{b2}
	96	16.40^{a1}	4.31^{a}	8.87^{a}	23.09^{a1}	47.32^{b}	Tr^{a1}	3.1^{b2}
1,8 Cineole	24	17.54^{a1}	2.29^{a}	4.98^{a}	33.41^{b1}	41.79^{b}	Tr^{a1}	3.5^{a2}
	48	28.89^{b1}	1.79^{a}	9.02^{a}	31.60^{a1}	28.70^{a}	Tr^{a1}	1.6^{a2}
	96	17.62^{a1}	2.93^{a}	9.97^{a}	28.61^{a1}	40.88^{b}	Tr^{a1}	2.7^{b2}
Thymol	24	19.42^{a1}	4.56^{a}	8.61^{a}	22.32^{a1}	45.09^{b}	Tr^{a1}	3.2^{a2}
	48	15.15^{a1}	4.1^{a}	5.68^{a}	22.89^{a1}	52.18^{b}	Tr^{a1}	4.0^{b2}
	96	18.23^{a1}	5.76^{a}	6.16^{a}	21.77^{a1}	48.08^{b}	Tr^{a1}	3.1^{b2}
Menthol	24	22.36^{a1}	9.06^{b}	11.56^{a}	20.14^{a1}	36.87^{b}	Tr^{a1}	2.1^{a2}
	48	23.78^{b1}	6.69^{a}	9.12^{a}	19.05^{a1}	43.59^{b}	Tr^{a1}	2.2^{a2}
	96	24.49^{b1}	9.75^{b}	11.39^{a}	27.87^{a1}	24.84^{a}	6.68^{b2}	1.7^{a2}
Geraniol	24	17.97^{a1}	6.58^{a}	8.03^{a}	30.39^{b1}	36.44^{b}	1.77^{b1}	2.9^{a2}
	48	29.49^{b1}	9.26^{b}	19.00^{b}	16.35^{a1}	25.07^{a}	1.66^{b1}	1.1^{a1}
	96	27.62^{b1}	8.90^{b}	15.31^{b}	22.72^{a1}	24.45^{a}	4.01^{b2}	1.3^{a1}
Camphor	24	27.44^{a1}	6.90^{a}	14.34^{b}	20.57^{a1}	30.74^{a}	Tr^{a1}	1.6^{a1}
	48	25.38^{b1}	5.38^{a}	20.41^{b}	21.96^{a1}	25.69^{a}	2.38^{b2}	1.0^{a1}
	96	32.97^{b1}	5.02^{aa}	20.83^{b}	22.20^{a1}	18.98^{a}	Tr^{a1}	1.1^{a1}

Results are the means of three independent experiments. Tr, Trace (<0.5%).

*ANOVA with interaction. Values having different letters are significantly different from each other according to DGC multiple range test at $P \leq 0.05$.

**ANOVA without interaction. For each monoterpene, value in columns followed by different numbers and for each time value in rows followed by different letters are significantly different, according to DGC multiple range test at $P \leq 0.05$.

higher values than controls at 48 and 96 hr), 1,8-Cineole also produced higher values of 16:1 than controls, occurring at 24 and 48 hr (Table 1).

Regarding the unsaturated to saturated FA ratio, a decrease from 0 to 96 hr was shown in control roots and the 1,8-cineole treatment. Nevertheless, the rest of the monoterpenes did not show changes over time but showed higher unsaturated to saturated ratio values than controls at 96 hr (Table 1).

Effect of Monoterpenes on Composition of Sterol Ester Fatty Acids. The main FA of SE, like PL, were 16:0, 18:1, and 18:2 (Table 2). The saturated FA 16:0 showed higher values than controls at 48 hr with 1,8-cineole, and at 48 and 96 hr with menthol, geraniol, and camphor. FA 18:0, on the other hand, increased with geraniol at 48 and 96 hr, and with camphor treatment at all time points studied (Table 2). Among unsaturated FAs, 16:1 and 18:3 showed increases preferentially with menthol and geraniol monoterpenes. With camphor, FA 18:3 increased at 48 hr. In contrast, the percentage of the major compounds FA 18:1 and 18:2 showed lower values than controls: 18:1 at 24 hr with monoterpenes thymol, menthol, and camphor, and FA 18:2 with 1,8-

TABLE 3. EFFECT OF 1,8-CINEOLE, THYMOL, MENTHOL, GERANIOL, AND CAMPHOR ON FS COMPOSITION OF MAIZE (*Zea mays*) ROOTS

		FS (% of total)		
Monoterpenes	Time (hr)	Campesterol	Stigmasterol	Sitosterol
Control	0	19.74[b]	41.56[a]	38.74[d]
	24	25.57[b]	36.50[a]	37.93[d]
	48	17.74[b]	53.90[b]	28.36[c]
	96	25.17[b]	42.03[a]	32.70[c]
1,8 Cineole	24	21.27[b]	45.87[a]	32.90[c]
	48	15.70[a]	59.14[b]	25.17[c]
	96	30.40[c]	50.67[a]	18.97[b]
Thymol	24	22.23[b]	47.25[a]	30.55[c]
	48	13.68[a]	55.84[b]	30.48[c]
	96	24.35[b]	48.88[a]	26.77[c]
Menthol	24	20.35[b]	61.93[b]	17.72[b]
	48	22.73[b]	57.79[b]	19.48[b]
	96	28.20[c]	63.60[b]	8.19[a]
Geraniol	24	21.38[b]	64.91[b]	13.71[b]
	48	17.85[b]	58.23[b]	23.92[c]
	96	17.59[b]	67.78[b]	14.63[b]
Camphor	24	26.51[b]	50.35[a]	23.14[c]
	48	23.13[b]	60.23[b]	16.64[b]
	96	23.40[b]	56.86[b]	19.74[b]

Results are the means of three independent experiments.
ANOVA with interaction. Values having different letters are significantly different from each other according to DGC multiple range test at $P \leq 0.05$.

cineole (at 48 hr), menthol (at 96 hr), geraniol (at 48 and 96 hr), and camphor (at all times). These changes in FA composition are shown in the unsaturated to saturated ratios, which were lower than controls at every time studied for all monoterpenes—with the exception of 1,8-cineole at 96 hr and thymol at 48 and 96 hr (Table 2).

Effect of Monoterpenes on Composition of Free Sterols. The main FS constituent in the FS maize root fraction was stigmasterol (36.5–67.8%), as observed in the control and all monoterpene treatments (Table 3). Treatment with 1,8-cineole and menthol showed an increase in campesterol at 96 hr, whereas at 48 hr it decreased with 1,8-cineole and thymol monoterpenes. Stigmasterol values increased from 0 to 48 hr, decreasing at 96 hr with controls and 1,8-cineole and thymol treatments. At 24 and 96 hr, there were increases in stigmasterol with the menthol and geraniol treatments. With camphor, this FS increased at 96 hr, too (Table 3). In the control and every monoterpene studied, sitosterol decreased through time. At 96 hr, only with thymol were there no significant differences from control, and at 48 hr this FS was unchanged with

TABLE 4. EFFECT OF 1,8-CINEOLE, THYMOL, MENTHOL, GERANIOL, AND CAMPHOR ON SE COMPOSITION OF MAIZE (*Zea mays*) ROOTS

		SE (% of total)		
Monoterpenes	Time (hr)	Campesterol	Stigmasterol	Sitosterol
Control	0	14.66[a]	41.87[b]	43.47[a]
	24	17.44[a]	27.12[a]	55.44[b]
	48	16.36[a]	14.53[a]	69.11[c]
	96	20.70[b]	28.17[a]	51.10[b]
1,8 Cineole	24	19.21[a]	20.52[a]	60.14[b]
	48	13.37[a]	15.80[a]	70.83[c]
	96	22.88[b]	22.32[a]	54.80[b]
Thymol	24	18.22[a]	21.82[a]	60.02[b]
	48	19.27[a]	17.83[a]	62.90[c]
	96	25.72[b]	28.29[a]	45.99[a]
Menthol	24	18.69[a]	26.36[a]	54.96[b]
	48	14.06[a]	19.66[a]	66.28[c]
	96	23.43[b]	23.82[a]	52.74[b]
Geraniol	24	17.30[a]	18.37[a]	64.32[c]
	48	21.89[b]	24.15[a]	53.95[b]
	96	13.83[a]	20.03[a]	66.14[c]
Camphor	24	16.01[a]	25.51[a]	58.48[b]
	48	18.66[a]	23.71[a]	57.63[b]
	96	23.91[b]	41.15[b]	34.94[a]

Results are the means of three independent experiments.
ANOVA with interaction. Values having different letters are significantly different from each other according to DGC multiple range test at $P \leq 0.05$.

1,8-cineole, geraniol, and thymol treatments. The rest of the studied monoterpenes showed lower sitosterol values than those of control. This is shown especially with menthol treatment, which produced the small value of 8.19% (Table 3).

Effect of Monoterpenes on Composition of Sterols of Sterol Esters. The main sterol in the SE maize root fraction observed in all treatments and controls was sitosterol (43.5–70.8%; Table 4), which is in accordance with the levels expected for this sterol in maize roots (Kemp et al., 1967). Sitosterol increased with time, reaching a maximum level at 48 hr in the control experiment, as well as in the 1,8-cineole, thymol, and menthol treatments. It decreased significantly

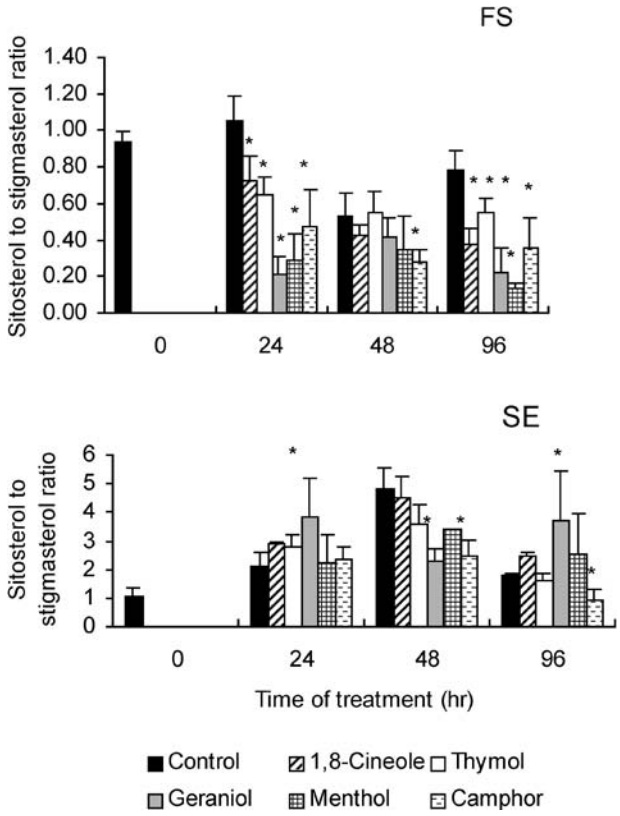

FIG. 2. Effect of monoterpenes on sitosterol to stigmasterol ratio of free sterol (FS) and steryl ester (SE) fractions in maize roots. Values are means and SD. *Denotes significant differences between treatment and control for each time of treatment, according to DGC multiple range test at $P \leq 0.05$ ($n = 3$).

in only the thymol and camphor treatments to levels of 45.99% and 34.94%, respectively, at 96 hr (Table 4). A different result was observed with geraniol treatments. It showed maximum values at 24 and 96 hr (64.32% and 66.14%, respectively) that were higher than controls.

Meanwhile, stigmasterol decreased from 0 to 96 hr in controls, and with all monoterpene treatments showed no significant differences. Only with camphor did this sterol increase at 96 hr—similarly to 0 hr (Table 4).

Geraniol treatment increased campesterol at 48 hr and decreased later (Table 4).

Effect of Monoterpenes on Sitosterol to Stigmasterol Ratio. The SE fraction presented a higher sitosterol to stigmasterol ratio (values >1) than that of FS fraction (values <1) (Figure 2), which is in agreement with previously

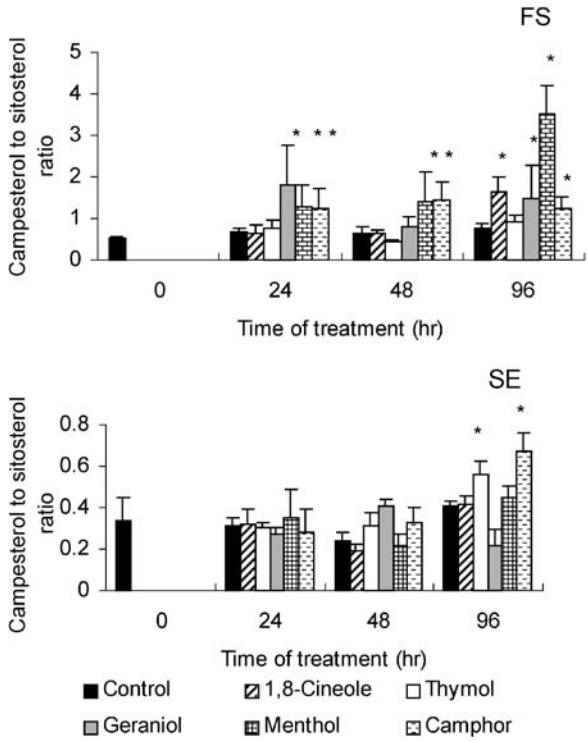

FIG. 3. Effect of monoterpenes on campesterol to sitosterol ratio of free sterol (FS) and steryl ester (SE) fractions in maize roots. Values are means and SD. *Denotes significant differences between treatment and control for each time of treatment, according to DGC multiple range test at $P \leq 0.05$ ($n = 3$).

published data and documents the discriminating character of sterol esterification in plants (Kemp et al., 1967, 1968; Dyas and Goad, 1993). The sitosterol to stigmasterol ratio was more affected by monoterpene treatments in the FS than in the SE fraction. In the FS fraction, this ratio decreased in the studied monoterpenes, mainly at 24 and 96 hr due to an increase in stigmasterol and decrease in sitosterol percentages (Figure 2). In the SE fraction, monoterpenes produced a different result, only geraniol and camphor treatments showing significant differences from controls (Figure 2).

Effect of Monoterpenes on Campesterol to Sitosterol Ratios. The FS fraction produced a higher campesterol to sitosterol ratio than that of the SE fraction (values <1) (Figure 3). Only thymol and camphor treatments increased this sterol ratio in the SE fraction at 96 hr, whereas in the FS fraction it was increased mainly by geraniol, menthol, and camphor. These terpenes increased by about 166%, 92%, and 84%, respectively, at 24 hr. Menthol treatment at 96 hr caused a 351% increase in that sterol ratio (Figure 3).

DISCUSSION

The growth of seedlings in darkness could explain the low content shown in the total PLFA fraction of the untreated control roots (Figure 1) because lipid biosynthesis slows in the dark (Somerville and Browse, 1991). Moreover, plants growing in prolonged darkness reactivate the β-oxidation cycle necessary for carbohydrate synthesis from fatty acids (Poirier et al., 1999). The fluctuation observed in control roots, in which the total PLFA content decreased at 24 hr and then increased to almost the original level at 48 hr (Figure 1), could be explained by the stress that the plant suffers when transferred to the bioassay.

Monoterpenes, which are lipophilic compounds, could alter the packing, fluidity, and/or physical arrangement of phospholipids in the membrane (García et al., 1995; Sikkema et al., 1995; Perillo et al., 1999; Turina and Perillo, 2003). Plants treated with monoterpenes showed an increase in the unsaturated to saturated PLFA ratio at 96 hr, with the exception of 1,8-cineole. This increase is mainly due to the high percentage of FA 18:2 and reduction of FA 18:0 (Table 1). Variations in PLFA unsaturation may produce drastic effects on physical and functional membrane properties, and membrane fluidity may increase (Stubbs and Smith, 1984; Karp, 1987; Borst et al., 2000).

Monoterpenes increased the total unsaturated FA of maize root (Zunino and Zygadlo, 2004). In contrast, saturated FAs of the SEFA fraction were increased at 96 hr, mainly in the menthol, geraniol, and camphor treatments (Table 2). This increase might be related to the specificity and increased activity of the acyltransferase enzyme, which esterifies sterols (Dyas and Goad, 1993).

Most of the higher plant sterols are found as FS, which serve as membrane components and reside predominantly in the plasma membranes (Hartmann and Benveniste, 1987; Schaller, 2003), whereas the greatest amount of SE in *Z. mays* roots are located in mitochondrial and microsomal fractions and in the nucleus (Dyas and Goad, 1993). The SE could operate as a supply of FS, as a transport form of sterols in terms of both intracellular movement and movement between tissues, and as a stored form in which sterols present in amounts greater than immediately needed by the plant are sequestered. There is a freely interconvertible pool of FS and SE (Dyas and Goad, 1993; Gondet et al., 1994).

In both the FS and the SE fractions, there was a high negative correlation between sitosterol and stigmasterol in time (see values in Tables 3 and 4). This is indicative of the biosynthetic relationship of these sterols (Kemp et al., 1967; Benveniste, 1986; Izzo and Navari-Izzo, 1993). The major changes observed in the sterol fraction corresponded to the FS fraction, which produced a decrease in the sitosterol to stigmasterol ratio (Figure 2) and an increase in the campesterol to sitosterol ratio (Figure 3). These changes were shown principally with geraniol, menthol, and camphor monoterpenes.

From the "membrane environment" point of view, an appropriate composition of sterols in the cell membranes is crucial for optimal enzymatic activity, ion and metabolite transport or channeling, protein–protein and protein–lipid interactions, signal transduction, and finally to face fluctuating environmental conditions (Schaller, 2003). It is known that membrane fluidity and permeability is regulated by the relative proportion of lipids, as well as by the length and the unsaturation of fatty acids, and that sterols can also participate in the control of membrane-associated metabolic processes (Ros et al., 1990; Schuler et al., 1991; Hartmann, 1998; Hellgren and Sandelius, 2001). Our results indicate that the treatment of maize roots with monoterpenes induces a modification of the sterol proportions, mainly in the FS fraction, and a change in the unsaturation of fatty acids. So it can be suggested that alcoholic monoterpenes (geraniol and menthol) together with camphor would be the most important monoterpenes affecting membrane permeability and fluidity according to the lipid changes shown, i.e., increased percentages of unsaturated PLFA at 96 hr (Table 1) together with an increase on stigmasterol of FS fraction (Table 3).

It has been shown that during senescence the stigmasterol to other sterols ratio increases (McKersie et al., 1978; Lees and Thompson, 1980), and several environmental stresses alter the FS composition as well (Hellgren et al., 2001). Equally, the sitosterol to stigmasterol and/or the stigmasterol to campesterol ratios are modified (Navari-Izzo et al., 1989; Mansour et al., 1994; Hellgren et al., 2001). The central role of campesterol as a precursor of the plant-growth regulators, called brassinosteroids, has been established (Yokota, 1997; Schaller, 2003). The ratio of campesterol to sitosterol is associated with growth and development modifications and with the activity of the enzyme sterol

methyltransferase 2 (SMT2) (Schaeffer et al., 2001). The campesterol to sitosterol ratio in our FS fraction of menthol, geraniol, and camphor treatments increased two times, mainly at 24 and 96 hr (note that this ratio in menthol treatment increased 4.5 times compared to controls) (Figure 3). Thus, the activity of the enzyme SMT2 might be reduced with the above-mentioned treatments. Additionally, the synthesis of 24-ethyl-sterols (sitosterol and stigmasterol) is kinetically favored over 24-methyl-sterols (campesterol) at some stages of corn development (Guo et al., 1995), and, thus, the first sterols are the principal ones. At 96 hr, the campesterol percentage was higher than that of sitosterol with every monoterpene studied, except thymol (Table 3). It may be that the reduced activity of SMT2 is favorable to the seedlings to support some physiological concentration of campesterol.

In summary, from the results obtained, it appears that every monoterpene affects the maize root lipid composition in a different way.

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CHANGED ISOFLAVONE LEVELS IN RED CLOVER (*Trifolium pratense* L.) LEAVES WITH DISTURBED ROOT NODULATION IN RESPONSE TO WATERLOGGING

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Abstract—The effect of disturbed root nodulation on the quantitative and qualitative composition of the main isoflavonoid glucoside–malonates, glucosides, and aglycones in the leaves of *Trifolium pratense* L. grown under waterlogging conditions was investigated. Isoflavonoids are involved in the regulation of root nodule activity and the establishment of the mycorrhizal association. Isoflavonoid determination was performed using reversed-phase liquid chromatography coupled to mass spectrometric and UV absorbance detection. In response to waterlogging, the concentrations of biochanin A and biochanin A–7-*O*-glucoside–malonate, biochanin A–7-*O*-glucoside, and genistein–7-*O*-glucoside in the leaves increased two- to threefold after a lag period of 3 wk because of disturbed root nodulation. The other isoflavones detected—formononetin, formononetin–7-*O*-glucoside–malonate, and formononetin–7-*O*-glucoside—did not show any significant changes related to waterlogging. After restoring normal soil water conditions, the concentrations of biochanin A and its glucoside and glucoside–malonate rapidly returned to the initial values, whereas the concentration of genistein–7-*O*-glucoside remained high.

Key Words—*Trifolium pratense* L., glucoside–malonates, isoflavones, Leguminosae, mass spectrometry, red clover, nodulation, waterlogging.

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INTRODUCTION

Because of their importance for the conservation of biodiversity, the restoration of wetlands is a goal in many countries worldwide. Restoration activities include changes in grassland management and rewetting dry (polder) areas and can have great impact on ecological processes, especially affecting those plant species that are sensitive to waterlogging and/or flooding. Flood-resistant plants can easily acclimatize to wet conditions, but in nonflood-resistant species, flooding may cause metabolic problems because of oxygen deficiency and enhanced availability of reduced metals (Lupwayi et al., 1997).

Red clover (*Trifolium pratense*, Leguminosae) is a nonflood-resistant species that commonly occurs in moist grasslands. Trifolium species are known symbiotic nitrogen-fixating crops that are used in sustainable agriculture to restore N fertility of a soil (Warembourg et al., 1997; Kumar and Goh, 2000). In symbiotic plant–rhizobium interactions, flavonoids play a role as signal transducing compounds. The isoflavonoids, for example, are involved as nodulation inducers (Edwards et al., 1997). In a variety of leguminous plants, they can influence the early stages of the establishment of biological nitrogen fixation in root nodules after infection by *Rhizobium* bacteria (Paiva, 2000).

In *T. pratense*, over 40 flavonoids have been identified (Klejdus et al., 2001; de Rijke et al., 2001, 2004a). They constitute one of the most important groups of natural products found in plants—in total, more than 6000 flavonoids have been identified and documented (Harborne and Baxter, 1999). In plants, they are usually present as glycosides, i.e., provided with one or more sugar groups such as glucose, galactose, or rhamnose. The sugars are often further substituted by acyl residues, such as malonate, acetate, 4-coumarate, caffeate, or ferulate (Stumpf and Conn, 1981). Glycosylated compounds, rather than free aglycones, are accumulated in plants; they are better stored within plant vacuoles and are less reactive towards other cellular components than aglycones. They are often thought of as detoxification products or as physiologically inactive plant storage forms (Stumpf and Conn, 1981). From a physiological point of view, flavonoids are of interest because of their various biological activities: as screens against elevated UV-B radiation (Rozema et al., 1997; Olsson et al., 1998; Logemann et al., 2000), infection resistance to microorganisms (Bednarek et al., 2003), protection from herbivore attack (Wang et al., 1999), and adaptation to water stress (Francis and Devitt, 1969; Stafford and Ibrahim, 1992). Flavonoids—and isoflavonoids in particular—are also involved in the induction of the expression of *Rhizobium* nodulation genes in leguminous plants (Smit et al., 1992; Gagnon and Ibrahim, 1997).

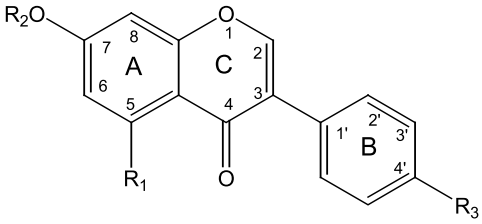
The aim of this study was to investigate the effect of disturbed root nodulation on the concentrations of the main isoflavones of *T. pratense*, including aglycones, glucosides, and glucoside–malonates. To that means *T. pratense*

plants were subjected to two different soil water regimes to study the effect of disturbed root nodulation in response to waterlogging on the levels of the main isoflavonoids (for structures and abbreviations, see Table 1). The study focuses on isoflavonoids in the leaves because isoflavonoids are known to respond to environmental disturbances (Edwards et al., 1997), and the leaves of *T. pratense* have the highest concentrations. In roots, the flavonoid concentrations are lower, and less root mass is available for analysis. To study isoflavonoid profiles and their changes due to environmental effects, a sensitive method is needed. Therefore, a previously reported reversed-phase liquid chromatographic (RPLC) procedure was used, coupled to negative atmospheric pressure chemical ionization mass spectrometry (NI APCI-MS) and UV absorbance detection (de Rijke et al., 2001, 2003).

METHODS AND MATERIALS

Flooding Studies. Flooding experiments were performed using *T. pratense* plants grown from commercially obtained seeds (Duchefa, Haarlem, The Netherlands). Seedlings were grown in a greenhouse for 4 mo. Plants were grown in a controlled-environment greenhouse maintained at 25/20°C day/night temperatures and 60–70% relative humidity. Light conditions were those of the

TABLE 1. STRUCTURES AND ACRONYMS OF THE ISOFLAVONE AGLYCONES, GLUCOSIDES AND GLUCOSIDE–MALONATES STUDIED



Isoflavone	Abbrev.	R_1	R_2	R_3	MW
Formononetin	F	H	H	OCH_3	268
Ononin	FG	H	7-*O*-β-D-Glc	OCH_3	430
Formononetin–7-*O*-glucoside–malonate	FGM	H	7-*O*-β-D-Glc−Mal	OCH_3	516
Genistein	GG	OH	7-*O*-β-D-Glc	H	432
Biochanin A	B	OH	H	OCH_3	284
Sissotrin	BG	OH	7-*O*-β-D-Glc	OCH_3	446
Biochanin A–7-*O*-glucoside–malonate	BGM	OH	7-*O*-β-D-Glc−Mal	OCH_3	532

natural glass-filtered (UV B) radiation in the period from 1 May to 24 June. Plants were approximately 25 cm high, with about 120 leaves per plant and an area of approximately 3 cm^2 per leaf. During the experiments, they were in the flowering stage. Plants were placed in PVC cylinders filled with potting compost containing 22 wt.% organic material (Jongkind, Aalsmeer, The Netherlands) and a wire mesh bottom. The cylinders were placed in plastic pots to enable soil water management. As sampling and extraction can affect flavonoid concentrations (de Rijke et al., 2001), sampling was considered destructive. Therefore, 10 separate *T. pratense* plants were placed in each pot. At each sampling date, fresh plants were sampled. Plants were removed from the experiment after sampling. Because all plants in one pot were subjected to the same environmental conditions, they are considered as one block that was sampled repeatedly over time.

The experimental setup followed a single-factor ANOVA design with two levels, waterlogged (W) and normal (N) conditions, and seven replicates per level. At t = 1 day, the soil water level of the W plants was raised to ensure complete waterlogging of the soil, i.e., water reached the soil surface. Throughout the study, the N plants were watered daily to field capacity. Plants were sampled at t = 0, 7, 14, 21, 35, and 42 days, and the flavonoid composition of their leaves was determined as described below. At t = 35 days, the soil water level of the W plants was changed to the level of the N plants and kept at this level until the end of the study.

Materials. Biochanin A and biochanin A–7-*O*-glucoside were purchased from Indofine (Somerville, NJ, USA), and genistein–7-*O*-glucoside, formononetin, and formononetin–7-*O*-glucoside from Roth (Karlsruhe, Germany). Methanol and formic acid were from J.T. Baker (Deventer, The Netherlands), and ammonium formate and white quartz sand were from Aldrich (Steinheim, Germany). Tris(hydroxymethyl)aminomethane (TRIS) was purchased from Merck (Darmstadt, Germany). Ultrapure water was prepared with a Millipore-Academic system (Etten-Leur, The Netherlands).

Extraction. Extraction of *T. pratense* leaves was performed as described earlier (de Rijke et al., 2003). Fresh leaves (1 g) were ground with 3 ml of aqueous 350 mM TRIS solution, pH 7.9, for 2 min. One gram of white quartz sand and 10 ml of methanol–water (9:1, v/v) were added, and the extract was stirred for 1 min. The extract was filtered over a Büchner funnel, and 10 ml of methanol–water (9:1, v/v) were added to the residue. The procedure was repeated once, and the combined extracts were filtered over a 0.45-μm filter and stored at −20°C until use.

Care must be taken during the extraction procedure, as the isoflavone–glycoside–malonates are readily converted to their corresponding glycosides and aglycones when the plant material is crushed (e.g., Saloniemi et al., 1995; Edwards et al., 1997). This is caused by high enzyme activity (β-glucosidase) in

the plant parts; because they are not substrate specific, hydrolysis easily occurs. In our study, TRIS solution, which functions as an inhibitor of these enzymes (Larner and Gillespie, 1956; Dale et al., 1985), was added to prevent this hydrolysis. In a previous paper (de Rijke et al., 2001), the suitability of this approach was demonstrated: we tested the stability of the isoflavone–glucoside–malonates in the extract under the extraction conditions described above; at room temperature, conversion of glucoside–malonates to their corresponding glucosides was only observed upon prolonged storage (ca. 8% after 1 wk of storage). In the current study, the extracts were prepared in a few minutes, directly stored at −20°C (which slows down the hydrolysis even further), and analyzed within a day; under these conditions, no measurable loss of the glucoside–malonates occurs.

Instrumentation. LC–DAD UV. Liquid chromatography was performed on an HP 1050 LC system (Hewlett Packard, Waldbronn, Germany) with a photodiode array UV detector (DAD) set at 250 nm. For separation, a 250 × 4.6 mm I.D. 5 μm Zorbax SB-C18 LC column was used, with a methanol–10 mM ammonium formate buffer (pH 4.0) gradient at a flow rate of 1.0 ml/min (see (de Rijke et al., 2003)). Before use, all solvents were filtered over a 0.45-μm filter and degassed with helium. Sample injection (10 μl) was performed with a Midas autosampler (Spark Instruments, Emmen, The Netherlands).

LC–APCI-MS. For LC–MS, a Shimadzu (Princeton, NJ, USA) LC system was used consisting of two LC-10A pumps, a SCL 10A controller unit, a DGU-14A degasser, a SIL-10AD autoinjector, and a SPD-10A UV detector (set at 250 nm). The autosampler was cooled to prevent hydrolysis of flavonoid glucoside–malonates in the extracts. The setup was coupled to a Thermo Quest Finnigan (San Jose, CA, USA) LCQ deca ion trap mass spectrometer. The injection volume, column, and gradients were the same as in LC–DAD UV. Atmospheric pressure chemical ionization spectra were obtained in the negative ionization mode in the *m*/*z* 150–650 range. Capillary and vaporizer temperatures were 250 and 450°C, respectively. Capillary voltage was set at −12 V, and the corona discharge current was 10 μA (see (de Rijke et al., 2003)). The sheath-gas (nitrogen) flow rate was approximately 200 l/hr, and the auxiliary gas (nitrogen) flow rate was approximately 50 l/hr. The LC flow was directed to the mass spectrometer without stream splitting.

Statistical Data Analysis. A single-factor repeated-measures ANOVA was performed to test for effects of soil water level, time of exposure, and their possible interaction on isoflavone concentrations, i.e., to test whether soil water level and time of exposure are independent parameters. Data requirements for the multivariate analysis concerning equality of the covariance matrix were checked using Box's *M* test. To test whether isoflavone concentrations decreased to the level of the control after restoring the original soil moisture conditions, isoflavone concentrations of both treatments were compared at

t = 42 days using an independent samples t-test. Data were checked for normality using Shapiro–Wilk's test. All analyses were performed using the program SPSS version 10.1.0.

RESULTS

LC–UV/APCI-MS. Figure 1 shows typical LC–UV and LC–MS chromatograms of an extract of *T. pratense* leaves. The advantage of an ion-trap MS instrument is that full-scan (FS) and time-scheduled selected ion monitoring (TS SIM) can be performed simultaneously. Full scan is used to scan the extracts and TS SIM for quantification of selected (see below) isoflavones. To select the masses to be used in SIM, a 10-mg/l standard mixture of five isoflavone

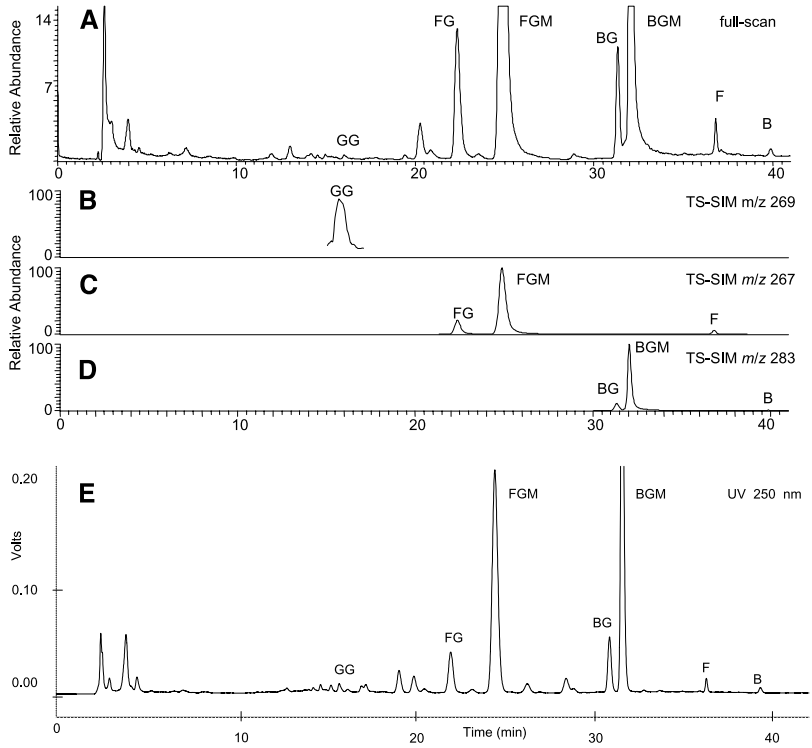


FIG. 1. Reversed-phase LC with **(**A**)** full-scan and **(**B–D**)** TS SIM (*m*/*z* 269 for GG, *m*/*z* 267 for F, FG, and FGM, and *m*/*z* 283 for B, BG, and BGM) NI APCI-MS and **(**E**)** UV_{250} of a *T. pratense* leaf extract, recorded at t = 35 d. For details, see text.

aglycones and glucosides [formononetin–7-*O*-glucoside (FG), biochanin A–7-*O*-glucoside (BG), genistein–7-*O*-glucoside (GG), formononetin (F), and biochanin A (B); cf. Table 1] was analyzed in the FS MS mode (*m*/*z* 150–650). This enabled us to attribute five of the main peaks that show up in Figure 1 to FG, BG, GG, F, and B, based on their mass spectra and retention times. In addition, the strong peaks at 25 and 32 min can be attributed to formononetin–7-*O*-glucoside–malonate (FGM) and biochanin A–7-*O*-glucoside–malonate (BGM), respectively (de Rijke et al., 2003). Compounds were identified based on their aglycone mass and their characteristic fragmentation pattern, using TS SIM (Figure 1B–D) and FS chromatograms (Figure 1A), respectively. The main ions that were generated in NI APCI-MS were the pseudomolecular ions $[M–H]^-$, formic acid adducts $[M + 45]^-$, $[M–Glc]^-$, and, when the analytes were glucoside–malonates, $[M–Mal]^-$ and $[M–Glc–Mal]^-$ ions. In all cases, the aglycone ions were selected for TS SIM because they had the highest intensities with the MS settings used. In an independent study, the structures of these compounds were confirmed by means of LC–UV–NMR and stand-alone COESY and NOESY NMR experiments of LC-separated fractions (de Rijke et al., 2004b).

In a previous paper (de Rijke et al., 2003), four ionization modes (PI and NI APCI; PI and NI ESI) were compared using a *T. pratense* extract and optimized LC conditions. In the PI modes, background signals were higher than in the NI modes. Overall, the analyte responses were best in the NI APCI modes. In the mass spectra, differences in relative intensities of the fragment ions were observed depending on the ionization mode and eluent composition.

Analyte detectability was the same for the simultaneous FS–TS SIM method and the conventional TS SIM method, whereas the gain in sensitivity of TS SIM compared with FS was about 100-fold. Detection limits (LODs; $S/N = 3$) in the TS SIM mode were found to be 1–45 μg/l (R^2 values of calibration plots, 0.993–0.999 in all cases). Because no standards of FGM and BGM—the malonates of FG and BG—are available, their concentrations were calculated based on the calibration curves of FG and BG, assuming equal MS response of the aglycone ions in SIM. This was confirmed on the basis of LC–UV standard curves; the molar extinction coefficients of the glucosides and the glucoside–malonates are identical (de Rijke et al., 2004a). With UV detection, LODs were higher than in TS SIM MS (40–530 μg/l; R^2 values 0.998–0.999); for that reason, TS SIM MS was used to identify and quantify the isoflavone aglycone, glucoside, and acylated glucoside peaks in the extracts. The simultaneous FS and TS SIM method was used throughout the study.

Flooding Studies. Leaves and roots of the two series of full-grown plants (4 mo-old) under N and W conditions were compared during the experiment. After 3 wk, the areas of the leaves of the W plants were slightly smaller (ca. 2 cm^2) than leaves of the N plants (ca. 3 cm^2), and some leaves of the W plants

had turned yellow. We also observed that the roots of W plants were gradually reaching the soil surface, and that the amount of root nodules on the submerged roots of W plants was decreasing over the course of the study. These qualitative observations are summarized in Table 2. After 35 d, an approximately twofold decrease was observed. At t = 42 d, the root nodule level was approximately restored to its initial value. N plants did not show any changes.

Concentrations of the seven isoflavone aglycones, glucosides, and acylated glucosides during the experiment are displayed in Figure 2. As expected, at t = 0 d, the concentrations of all isoflavones in the N and W plants were the same and, therefore, the two groups were pooled together (N = 14). Concentrations of all isoflavones varied in a compound-specific way. For F, FG, and FGM, the changes were essentially independent of water level conditions, with a constant increase in FG during the experimental period. For B, BG, BGM, and GG, a significant increase of the concentrations was observed. Biochanin A–7-*O*-glucoside and GG remained virtually constant up to 21 d, then, between 21 and 35 d, they increased two- to threefold in the W plants. In contrast, B and BGM differences occurred already after 10 d. The data set was evaluated with repeated-measures ANOVA. The results are shown in Table 3. The level of significance is displayed for the effects of soil water level, time of exposure, and their interaction. Soil water level significantly affects B, BG, BGM, and GG concentrations over the period up to 35 d. Time significantly affects BG, BGM, FG, and FGM concentrations, whereas for B and GG, a minor effect is observed. The effects of soil water level hardly interact with effects of time. In other words, a change of soil water level does not change the response over time and vice versa. A low significance tells us that most variation in the data is accounted for by the separate effects of soil water level and time.

At t = 35 d, normal soil water conditions were restored for the W plants, and at t = 42 d, the concentrations of all isoflavone aglycones, glucosides, and

TABLE 2. ROOT NODULE LEVEL OF *T. pratense* ROOTS AFTER NUMBER OF DAYS EXPOSED TO WATERLOGGING

Time (days)	Root nodule level
0	++
7	+
14	+
21	−
35	−−
42	++

The root nodule level of the roots of the *N* plants remained ++ during the course of the study.

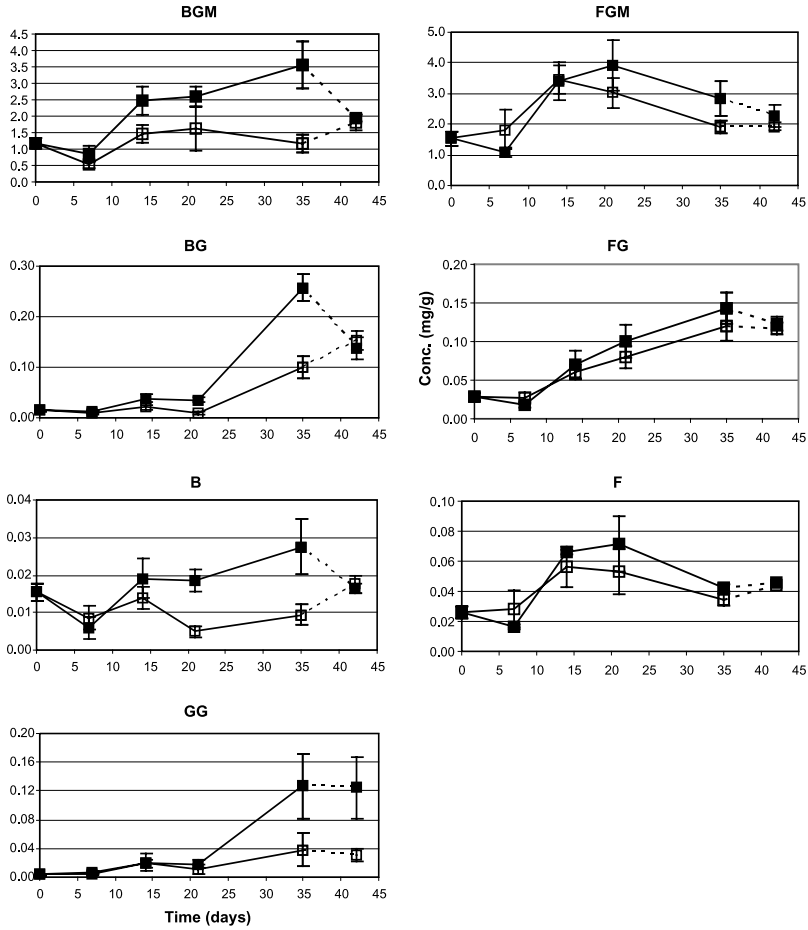


FIG. 2. Effects of waterlogging (□ = normal; ■ = waterlogged) over time on the concentrations (mg/g fresh wt) of seven isoflavone aglycones, glucosides, and glucoside–malonates in *T. pratense* leaves in LC−TS SIM MS, corrected for weight of leaves. Mean values and standard deviations of the mean are given ($N = 7$; 0 d, $N = 14$). Soil water levels were raised at $t = 1$ d and restored to normal at $t = 35$ d.

glucoside–malonates except GG had returned to values equal to those for the N plants (cf. Figure 2). In all cases, except for FG and BG, these values were equal to the values of $t = 0$ d, and even for these two analytes, the differences were not large. The results of the independent samples *t*-test to check whether isoflavone concentrations decreased to the level of the control treatment after restoring the original soil moisture conditions confirm this. There was a significant difference

TABLE 3. LEVEL OF SIGNIFICANCE FOR THE EFFECTS OF SOIL WATER LEVEL, TIME OF EXPOSURE, AND INTERACTION

Flavonoid	Soil water level	Time	Interaction
B	**	*	*
BG	**	***	n.s.
BGM	***	***	n.s.
GG	***	*	*
F	n.s.	**	n.s.
FG	n.s.	***	n.s.
FGM	n.s.	***	n.s.

n.s.: Not significant.
*$P \leq 0.05$.
**$P \leq 0.01$.
***$P \leq 0.001$.

between the W and N plants only for GG, which means that at t = 42 d, the effect of waterlogging on the concentration of GG continued. For B, BG, and BGM, there was no significant difference at t = 42 d between the W and N plants: the concentrations of the W plants decreased to the level of the N plants.

DISCUSSION

The observed changes in the roots and color of the leaves between W and N plants indicate that the W plants suffered from oxygen shortage. Nonflood-resistant plants—such as *T. pratense*—under water stress can avoid oxygen shortage, either by having their lateral roots close to better oxygenated soil surfaces or by developing superficial adventitious roots in response to flooding. Flood-resistant plants can tolerate the stress because they have morphological and anatomical features, e.g., aerenchyma, which allow internal oxygen transport to satisfy the needs of root respiration and also the oxidation of chemically reduced toxins (Etherington, 1983; Gibberd et al., 2001).

The increase in isoflavone levels in *T. pratense* under waterlogged conditions is comparable to the behavior of three Australian clover species (Francis and Devitt, 1969). Because the processes in roots such as *Rhizobium* infection and formation of root nodules are disturbed in response to waterlogging, it is likely that the isoflavones that are usually involved in the nodulation process and in the arbuscular mycorrhizal symbiosis accumulate in the leaves of the plant. Earlier studies with *Trifolium semipilosum* (Lupwayi et al., 1997) and *T. repens* (Pugh et al., 1995) also show that processes in the roots are affected under waterlogged conditions. Flavonoids are involved in response mechanisms to stress conditions in plants (Etherington, 1983; Stafford

and Ibrahim, 1992; Reuber et al., 1996; Strack, 1997; Rozema et al., 1997; Olsson et al., 1998; Wang et al., 1999) and in biological interactions of clover species with microorganisms. In the symbiosis of clover with arbuscular mycorrhizal fungi, flavonoids can stimulate spore germination and hyphal growth (Chen et al., 2001; Fracchia et al., 2003). Bacterial nodulation (*nod*) genes are essential for infection of the host root and the establishment of the nodule. The expression of these genes requires the presence of flavonoids as signal compounds, released from the host plant roots, and the *nodD* regulatory protein (Rossen et al., 1987). Several authors report the involvement of flavonoids in the induction of the expression of *Rhizobium* nodulation genes from leguminous plants, e.g., *T. pratense* (Janczarek et al., 1997), *Galega orientalis* (Suominen et al., 2003), and *Lupinus albus* (Gagnon and Ibrahim, 1997).

Edwards et al. (1997) studied the effect of nodulation on isoflavonoid concentration in red clover. Red clover plants that were inoculated with *Rhizobium trifolii* were compared with noninoculated plants. In the leaves of noninoculated plants, the concentrations of BGM and FGM increased with age, and the accumulation of BGM in the leaves was suppressed by nodulation, whereas FGM remained constant. In their study, a 3-wk lag period was observed. These observations are in accordance with our findings: because of waterlogging, nodulation decreases, and this coincides with accumulation of BGM, BG, B, and GG, whereas the concentrations of FGM, FG, and F remain virtually unaffected. The observed changes of isoflavonoid accumulation are most likely caused by signals from the roots that are indicative of the extent of root nodulation.

After restoration of normal soil water conditions, oxidation processes are stimulated, and processes in the root zone such as mycorrhiza infection and root nodule formation are restored. As Figure 2 suggests, this regeneration process is much faster than the initial response of the isoflavone aglycones, glucosides, and glucoside–malonates, viz., about 1 vs. over 3 wk. After 1 wk, the concentrations of all isoflavones except GG returned to the values found for the N plants. For GG, the concentration remained higher: there was no decrease after restoring the normal soil water conditions. An explanation for this may be that GG is biosynthetically the simplest of the isoflavonoids and, therefore, a key intermediate in the biosynthesis of more complex isoflavonoids (Dixon and Ferreira, 2002). Whereas the other isoflavones may be used to restore the nodulation processes in the roots, the production of G and GG may remain high for the formation of other isoflavonoids such as coumestans and pterocarpans.

As regards the much faster recovery of oxygenation of the soil when restored to normal conditions compared with the change because of waterlogging, it takes some time before all the oxygen is removed from the soil (Pugh et al., 1995). Apparently, after 3 wk, the soil is completely anaerobic, and processes in the roots and in the rhizosphere are being disturbed. When the soil

water level is restored to normal, the diffusion of oxygen into the soil will be relatively fast, and the processes in the roots are restored within 1 wk. A rapid recovery of rhizobial nitrogen fixation was found earlier in soybean (Sung, 1993). The activity of rhizosphere microorganisms may strongly increase as well (Ozan et al., 1997).

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INCREASED SULFUR PRECURSORS AND VOLATILES PRODUCTION BY THE LEEK *Allium porrum* IN RESPONSE TO SPECIALIST INSECT ATTACK

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Abstract—To defend themselves against herbivory, plants use a variety of direct and indirect strategies involving induced increases in secondary substances. Species of the *Allium* genus (Alliaceae), such as the leek *Allium porrum* (L.), produce nonprotein sulfur amino acids derived from cysteine, i.e., alk(en)yl-cysteine sulfoxides that are precursors of volatile thiosulfinates and disulfides. These defend most species including the specialist leek moth, *Acrolepiopsis assectella*. We determined by measuring the increase in the sulfur precursor propyl-cysteine sulfoxide (PCSO) if production of this precursor is induced in response to moth attack and mechanical wounding. The concentration of PCSO was determined by HPLC in 2- or 6-mo-old leeks after attacks of various intensity either by the specialist leek moth or by a generalist moth, *Agrotis ipsilon*. Injury-induced release of sulfur volatiles was measured by GC/MS after the attacks. Results showed an increase in the production of sulfur compounds in both the precursor and volatile form, occurring only in association with intensive attacks by leek moths. The increase in sulfur precursors also led to an increase in the release of sulfur volatiles. This induced response may provide an effective defense strategy against the plant's main natural enemy, both directly and indirectly by attracting entomophagous insects.

Key Words—*Acrolepiopsis assectella*, *Allium porrum*, constitutive defenses, induced defenses, sulfur compounds, disulfides, thiosulfinates, propyl-cysteine sulfoxide, specialist insect, tritrophic interactions.

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INTRODUCTION

Through evolution, plants have developed various defense strategies against pathogens or herbivores. Most are based on production of defensive chemicals (Karban and Baldwin, 1997) with possible toxic or repellent effects on insects (Fraenkel, 1959). In some cases, production is constitutive and occurs independently of injury to the plant. However, an abiotic or biotic wounding such as a herbivore damage often triggers increased defensive chemical production (Karban and Baldwin, 1997; Constabel, 1999). Injury can also modify secondary metabolic processes resulting in increased production of nonvolatile toxic compounds such as nicotine in tobacco plants (Baldwin, 1988a) or glucosinolates in crucifers (Agrawal and Kurashige, 2003). These strategies can be classified as induced direct defense systems, and can lead to increased release of volatile substances such as terpenes in corn (Turlings et al., 1990) that attract entomophagous insects (Turlings et al., 1995; Paré and Tumlinson, 1999). Although direct and indirect induced defense systems have been observed in numerous species, it is not possible to conclude that they occur in all plants.

Plants of the *Allium* genus (Alliaceae), such as the leek *Allium porrum* (L.), produce nonprotein sulfur amino acids derived from cystein, i.e., alk(en)yl-cysteine sulfoxides (Virtanen, 1965; Block, 1992). These amino acids are considered precursors of sulfur volatiles because their contact with the enzyme alliinase produced after the rupture of plant tissue cells leads to the formation of sulfur volatiles mainly in the form of thiosulfinates that subsequently break down and rearrange into disulfides and trisulfides (Auger et al., 1989; Jaillais et al., 1999). This complex involving alk(en)yl-cystein sulfoxides–alliinase–thiosulfinates and di- or trisulfides is probably involved in the plant's defense strategy. Tests in confined spaces have demonstrated that these products are highly toxic for various species (Auger and Thibout, 2002). One, dimethyl disulfide, interferes with the cytochrome oxidase mitochondrial complex involved in the production of ATP, thus causing a loss of neuronal activity and death in some species (Dugravot et al., 2003). Moreover, tests in natural settings on crucifers (Chew, 1988) have shown that sulfur compounds can act as chemical repellents against herbivores (Auger et al., 1989).

In leeks, constitutive concentrations of sulfur precursors appear to provide an effective defense against nonspecialized insects. The main pest for field-produced leeks is the specialist leek moth *Acrolepiopsis assectella* (Zeller) (Lepidoptera: Acrolepiidae) (Arnault et al., 1986), which is particularly well adapted to the organosulfur chemistry of the host plant (Dugravot et al., 2004). The biosynthesis of secondary products in leek may change when damaged. To ascertain this possibility, the response induced in leeks damaged by the specialist herbivore moth was characterized by measuring the concentration of

sulfur compounds in both the nonvolatile precursor form, i.e., mainly propyl-cysteine sulfoxide (PCSO), and the volatile form, i.e., mainly thiosulfinates and disulfides. Response was studied in plants of various ages under several damage conditions inflicted by the moth and compared with responses elicited by mechanical wounding or by damage of the generalist moth *Agrotis ipsilon* (Lepidoptera: Noctuidae).

METHODS AND MATERIALS

Insects. A. assectella and *A. ipsilon* were reared on leek plants in the laboratory with a photoperiod of 16-hr light/8-hr dark at 25°C during the photophase and 16°C during the scotophase with 60–80% relative humidity. The *A. ipsilon* stock was obtained from the UMR Santé Végétale, INRA Bordeaux, France. The *A. assectella* stock was renewed every year in July by collecting caterpillars and chrysalids in leek fields around Tours, France.

Plants. In a first experiment, healthy leeks with no evidence of attack and damaged leeks with visible evidence of attack by leek moth caterpillars were picked in the field of a local grower practicing organic farming near Tours. The intensity of attack and time lapse since its end (more than 2 wk) could not be determined precisely. In other experiments, leek plants (Parlton variety) used were obtained from the Centre Technique Interprofessionel des Fruits et Légumes at Carquefou, France. When 4-months-old, they were transplanted to 10-cm diam pots and maintained in a greenhouse for 6–8 wk. Plants were randomly assigned to control or experimental groups. Most experiments were performed on 6-mo-old leeks, but smaller 2-mo-old leeks were also used to study the effect of plant age on the induced response.

Experimental Setup. All experiments were carried out on whole plants. Leaves or other plant parts had no other injuries than those inflicted for experimental purposes as described. Herbivore attacks were realized by placing third instar leek moth caterpillars on plants in the greenhouse. Caterpillars that escaped or underwent nymphosis were replaced.

The effect of attack intensity was studied by varying the number of caterpillars used and the duration of their presence on the plant: five caterpillars for 8 d, 15 caterpillars for 3 d, and 15 caterpillars for 8 d. Plants submitted to attacks under these three conditions were compared with plants submitted to mechanical wounding and healthy controls.

Mechanical wounding consisted of 10 1-cm incisions on 6-mo-old leek plants twice a day for 8 d on a new leaf everyday. This technique was not intended to reproduce an attack by *A. assectella* but to demonstrate the possible effect of a stress induced by abiotic cell destruction.

The systemic reaction of the leek plants was studied using 6-mo-old plants as follows. Fifteen *A. assectella* caterpillars were confined for 8 d in a transparent, ventilated chamber containing a portion of a green leaf. In this way, caterpillars were forced to consume only the available part of the green leaf. The response of the damaged green leaf was compared with the response on healthy green leaves on the same leek plant and healthy green leaves on control plants.

The specificity of leek plant response to herbivory was determined by comparing the effects of damage by *A. assectella* caterpillars to damage by generalist *A. ipsilon* caterpillars. These experiments were also performed using chambers to confine the caterpillars to certain parts of the plant. Unlike *A. assectella*, *A. ipsilon* is highly mobile with a tendency to consume only leaf tips and then leave the plant. Because of their larger size, five second-stage *A. ipsilon* caterpillars caused as much (or more) damage to the plant as 15 *A. assectella* caterpillars in 8 d.

To study the persistence of the response to damage, results in undamaged control leeks were compared with results in three batches of injured leeks immediately after and 2 or 4 wk after damage by 15 *A. assectella* caterpillars for 8 d. The number of caterpillars used, duration of damage, and number of replications in each experiment are indicated in figures.

Measurement of Sulfur Precursor. The level of PCSO, the main sulfur precursor in leek (Freeman and Whenham, 1975), was measured on a 10-cm segment of the stalk using the Waters Pico-Tag technique (Bidlingmeyer et al., 1984). The sample was weighed and ground in methanol for 40 sec at atmospheric pressure. The resulting mixture was filtered first through Whatman 114V paper and then through 0.45-μm Millex. Ten μl of the resulting extract was used for UV detection of all derived amino acids. Phenylisothiocyanate was used as the derivatization reagent. The HPLC system consisted of a Waters (Milford, MA, USA) chromatograph with a two-pump delivery system (Waters 600E) and a UV detector with the wavelength set at 254 nm (Waters 488). The column was a Waters Novapack C18 (3.9 × 300 mm). The solvent consisted of two Waters eluents, and the flow rate was 1 ml/min. Precise amounts of PCSO synthesized in the laboratory (Auger and Thibout, 1981) were added to allow quantification by comparing retention time and peak areas. Concentrations were expressed in nmol/mg of fresh leek.

Measurement of Sulfur Volatiles. Sulfur volatiles released by damaged leek were studied using a specially designed dual technique (Mondy et al., 2002). In the first step, the leek either undamaged or that had undergone an 8-d attack by 15 caterpillars was placed for 30 min under light conditions in an air-conditioned chamber (26°C, 70% relative humidity). A leaf was placed inside a glass tube (20 cm long × 2.6 cm in diam) closed at both ends using cotton to avoid damaging the plant. The tube had two side openings. Through one side

opening, 10 1-cm incisions were made in the leaf. Through a Teflon membrane placed over the other side opening, a polyacrylate solid phase microextraction (SPME) fiber (85-μm diam; Supelco, Bellefonte, PA, USA) was introduced and used to trap odors released in the headspace for 15 min. The second step used an electric crusher to grind the preceding leeks to compensate for natural degradation of some sulfur volatiles on the SPME fiber (Jaillais et al., 1999; Arnault et al., 2000). The resulting liquid was filtered through a blutter cloth. After a 60-min standing period, a 5-ml specimen was collected, extracted in 1 ml of ether, and centrifuged at 8000 $\times$ *g* for 5 min. The ether phase was passed through a 0.4-μm filter, dried with NaCl, and injected directly into the GC/MS to quantify all volatiles produced by the leek during the crushing process. GC/MS analyses were carried out using a Perkin-Elmer Turbomass benchtop system (Perkin-Elmer, Norwalk, CT, USA) fitted with a split–splitless injector and a fused-silica capillary column (10 m $\times$ 0.32 mm) with a 4-μm methylsilicone coating. Helium (99.999%) was used as carrier gas, and the column temperature rate was programmed for 5°C/min from 70 to 250°C. The injection port temperature was 200°C. Total ion chromatograms and mass spectra were recorded in the electron impact ionization mode at 70 eV. The transfer line and source temperature were maintained at 150°C. Dipropyl disulfide was used as the external calibration standard for quantification. Data processing was performed using the full-screen mode for the SPME fiber and the selected ion recorded (SIR) mode for ether extracts. Compound identification was based on retention time and comparison with spectra of available authentic compounds or with published data (Arnault et al., 2000; Mondy et al., 2002).

Statistical Analysis. Data recorded for sulfur precursors and compounds trapped by the SPME fiber were compared in pairs using the nonparametric Mann–Whitney *U*-test for independent samples ($P < 0.05$). Means of more than two specimens were compared using a nonparametric Kruskal–Wallis one-way analysis of variance ($P < 0.05$) followed if necessary by a classification test (Dunn test). For each chromatographic profile, the relative value of each identified peak with respect to the total was calculated and expressed as a percentage. Peaks of volatiles were analyzed with principal component analysis (PCA).

RESULTS

Evidence of Propyl-Cysteine Sulfoxide Production. Concentration of PCSO under natural conditions was significantly higher in damaged than healthy leeks (Mann–Whitney *U*-test, $P = 0.012$; Figure 1). Concentration was almost three times higher in damaged than in healthy leeks: 0.21 ± 0.09 vs. 0.59 ± 0.15 nmol/mg (means ± SE).

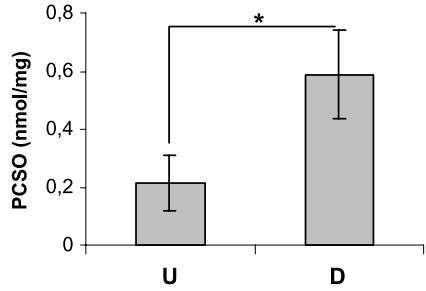

FIG. 1. Mean (±SEM) propyl-cysteine sulfoxide concentration (PCSO) in undamaged and damaged leek *A. porrum* collected in the field. U: undamaged leeks ($N = 6$); D: leeks damaged by *A. assectella* ($N = 6$); *$P < 0.05$ (Mann–Whitney *U*-test).

Mechanical Injury and Intensity of Attack. Cutting the leaves daily did not induce a significant change in PCSO concentration (Figure 2). Only damage by 15 caterpillars for 8 d triggered an increase in PCSO concentration threefold higher than in undamaged leeks (Kruskal–Wallis test, $P < 0.001$; Figure 2). Damages of lower intensity, i.e., by five caterpillars for 8 d or 15 caterpillars for 3 d, caused no significant increase in PCSO concentration (Figure 2).

Plant Age. In 2-mo-old leek plants, damage by three caterpillars for 8 d was sufficient to induce a significant increase in PCSO concentration (Mann–Whitney *U*-test, $P < 0.001$; Figure 3). In this case, PCSO concentration was also three times higher in the stalks of the damaged than undamaged leeks.

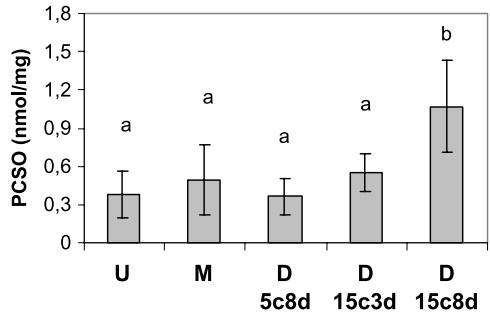

FIG. 2. Mean (±SEM) propyl-cysteine sulfoxide concentration (PCSO) in 6-mo-old undamaged leek *A. porrum* or damaged leek that had undergone mechanical wounds or attacks of various intensity by *A. assectella* caterpillars. U: undamaged ($N = 22$); M: mechanically wounded ($N = 12$); D 5c8d: damaged by five caterpillars for 8 d ($N = 12$); D 15c3d: damaged by 15 caterpillars for 3 d ($N = 12$); D 15c8d: damaged by 15 caterpillars for 8 d ($N = 15$). Equal letters indicate no significant differences among treatments ($P < 0.001$, Kruskal–Wallis test).

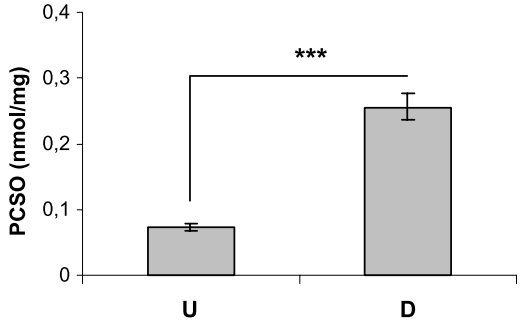

FIG. 3. Mean (±SEM) propyl-cysteine sulfoxide concentration (PCSO) in undamaged and damaged 2-mo-old leek *A. porrum*. U: undamaged ($N = 12$); D: damaged by three *A. assectella* caterpillars for 8 d ($N = 12$). ***$P < 0.005$ (Mann–Whitney *U*-test).

Furthermore, PCSO concentration was nearly four times lower in 2-mo-old leeks than in the older leeks (0.07 ± 0.01 and 0.37 ± 0.18 nmol/mg in healthy leeks vs. 0.26 ± 0.02 and 1.07 ± 0.35 nmol/mg in damaged leeks; Figures 2 and 3). However, low-intensity damage with no effect on 6-mo-old leeks sometimes caused a response in younger, smaller leeks.

Systemic Response. Green leaves from undamaged leek showed significantly lower PCSO concentrations than healthy or wounded leaves from damaged leeks (Figure 4). However, healthy leaves from damaged plants also showed significantly lower PCSO concentrations than leaves directly damaged by caterpillars (Figure 4). The PCSO concentration was 0.09 ± 0.01 nmol/mg in

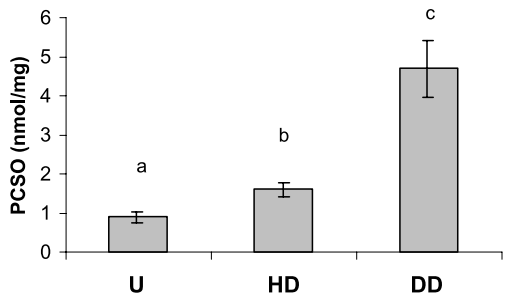

FIG. 4. Mean (±SEM) propyl-cysteine sulfoxide concentration (PCSO) in leaves from undamaged and damaged 6-mo-old leek *A. porrum*. U: healthy leaves from undamaged leeks ($N = 12$); HD: healthy leaves from leeks damaged by 15 *A. assectella* caterpillars for 8 d ($N = 12$); DD: damaged leaves from leeks attacked by 15 *A. assectella* caterpillars for 8 d ($N = 12$). Equal letters indicate no significant differences among treatments ($P < 0.001$, Kruskal–Wallis test).

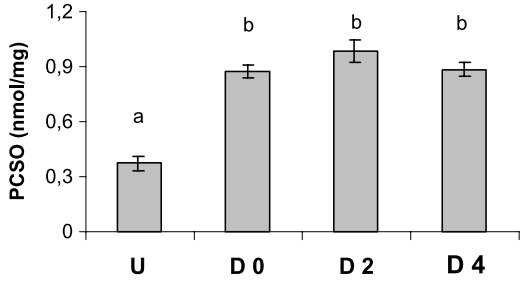

FIG. 5. Mean (±SEM) propyl-cysteine sulfoxide concentration (PCSO) according to the time in undamaged and damaged leek *A. porrum*. U: undamaged (N = 22); D0: immediately after end of attack by *A. assectella* caterpillars (N = 12); D2: 2 wk after end of attack by *A. assectella* caterpillars (N = 12); D4: 4 wk after end of attack by *A. assectella* caterpillars (N = 12). Equal letters indicate no significant differences among treatments ($P < 0.001$, Kruskal–Wallis test).

the leaves from undamaged plants as compared with 0.16 ± 0.02 nmol/mg in healthy leaves from damaged leeks and 0.47 ± 0.07 nmol/mg in the wounded leaves from the same damaged plants (Kruskal–Wallis test, $P < 0.001$).

Persistence of Plant Response. Regardless of the interval between the end of damage and date of measurement, PCSO concentrations were significantly higher in damaged than in undamaged control leeks (Figure 5). The concentration, i.e., 0.37 ± 0.04 nmol/mg in undamaged leeks, rose sharply immediately after the damage and remained between 0.9 and 1.0 nmol/mg for at least 1 mo (Kruskal–Wallis test, $P < 0.001$).

Specificity of Plant Response. Mean PCSO concentration in leeks damaged by *A. ipsilon* caterpillars was 0.28 ± 0.04 nmol/mg of fresh leek (N = 12) and was not significantly different from the mean PCSO concentration in undamaged leeks, 0.38 ± 0.06 nmol/mg of fresh leek (N = 12; Mann–Whitney U-test, $P = 0.064$).

Effects on Release of Sulfur Volatiles. An undamaged 6-mo-old leek gave off no detectable odor under the experimental conditions used (data not shown). In contrast, leeks damaged by *A. assectella* caterpillars or submitted to mechanical wounding released general green leaf volatiles, mainly *trans*-2-hexenal, *cis*-2-hexen-1-ol, and *cis*-3-hexenyl-acetate, as well as sulfur volatiles that broke down mainly into dipropyl disulfide and propyl-propenyl disulfide on the SPME fiber.

The ether extraction technique allowed identification of substances actually produced by leeks during damages prior to their breakdown into disulfides. A total of seven sulfur volatiles were identified: methyl-propyl thiosulfinate (and its isomer propyl-methyl thiosulfinate), methyl-propenyl thiosulfinate, dipropyl

thiosulfinate, propyl-propenyl thiosulfinate, and two *cis* and *trans* isomers of zwiebelanes. The last two compounds resulted from rearrangement of dipropyl thiosulfinate. There was no significant qualitative difference between undamaged and damaged leeks when crushed. Principal component analysis performed on these seven sulfur volatiles showed three principal components with eigenvalues higher than 1, explaining 80.9% of the total variance. On the basis of their proportions of sulfur volatiles, undamaged leeks were clearly distinguished from damaged leeks (Figure 6).

In addition, different degradation compounds of thiosulfinates and zwiebelanes were affected by insect attack. These compounds trapped by SPME were mainly dipropyl disulfide (DPDS) and propyl-propenyl disulfide (PPeDS) (Figure 7). During the formation of disulfides, the propyl moieties came from both the thiosulfinates and the zwiebelanes. After being cut, leek plants that had been damaged 8 d earlier released around twofold more disulfides than leek plants that had not been damaged (Mann–Whitney *U*-test, $P_{\mathrm{DPDS}} = 0.015$; $P_{\mathrm{PPeDS}} = 0.004$; Figure 7).

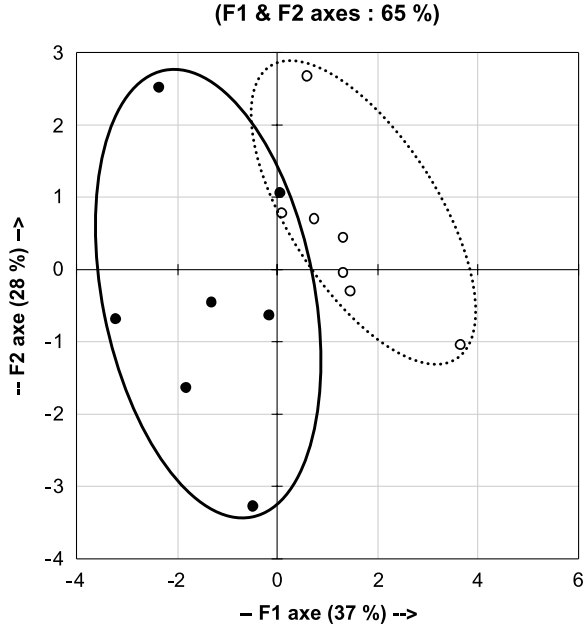

FIG. 6. Principal components analysis (PCA) of all sulfur volatiles released by undamaged leek *A. porrum* (black dots, $N = 7$) and by leek damaged by 15 *Acrolepiopsis assectella* caterpillars for 8 d (white dots, $N = 7$).

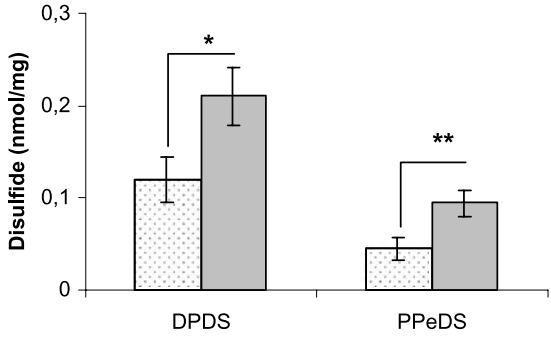

FIG. 7. Mean (±SEM) disulfide concentration released by mechanically wounded leek *A. porrum* that had or had not been previously damaged by 15 *A. assectella* caterpillars for 8 d. Dark bars: undamaged ($N = 7$); light bars: attacked ($N = 7$); DPDS = dipropyl disulfide; PPeDS = propyl propenyl disulfide. *$P < 0.05$; **$P < 0.01$ (Mann–Whitney *U*-test).

DISCUSSION

This study shows that the concentration of PCSO in leek plants increases in response to damage by the herbivore *Allium* specialist *A. assectella*, and that this increase is followed by a characteristic profile of sulfur volatile release.

In preliminary experiments using another liquid chromatography technique (Arnault et al., 2003), the concentrations of three sulfur precursors in leek plants were determined. Results showed that in addition to PCSO, the only precursor studied here, methyl-cysteine sulfoxide and propenyl-cysteine sulfoxide also increased sharply after intensive damage by *A. assectella* caterpillars (Auger and Arnault, personal communication). Without excluding possible repercussions on other biosynthetic pathways, these findings indicate that the concentration of all sulfur precursors in the leek increases in response to damage by *A. assectella* caterpillars.

The induced increase in the production of sulfur precursors depends on several factors including the intensity of attack. Under our study conditions, no response was observed after damages involving a small number of caterpillars or attacks of short duration. Similar findings have been reported in cotton *Gossypium hirsutum* in which the induced response to attack by *Spodoptera littoralis* depends on the extent of damage (Anderson et al., 2001). In *Brassica napus*, the increase in concentration of glucobrassicine (but not the other 13 glucosinolates analyzed) depends on the intensity of the damage inflicted by *Delia floralis* larvae (Hopkins et al., 1998).

The sensitivity of leeks to damage also varies with age. Young 2-mo-old plants respond to damages of lower intensity than 6-mo-old plants. This finding

suggests that production of sulfur precursors occurs only if the stimulus resulting from *A. assectella* caterpillar activity is sufficient. It seems that because of the possible metabolic costs involved, as observed in various plant species (Simms, 1992; Rausher, 1996), and the abundance of constitutive defenses, leeks invest in production of sulfur precursors only in cases of intensive attack. This may be especially true for older leeks that are less sensitive to attack than younger leeks because the same damage intensity from leek moth represents a lower threat in old leeks.

Repeated mechanical wounding did not trigger an induced response. This suggests that herbivore-specific cues may be involved in the leek response to caterpillar damage. Similar findings have been reported in the wild radish *Raphanus raphanistrum* in which response is induced by insect herbivory but not by mechanical wounding (Agrawal, 1998, 1999). Further experiments demonstrated that elicitors in larval oral secretions or feces were responsible for inducing the plant response (Turlings and Tumlinson, 1992; Turlings et al., 1993; Mattiacci et al., 1995; Alborn et al., 1997; Neveu et al., 2002).

The response induced in leek plants by *A. assectella* appears to be systemic. Healthy leaves on damaged leeks have higher PCSO concentrations than healthy leaves on undamaged plants. However, the PCSO concentrations observed in healthy leaves from damaged plants were not as high as those observed in injured leaves. Systemic induction of secondary compounds has been reported in numerous other plants (Green and Ryan, 1972; Baldwin, 1988b; Turlings and Tumlinson, 1992; Potting et al., 1995; Cortesero et al., 1997; McAuslane et al., 1997; Mattiacci et al., 2001; Neveu et al., 2002).

In leeks, a sustained induced increase in sulfur precursors persists for at least 1 mo after the attack. Neobiosynthesis can require a long time. In leeks, an 8-d damage is required to induce the response. Similar findings have been observed in tobacco in which the peak increase of nicotine production occurs 7–8 d after the beginning of the attack (Baldwin, 1989). In cotton, attack by *S. littoralis* induces a strong response 5–10 d after the beginning of the attack but not after only 1–3 d (Anderson and Alborn, 1999; Anderson et al., 2001). Previously attacked leeks may present higher precursor levels than never-attacked plants for a long time after injury. As a result, previously damaged plants can be better protected against herbivory and against the offspring of the insects that inflicted the first damage (Dugravot and Thibout, unpublished data). This type of protection is comparable to an immune response (Agrawal, 1998) that once induced would allow the plant to be better protected in case of further damage. Induced protection may persist for months or more. In the birch tree *Betula pubescens*, protection persists from 1 yr to another (Haukioja and Neuvonen, 1985; Haukioja et al., 1985). However, long-term protection is not consistently observed in all species. In tobacco, the concentration of nicotine returns to normal 14 d after induction (Baldwin, 1989), and induced resistance

in *Glycine max* soybean disappears 20 d after the end of damage by *Epilachna varivestis* (Underwood, 1998). These findings could indicate that duration of the induced resistance may be correlated with the life cycle of the plant. In annual species such as tobacco and soybean, the response lasts only a few days, whereas in pluriannual tree species such as the birch, the response lasts several months. We suggest that in biennial leek plants, the response duration of at least 1 mo could be intermediate between annual and pluriannual plants. In addition, the greater the number of generations that the attacking insect may develop, the longer the induced response could last.

In experiments involving a generalist herbivore, *A. ipsilon*, PCSO concentration in leek plants did not increase even after intense damage. This finding implies that the elicitors of the induced response to *A. assectella* caterpillars in leeks are not present in *A. ipsilon* caterpillars. Constitutive levels of sulfur precursor concentrations may be sufficient to protect the plant against this type of generalist herbivore. In the field, *A. ipsilon* caterpillars consume mainly the tips of old leek leaves, i.e., the site where the concentration of sulfur metabolites is the lowest (Dugravot, personal observations). The finding concerning the response of leeks to generalist attack is in contradiction with findings reported in the herbacean *Hypericum perforatum* that increases production of secondary compounds in response to attack by generalist insects, i.e., *Spilosoma virginica*, *S. congrua*, and *Spodoptera exigua*, but not in response to attack by the specialist *Chrysolina quadrigemina* that causes more extensive damage (Sirvent et al., 2003). Schultz (2002) reported that it was exceptional for herbivore insects not to trigger a response in the consumed plant. It cannot be ruled out that attack by *A. ipsilon* caterpillars induces a response using biosynthetic pathways other than those producing sulfur precursors.

Gas chromatography studies showed that healthy leeks release no detectable odor under our experimental conditions. Conversely, leeks that have been attacked by leek moth or mechanically wounded release volatiles, including numerous thiosulfinates and zwiebelanes, compounds previously identified in analytical conditions (Auger et al., 1989; Jaillais et al., 1999). The lack of qualitative difference between volatiles released after herbivore damage and mechanical wounding has also been reported in Solanaceae such as tomato and potato (Bolter et al., 1997) and in crucifers (Mattiacci et al., 1994). Like leeks, these plants generally exhibit nonvolatile secondary compounds that can be used for effective direct protection against herbivores (Dicke, 1999). In corn or bean plants that do not produce nonvolatile secondary compounds for direct defense (Dicke, 1999), the spectrum of volatiles released in response to herbivores is different from that released after mechanical wounding (Dicke et al., 1990; Turlings et al., 1990; Tumlinson et al., 1999). The differences observed between undamaged and damaged leeks suggest that the insects living on *Allium* plants will be able to distinguish between the two plant categories.

This study has also demonstrated that the increase in nonvolatile sulfur precursor production following a damage by *A. assectella* caterpillars leads to a different chemical profile from that of another attack or wound event. The main difference consists of a significant increase in breakdown disulfides and rearrangement of all released sulfur volatiles products. This response by leek plants to *A. assectella* damage persists for at least 1 mo and could be considered as a direct induced defense strategy involving substances that can act on the metabolism and behavior of the damaging insect as well as its offspring.

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PREFORMED AND INDUCED CHEMICAL RESISTANCE OF TEA LEAF AGAINST *Exobasidium vexans* INFECTION

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Abstract—Levels of (−)-epicatechin in tea cultivars resistant to blister blight leaf disease were significantly higher than those in susceptible cultivars, while the reverse was true for (−)-epigallocatechin gallate, suggesting that epicatechin was involved in the resistance mechanism. The content of the methylxanthines, caffeine and theobromine, in the leaf increased in the initial translucent stage of the disease, probably as a defense response to fungal attack. Epicatechin and epigallocatechin levels were less than in healthy tissues at this stage, but increases in the corresponding gallate esters suggested that they were being converted into esters. Although epicatechin and epigallocatechin levels decreased from translucent to mature blister stages, the decrease was not significant. The decrease in levels of epicatechin, epigallocatechin, and their esters on infection and the formation of cyanidin and delphinidin on oxidative depolymerization of the blisters suggests that proanthocyanidins may play a role in the defense mechanism. The high resistance of a purple green leafed cultivar is attributed to the additional catechin source provided by the high levels of anthocyanins present.

Key Words—Tea, *Camellia sinensis*, blister blight, *Exobasidium vexans*, resistance, catechins, proanthocyanidins, caffeine, theobromine.

INTRODUCTION

Blister blight caused by *Exobasidium vexans* Massee is a leaf disease in tea, *Camellia sinensis* (L.) O. Kuntze, that is important in tea-growing areas of Asia (Arulpragasam, 1992). *E. vexans* is an obligatory biotrophic fungus that com-

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pletes its life cycle of 11–28 d in the tea leaf (Gadd and Loos, 1948; Agnihothrudu and Moulli, 1991). The initial symptoms of the disease are lime green translucent spots, which appear 6–9 d after the infection. These spots develop into blisters that are ruptured releasing spores at the completion of the life cycle.

Tea cultivars have been categorized as resistant and susceptible to blister blight (Balasooriya et al., 1996), and some morphological and anatomical characters have been correlated with resistance (Martosupono, 1991). Little attempt has been made to correlate resistance with the chemical composition of tea leaf, although changes in saccharide metabolism (Pius et al., 1998) and increased activity of polyphenol oxidase and peroxidase, and decrease in chlorophylls and carotenoids (Rajalakshmi and Ramarethinam, 2000) induced by blister blight infection have been shown. A preliminary report (Nagahaulla et al., 1996) discusses the formation of phytoalexins, probably polyphenolic in nature, on blister blight infection. The variation in chemical composition and the quality of tea with increasing severity of blister blight has also been studied (Gulati et al., 1999).

Polyphenols are the major chemical constituents of tea, with the catechins being predominant. The role of phenolic substances in disease resistance is well documented(Vidhyasekaran,1988; Nicholson and Hammerschmidt, 1992). Polyphenols are fungitoxic and antibacterial, with varying levels of toxicity to spore germination, mycelial growth, and fungal enzyme production (Vidhyasekaran, 1988). Caffeine has been shown to play a role in the resistance of tea to attack by the shot-hole borer, *Xyleborus fornicatus* (Kumar et al., 1995).

The present study was initiated to investigate the role, of flavan-3-ols (catechins) and methylxanthines on preformed or induced chemical resistance of tea leaf against *E. vexans*.

METHODS AND MATERIALS

Plant Material. Tea plants were grown in experimental plots at the Tea Research Institute of Sri Lanka, Talawakelle, Sri Lanka. The plants were raised by vegetative propagation using standard procedures (Kathiravetpillai and Kulasegaram, 1986). Tender tea leaves (two apical leaves and the bud) from each tea cultivar, which belong to *E. vexans* resistant (DT1, TRI777, TRI2043, N2, TRI4067, TRI4052, NAY3, and TRI3073) and susceptible (TRI2025, TRI2024, TRI2023, TRI62/5, TRI3015, TRI3014, and TRI62/1) groups, were used for comparison of catechin and caffeine contents.

Plants selected for the infection study were those in an experimental plot that contained about 50 plants. They were naturally infected with *E. vexans* during the rainy season. About 100 leaves were randomly harvested, imme-

diately brought to the laboratory, and sorted into those that were healthy and those in the three stages of infection-translucent spot stage, mature blister stage 1 (convex lesions which are green in color), and mature blister stage 2 (convex lesions which are white in color due to sporulation). Leaf discs cut from each of the stages or leaves, depending on the requirement, were transferred to glass tubes and stored at −20°C until use within 48 hr.

Extraction and HPLC Analysis of Catechin and Methylxanthines. Analysis was carried out according to ISO procedure (ISO/TC34/SC8; ISO, 1999). Tender tea leaves (10 g) from each cultivar or leaf discs (1 g) from infected material were plunged into boiling 70% aqueous methanol and boiled for 10 min. The extract was cooled and homogenized for 3 min using a top-drive macerator (Ultra-Turrax, USA). The homogenate was centrifuged (4000 rpm × 15 min), and the supernatant was decanted into a volumetric flask (100 ml). The residue was reextracted with the solvent (40 ml), centrifuged, and the supernatant was transferred to a volumetric flask. The volume of the pooled extract was increased up to 100 ml with 70% aqueous methanol. After tenfold dilution, the sample was filtered through 0.45-μm filter, and 10 μl of this were injected into the high-performance liquid chromatography (HPLC) system (Waters Alliance 2690XE Separation module coupled to a Waters 996 photodiode array detector (PDA) and Waters Millennium 32 data system). The analysis was replicated eight times. Standards of caffeine, theobromine, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epigallocatechin gallate, and (−)-epicatechin gallate (Sigma, St. Louis, MO, USA) were prepared. A phenomenex phenyl hexyl column (4.6 × 250 mm) and a linear gradient with mobile phases A (9% acetonitrile containing 2% acetic acid) and B (80% acetonitrile) were used for the separation.

Histochemical Staining of Infected Leaf Tissues. A tea leaf infected with *E. vexans* was rinsed with distilled water and soaked in ethanol/glacial acetic acid (3:1 v/v) for 30 min. The decolorized leaf was stained with vanillin (5% vanillin in ethanol/4 N HCl; Broadhurst and Jones, 1978) and 4-dimethylaminocinnamaldehyde (0.3% DMACA in methanol/6 N HCl, Li et al., 1996) for 20 min at 25°C. Excess stains were washed away with distilled water, and the leaf was observed for color changes. The staining was replicated eight times, and a suitable specimen was chosen for photographic record.

Determination of Proanthocyanidins in Infected and Healthy Leaf Material. Oxidative cleavages were carried out as described by Porter et al. (1986) using leaf discs (1 g) from healthy tea leaves and the infected area of three stages of blister blight infected leaf tissues. The leaf tissues (1 g) were homogenized with 70% acetone (containing 0.1% ascorbic acid). The extract was centrifuged and the volume of the supernatant was increased up to 10 ml with the extracting solvent. To an aliquot of the above extract (1 ml), BuOH/concentrated HCl (95:5 v/v, 5 ml) and iron reagent [2% $NH_4Fe(SO_4)_2 \cdot H_2O$ in

2 M HCl, 0.2 ml] were added. The resultant solution was placed on a water bath and heated at 95°C for 40 min, cooled, and decanted into a volumetric flask, and the volume was increased up to 10 ml with BuOH/HCl (95:5 v/v). A small aliquot was filtered through 0.45-μm filter (Millipore, USA), and 10 μl were injected into the HPLC system. The experiment was replicated twelve times. Calibration curves were constructed using standards of cyanidin and delphinidin (Carl Roth Gmbh, Germany).

Determination of Moisture Content. The moisture content of leaf samples was determined by drying at 103°C for 6 hr (ISO 1573, 1980).

Identification of Anthocyanidins in TRI2043. Tender leaves (50 g) from the cultivar TRI2043 were homogenized with methanol containing 0.01% HCl (100 ml). The extract was centrifuged (4000 rpm, 10 min), and the volume of the supernatant was increased up to 100 ml with the extracting solvent. To an aliquot (5 ml) of the above homogenate, 2 N HCl (5 ml) were added, and the mixture was heated on a water bath at 95°C for 2 hr. The acid hydrolyzate obtained was analyzed initially by thin-layer chromatography (TLC) and paper chromatography and then by HPLC. An aliquot (50 μl) was chromatographed on analytical cellulose TLC plates (20 × 20 cm, E. Merck, Germany) and on Whatman no. 3 paper (Whatman Inc, USA) using acetic acid/water/HCl (30:10:3, Forestal solvent system, Harborne, 1967) as eluent in both cases. The chromatographic bands were separately removed, dissolved in methanol containing 0.01% HCl, and spectroscopically analyzed (Cintra-5-UV–Visible Spectrophotometer, GBC, Australia). An aliquot (10 μl) of the acid hydrolyzate was filtered through a 0.45-μm filter (Millipore, USA) and injected into the HPLC system. The anthocyanidins, cyanidin and delphinidin, were detected at 520 nm.

Statistical Analysis. Student's *t*-test was performed to identify the difference between the two means against the critical difference (LSD) using Statistical Analysis System Software, USA. GLM procedure was conducted to identify any significant difference among healthy, translucent, and mature blister stages for the catechins and methylxanthines, and Duncan's multiple-range test was used for the mean separation.

RESULTS AND DISCUSSION

A study of the flavan-3-ol and caffeine content of resistant and susceptible tea cultivars showed that significantly higher levels of (−)-epicatechin (22.4 mg/g) were present in cultivars of tea resistant to blister blight compared with those in susceptible cultivars (11.3 mg/g) (Tables 1 and 2). A significantly higher level of (−)-epigallocatechin gallate was present in susceptible cultivars compared with resistant cultivars. No significant differences were seen among

TABLE 1. EC AND EGCG CONTENT IN TEA CULTIVARS

Cultivar	R/S	EC (mg/g)	EGCG (mg/g)
DT1	R	20.15 ± 1.33	98.05 ± 3.01
TRI777	R	26.64 ± 3.38	107.15 ± 5.50
TRI2043	R	21.17 ± 0.75	68.87 ± 2.32
N2	R	19.08 ± 0.56	113.82 ± 8.26
TRI4067	R	20.98 ± 1.01	81.27 ± 8.03
TRI4052	R	21.37 ± 1.26	93.65 ± 10.34
NAY3	R	25.23 ± 0.55	130.13 ± 9.93
TRI3073	R	24.63 ± 0.52	90.14 ± 14.25
Mean		**22.41**	**97.88**
TRI2025	S	8.16 ± 0.13	142.72 ± 5.51
TRI2024	S	13.4 ± 0.83	141.73 ± 5.74
TRI2023	S	11.45 ± 0.40	152.77 ± 9.90
TRI62/5	S	13.3 ± 0.50	132.06 ± 8.09
TRI3015	S	13.92 ± 0.85	133.68 ± 4.50
TRI3014	S	9.23 ± 0.67	138.8 ± 6.52
TRI62/1	S	12.43 ± 1.16	113.95 ± 5.75
Mean		**11.70**	**136.53**
$P < 0.05$		**0.001**	**0.001**

R = Resistant to *E. vexans*, S = Susceptible to *E. vexans*, EC = (−)-epicatechin, EGCG = (−)-epigallocatehin gallate, ANOVA, $P < 0.05$.

cultivars in (+)-catechin, (−)-epigallocatechin, (−)-epicatechin gallate, or caffeine. Resistant cultivars with higher levels of epicatechin had lower levels of epigallocatechin gallate.

The resistance of apple cultivars (Treutter and Feucht, 1990) to *Venturia inaequalis*, and avocado (Prusky et al., 1996; Prusky, 1996) to anthracnose have been attributed to high levels of epicatechin, suggesting that epicatechin may be directly or indirectly involved in the resistance mechanism of tea against blister blight.

TABLE 2. MEANS OF CATECHIN AND CAFFEINE CONTENT IN TEA CULTIVARS

	C (mg/g)	EGC (mg/g)	EC (mg/g)	EGCG (mg/g)	ECG (mg/g)	Caffeine (mg/g)
Mean (resistant)	5.48 ± 1.52	47.96 ± 11.34	22.41 ± 1.88	97.89 ± 13.29	39.49 ± 7.66	37.02 ± 2.58
Mean (susceptible)	7.19 ± 1.33	38.87 ± 4.97	11.70 ± 1.64	136.53 ± 8.93	39.89 ± 5.87	38.51 ± 1.77
$P < 0.05$	NS	NS	0.001	0.001	NS	NS

NS = Not significant, C = (+)-catechin, EGC = (−)-epigallocatechin, EC = (−)-epicatechin, EGCG = (−)-epigallocatechin gallate, ECG = (−)-epicatechin gallate, ANOVA, $P < 0.05$.

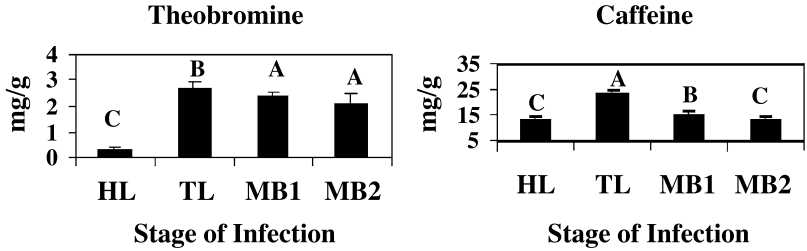

FIG. 1. Changes of theobromine and caffeine content (on dry weight basis) upon *E. vexans* infection, $N = 12$. HL, Healthy; TL, Translucent stage; MB1, Mature Blister 1; MB2, Mature Blister 2. Bars with the same letter are not significantly different.

Analysis of leaf material for flavanols and methylxanthines showed that significant changes occurred in flavan-3-ol and methylxanthine during infection. No significant difference was observed in catechin and methylxanthine between a disc sample (1 g) cut from fresh leaf and a similar sample from the same leaf stored at −20°C for 48 hr. The methylxanthines, caffeine and theobromine, increased significantly during the translucent stage of the disease, but the increase was not significant thereafter (Figure 1). The increase in methylxanthine at the first stage of infection could be the initial defense response of the plant to fungal attack, as reported to occur in infestation of tea by the shot-hole borer beetle, *Xyleborus fornicatus*, (Kumar et al., 1995) and in fungal attack on cocoa leaves (Aneja and Gianfagna, 2001).

Epicatechin and epigallocatechin significantly decreased upon infection (Figure 2). Epicatechin levels decreased in the translucent stage when compared with healthy tissues. A twofold reduction of epigallocatechin levels (from 42 to 21 mg/g) was observed in the translucent stage of the infection. However, the observed decrease in epicatechin and epigallocatechin content from the translucent stage to the mature blister stage was not significant. The content

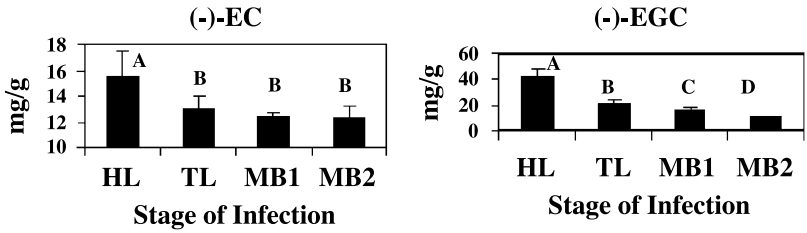

FIG. 2. Changes of catechins (on dry weight basis) upon *E. vexans* infection, $N = 8$. HL, Healthy; TL, Translucent stage; MB1, Mature Blister 1; MB2, Mature Blister 2. Bars with the same letter are not significantly different.

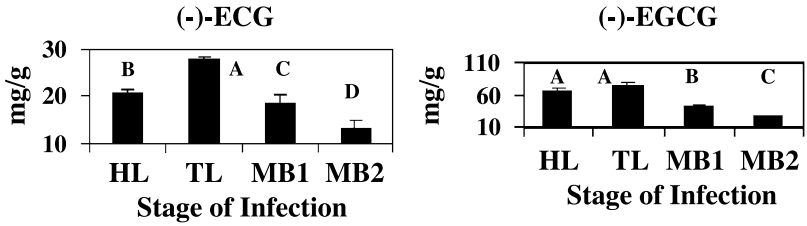

FIG. 3. Changes of catechins (on dry weight basis) upon *E. vexans* infection, $N = 8$. HL, Healthy; TL, Translucent stage; MB1, Mature Blister 1; MB2, Mature Blister 2. Bars with the same letter are not significantly different.

of epicatechin gallate and epigallocatechin gallate increased at the translucent stage of infection, but the increase was significant only for the former. The content of the gallate esters, however, decreased significantly during the mature blister stage (Figure 3).

Histochemical staining of tea leaves with vanillin reagent (Broadhurst and Jones, 1978) and 4-dimethylaminocinnamaldehyde (DMACA) reagent (Li et al., 1996) gave evidence for the accumulation of proanthocyanidins in the infected areas of the leaf tissue. Vanillin reagent produced the deep red color characteristic for flavan-3-ols or proanthocyanidins, and the blue green color shown by DMACA (Figure 4) indicated that proanthocyanidins were formed on infection. DMACA has been shown to be more sensitive to soluble proanthocyanidins (Joseph et al., 1998), reacting only with the terminal units of proanthocyanidins (Rohr, 1999).

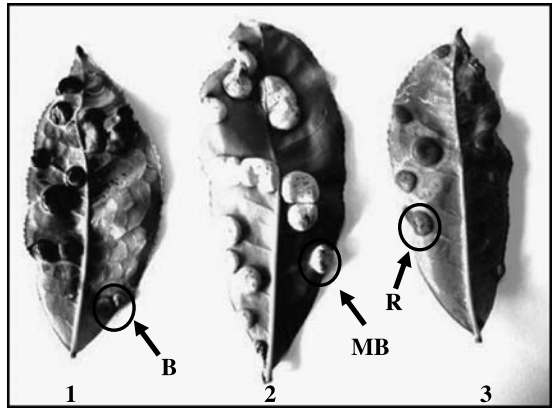

FIG. 4. Histochemical staining of tea leaves with mature *E. vexans* blisters. (1) Stained with DMACA, B = blue green in color. (2) Without staining, MB = mature *E. vexans* blisters. (3) Stained with vanillin, R = red in color.

Acid-catalyzed oxidative depolymerization of proanthocyanidins to the anthocyanidins, cyanidin, and delphinidin has been used to detect proanthocyanidin levels in plants (Porter et al., 1986). Depolymerization of infected tissue gave higher levels of cyanidin and delphinidin compared with healthy tissue (Figure 5).

It has been suggested that catechins condense with flavan-3,4-diols to give proanthocyanidins, which are known to be fungitoxic (Rao and Rao, 1986; Winkel-Shirley, 2002). The observed reduction in catechin levels and increase in proanthocyanidin levels on infection suggest a possible role for proanthocyanidins in the defense mechanism.

Of the cultivars studied here, TRI 2043 is the most resistant cultivar to blister blight. Substantially higher amounts of cyanidin and delphinidin were formed during the acid hydrolysis of the extract of the purple green leaf of this cultivar when compared with other cultivars. The identities of the anthocyanidins were confirmed by comparing their ultraviolet (UV)–visible spectra, paper chromatography, TLC, and HPLC values (Harborne, 1958, 1967) with those of standards. The color of the leaf was, therefore, attributed to the presence of high levels of anthocyanins. The higher tolerance of this cultivar may be explained as being due to the enhanced levels of catechins, which are proanthocyanidin precursors formed from the anthocyanins.

The increase in epicatechin gallate at the translucent stage could be due to the conversion of epicatechin into its gallic acid ester upon infection, since the translucent stage is accompanied by a significant decrease in epicatechin content. Similar observations were seen with epigallocatechin gallate and epigallocatechin content, although the increase in the former at the translucent stage was not significant (Figure 3). The increase in gallic acid esterification is important, as gallic esters of catechins have higher antibacterial, antiviral, and antioxidant activity than catechins (Kajiya et al., 2001, 2002). The decrease in

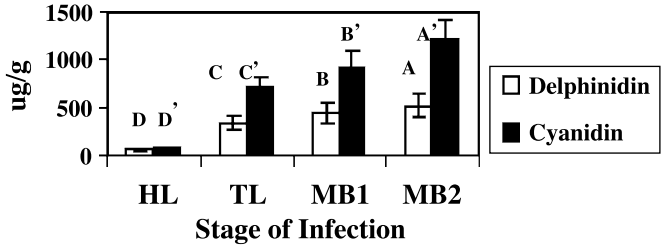

FIG. 5. Contents (on dry weight basis) of cyanidin and delphinidin liberated from proanthocyanidins upon acid-catalyzed oxidation of *E. vexans* infected leaf tissues, $N = 12$. HL, Healthy; TL, Translucent stage; MB1, Mature Blister 1; MB2, Mature Blister 2. Bars with the same letter are not significantly different.

catechin observed in the mature stage may be ascribed to the utilization of catechins for the formation of proanthocyanidins.

Epicatechin appears to play an important role in the resistance mechanism of tea against blister blight disease, while methylxanthines may play a role during initial attack by the fungus, *E. vexans*. The high resistance of the cultivar TRI 2043 is attributed to the high level of anthocyanins present.

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SOURCES OF CHEMICAL SIGNALS WHICH ENHANCE MULTIPARASITISM PREFERENCE BY A CLEPTOPARASITOID

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Abstract—The cleptoparasitoid *Eupelmus vuilleti* recognizes and prefers laying on hosts parasitized by *Dinarmus basalis* to unparasitized hosts. This recognition is based on the perception of a chemical substance deposited on the surface of the seed. Dufour's gland secretion and cuticular hydrocarbons of *D. basalis* are attractive and may mediate the host discrimination. This activity is linked to a mixture of linear and methyl alkanes whose source is apparently the Dufour's gland.

Key Words—*Eupelmus vuilleti*, *Dinarmus basalis*, hymenoptera, eupelmidae, pteromalidae, cleptoparasitoid, interspecific discrimination, Dufour's gland, venom gland, cuticle, hydrocarbons.

INTRODUCTION

Interspecific competition between two species exploiting the same resource induces negative interactions that reduce the fitness of each competitor. Some behaviors are selected for these species that reduce the negative effects of competition. More often than not, competing species position themselves on temporally or spatially distinct portions of the resource gradient and thus avoid competition (Price, 1971). Some species, in contrast, have selected indirect strategies that allow them to take advantage of interspecific competition. Individuals of competing species can, for example, physically eliminate a competitor (intraguild predation; Polis et al., 1989) or promote intervention of

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natural enemies (apparent competition; Holt, 1977). One of the most common strategies that allows a species to take benefits from interspecific competition is to "rob" resources foraged by a competitor (cleptoparasitism; Furness, 1987; Ronquist, 1994). These resources could be food or laying site.

In the parasitoid hymenoptera, hosts represent both a laying site and a food stock for the developing offspring. With the species named solitary parasitoid, hosts can allow the development of a unique parasitoid larvae, which induces an intensive competition both with conspecific and heterospecific females to gain access to these hosts. Some studies of parasitoid species mention a behavior that can be considered as cleptoparasitism (Arthur et al., 1964; Spradbery, 1969; Price, 1970; van Alebeek et al., 1993). Females of a species recognize the hosts parasitized by the competing species, reuse the hole drilled by the ovipositor, destroy the heterospecific egg, and lay their own egg instead. The stimulus implicated in the interspecific host recognition has never been identified. Potentially, this behavior is damaging for the challenged species, which would be expected to select adaptations to avoid the recognition of the hosts by the cleptoparasitoid.

How can a female recognize hosts parasitized by another species? Is the interspecific discrimination based on the diversion of an intraspecific signal or on the perception of exploitation cues left unintentionally by the competitor? Arthur et al. (1964) suspected that the cleptoparasitoid perceived the intraspecific marking pheromone of the competitor. In most species where the origin of the marking pheromone has been studied, the active product is produced by the Dufour's gland, one of the two accessory gland of the female genital apparatus (Guillot and Vinson, 1972; Mudd et al., 1982; Harrison et al., 1985; Hubbard et al., 1987). This gland produces an oily secretion containing mainly hydrocarbons, nearly identical to the cuticular secretion of the female (Syvertsen et al., 1995; Howard and Baker, 2003). After the laying, this secretion is deposited on the surface of the host or of the plant structure containing the host, during a typical marking posture (Salt, 1937; Bosque and Rabinovitch, 1979).

Eupelmus vuilleti (CRW.) (Hymenoptera: Eupelmidae) and *Dinarmus basalis* (ROND.) (Hymenoptera: Pteromalidae) are two larval and nymphal solitary ectoparasitoids. They have for hosts several bruchid species (Coleoptera: Bruchidae), including *Callosobruchus maculatus*, which at larval stages destroys *Vigna unguiculata* (Fabaceae) seeds (Monge et al., 1995). They are sympatric in West Africa, and they compete with each other for unparasitized hosts. In the presence of seeds containing unparasitized and hosts parasitized by *D. basalis*, *E. vuilleti* females prefer laying their egg on parasitized hosts (van Alebeek et al., 1993; Lévêque et al., 1994; Jaloux et al., 2004). The recognition of the host seems to be attributable to the female perception of a stimulus, present on the surface of the seed, and linked to the host exploitation by the *D. basalis* female (Jaloux et al., 2004).

The research reported here was designed to determine the nature, origin, and chemical composition of the stimulus produced by the *D. basalis* parasitized host and recognized by cleptoparasitoid *E. vuilleti* female.

METHODS AND MATERIALS

Parasitoid Rearing. E. vuilleti and *D. basalis* populations originated from the area of Dapaong, an important cultivation area of *V. unguiculata* in the North of Togo. These two populations were reared separately in the laboratory. Rearing and experimentations were run under the same conditions: 35°C:25°C; L–D 12:12 hr; RH 70% (Jaloux et al., 2004).

Choice Tests. A choice test was done to determine the preference of *E. vuilleti* females for the hosts treated with different substances. A two-days-old mated *E. vuilleti* female was placed in a Petri dish containing two hosts: a control and a treated one fixed at the bottom of the dish. The position of the female was recorded periodically for 30 min (at 10 sec, 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 20, 25, and 30 min) in order to build a preference index. The position of the female at each observation time was encoded: 0 at the bottom of the Petri dish, 1 on the treated host, and −1 on the control. The average position of one female gave an individual preference index (PI_i), between −1 and 1. For each experiment, 60 *E. vuilleti* females were tested, leading to calculation of the mean preference index (PI). This index value, obtained in test conditions, was compared to the one obtained in control conditions, presenting a choice between two identical healthy hosts. In the control condition, 0 was encoded when the female was observed at the bottom of the Petri dish, 1 on the first host, and −1 on the second host (the position of the first and second hosts was determined randomly before the experience). Statistical analysis required a nonparametric Mann–Whitney *U* test ($\alpha = 0.05$) under Xlstat 6.1.9.

Stimulus Nature. In order to check that the stimulus involved in the interspecific recognition was chemical, we studied its transfer through dichloromethane. The hosts used were contained in artificial systems, transparent capsules 2 cm long and 0.6 cm in diam. These capsules were modified to create a lodge in which a *C. maculatus* nymphae was introduced (Jaloux et al., 2004). Sixty capsules containing a host were placed in Petri dishes with 60 mated and experienced *D. basalis* females. Forty-eight hours later, *D. basalis* females were removed, and the parasitism was verified. The upper parts of parasitized capsules were taken and plunged about 10 sec into 2 ml of dichloromethane. A control extract was carried out immersing the upper parts of 60 clean capsules containing healthy hosts into 2 ml of dichloromethane. Five microliters of each extract were deposited on the surface of 60 capsules containing a healthy host. Sixty *E. vuilleti* females were submitted to a choice test between a capsule

treated with parasitized capsules extract and a capsule treated with clean unparasitized capsule extract (Exp. 1).

Stimulus Origin. In order to determine the role of *D. basalis* accessory glands and cuticular secretions in the interspecific recognition, we studied the preference of *E. vuilleti* females in choice tests for hosts treated with extracts of these structures. For these experiments, we used a simpler artificial system, a capsule without lodge or host. This system allowed us to study the activity of *D. basalis* secretions regardless of plant and host stimuli. First, 120 *D. basalis* females were sacrificed and dissected into saline solution (0.9% NaCl). Accessory glands of 60 females were removed and crushed on the surface of 60 clean capsules. The rest of the genital apparatus was deposited on the surface of a second group of 60 clean capsules. The Dufour's gland of the 60 other females was isolated from the venom gland-reservoir complex. Dufour's and venom gland extracts were carried out by crushing 60 glands into 2 ml of dichloromethane. These extracts were evaporated at 25°C and then taken up again into 5 ml of dichloromethane. Sixty *E. vuilleti* females were submitted to a choice test between a capsule on which were crushed *D. basalis* accessory glands (Exp. 2) or the rest of the genital apparatus (Exp. 3), and a capsule on which was deposited an equivalent quantity of body fat taken in the abdomen during the dissection. Sixty *E. vuilleti* females were submitted to a choice test between a capsule on which were deposited 5 µl of Dufour's gland extract (Exp. 4) or 5 µl of venom complex extract (Exp. 5), and a capsule on which were deposited 5 µl of pure dichloromethane. Second, 20 other emerging *D. basalis* females were placed separately into Petri dishes without hosts for 3 days. The 4th day, these females were plunged directly into 5 ml of dichloromethane for 10 sec. Five microliters of this extract were deposited on the surface of 60 clean capsules. These capsules were then presented in a choice test to 60 *E. vuilleti* females with 60 clean capsules on which were deposited 5 µl of pure dichloromethane (Exp. 6).

Chemical Analysis. In order to avoid contamination by the plant or the host, *D. basalis* females were placed into capsules for larval development, first containing a host, then without the host for nymphal stage. Five cuticular extracts were carried out with five groups of 10 *D. basalis* females, each into 100 µl of pentane (GC grade, 99.9%) for 10 sec. Females were removed and dissected. Five Dufour's gland extracts were prepared by crushing the glands of 10 females into 50 µl of pentane. Two microliters of each extract were injected on-column into the GC/MS Perkin-Elmer Turbomass system equipped with a capillary column (BP, 1.25 m long, 0.32 mm ID) that was temperature-programmed from 50 to 280°C at 10°C per min, maintained at 280°C for 10 min. The compounds were identified by using their retention times compared with reference products and by matching their mass spectra at 70 eV to the US National Institute of Standards and Technology (NIST) spectrum library.

RESULTS

Stimulus Nature. In experiment 1, *E. vuilleti* females preferred the capsules on which were deposited an extract of a capsule exploited by *D. basalis* to the clean capsules. The preference index was significantly different from the one obtained in the control experiment (Figure 1; PI = 0.24; *U*: $P < 0.001$). The stimulus can be transferred from a parasitized host to an unparasitized one through dichloromethane, and, therefore, can be considered a chemical substance.

Stimulus Origin. In experiment 2, *E. vuilleti* females preferred capsules on which were crushed the accessory glands of *D. basalis* to the ones on which were deposited an equivalent quantity of body fat (Figure 2) even though these capsules did not contain any host. The preference index obtained in this experiment was positive and significantly different from the one obtained in control experiment (PI = 0.33; *U*: $P < 0.01$). Experiment 3 showed that the rest of the genital apparatus was not active (Figure 2). The preference index obtained in this experiment, although positive, did not differ from the one observed in the control (PI = 0.09; *U*: $P = 0.06$). The ovaries, oviducts, collateral glands, and spermatheca do not seem to be involved in synthesis or in storage of chemical substances recognized by *E. vuilleti* females. In experiment 4, *D. basalis* Dufour's gland secretion was active and apparently caused the preference of *E. vuilleti* females for capsules on which it had been deposited. The preference index differed from the control (PI = 0.23; *U*: $P < 0.001$). *D. basalis* Dufour's gland seems to be involved in the production of or in the

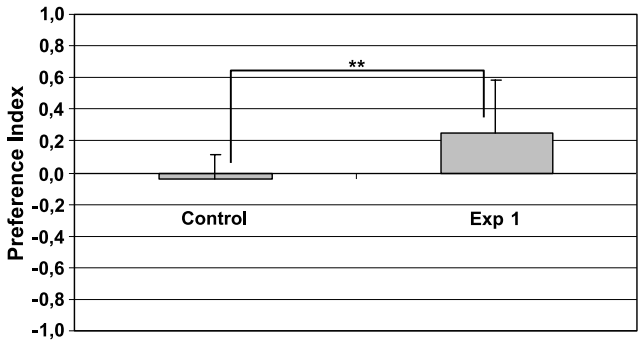

FIG. 1. Mean preference index (±SEM) of *E. vuilleti* females in choice condition between two clean capsules containing a healthy host (Control) or between a capsule on which were deposited 5 µl of an extract obtaining by washing capsules exploited by *D. basalis* in dichloromethane and a capsule on which were deposited the same quantity of pure dichloromethane (Exp. 1). * : Highly significant (*U*; $P < 0.001$).

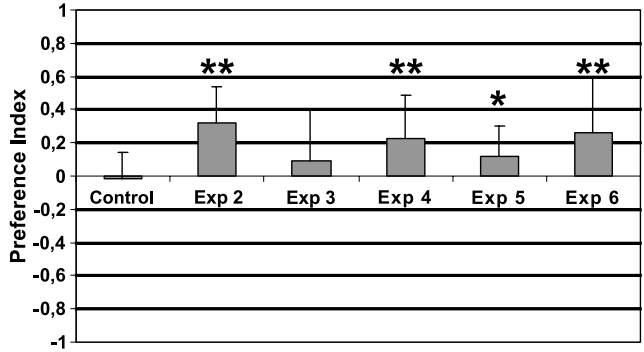

FIG. 2. Mean preference index (±SEM) of *E. vuilleti* females in choice condition between two empty clean capsules (Control); between a capsule on which were crushed *D. basalis* accessory glands and a capsule on which was deposited fat body (Exp. 2); between a capsule on which was crushed the rest of the genital apparatus and a capsule on which was deposited fat body (Exp. 3); between a capsule on which were deposited 5 µl of a Dufour's gland extract and a capsule on which were deposited 5 µl of pure dichloromethane (Exp. 4); between a capsule on which were deposited 5 µl of a venom gland extract and a capsule on which was deposited 5 µl of pure dichloromethane (Exp. 5); between a capsule on which were deposited 5 µl of a cuticular extract and a capsule on which were deposited 5 µl of pure dichloromethane (Exp. 6). ** : Highly significant (U; $P < 0.001$); * : Significant (U; $P < 0.05$).

storage of the substance perceived by the *E. vuilleti* female. Venom gland secretion was also active (Exp. 5: PI = 0.12; U: $P < 0.05$). However, the corresponding preference index was weaker than the one obtained with the Dufour's gland secretion extract. In experiment 6, *E. vuilleti* females showed a preference for the capsules treated with a cuticular secretion extract (PI = 0.27; U: $P < 0.001$). The preference index was similar to the one observed with Dufour's gland extract.

Chemical Analysis. The *D. basalis* Dufour's gland secretion is made up of saturated hydrocarbons, linear, mono, or dimethyl branched alkanes, and of three esters in low amount (3% of the total) including the decylpalmitate (Figure 3, Table 1). The *n*-alkanes (C_{21} to C_{34}) are the most common hydrocarbons (74%), followed by the methyl branched alkanes (2% of the total) in position 2-, 3-, 10-, 11-, 12-, 13-, 15-, and 17-, and by the dimethyl branched alkanes (1%) in position 3,9-, 9,13-, 10,12-, 11,15-. No unsaturated hydrocarbon were detected. The cuticular profiles are similar to those of Dufour's gland (Figure 4). Cuticular secretion is mainly made up of *n*-alkanes (73%), and contains monomethyl branched alkanes (8%), dimethyl branched alkanes (3%), and esters (3%). The compounds identified in Dufour's gland secretion and on the cuticle are the same. No compound was identified as characteristic of the

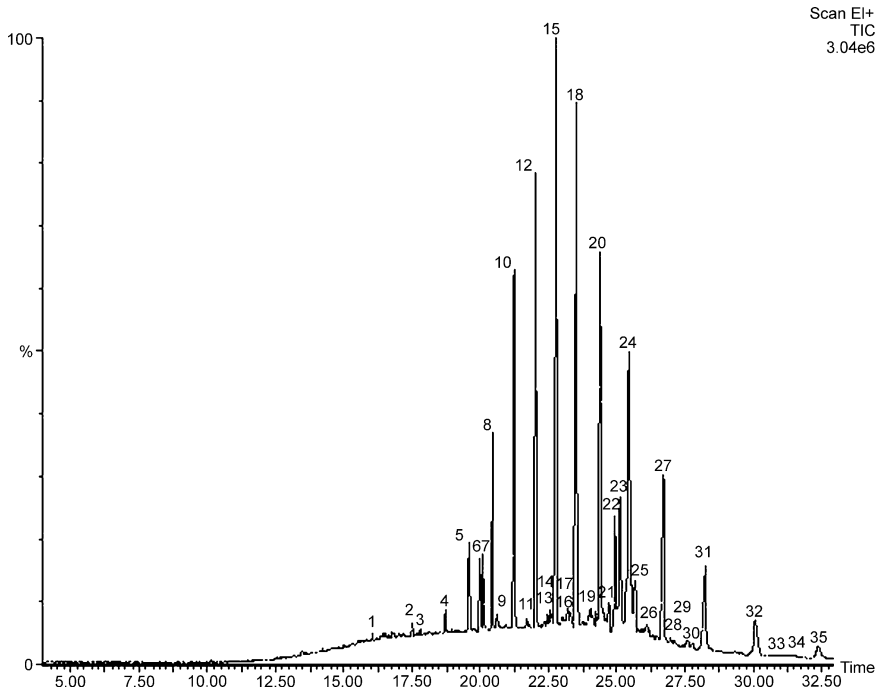

FIG. 3. Gas chromatogram of Dufour's gland secretion of *D. basalis* females. The peak numbers refer to compounds identified in Table 1.

cuticle or of the Dufour's gland. Only slight quantitative differences in the relative amounts of some compounds between the cuticular profile and the Dufour's gland profile were found (Table 2). Dufour's gland extracts are characterized by a significantly higher amount of the compounds corresponding to the peaks 7 (unidentified), 14 (decylpalmitate), and 19 (unidentified) (U; $P < 0.05$). Cuticular extracts are characterized by a significantly higher amount of the compounds corresponding to the peaks 17 (3-MeC_{27}), and above all the most heavy alkanes, 28 (11-+13-+15-MeC_{31}), and from 30 to 35 (3-MeC_{31}, C_{32}, C_{33}, 11-+13-+15-+17-MeC_{33}, 11,15-+13,17-DiMeC_{33} and C_{34}).

DISCUSSION

The stimulus allowing *E. vuilleti* females to recognize hosts parasitized by *D. basalis* is a dichloromethane-soluble chemical substance deposited by the *D. basalis* female on the surface of the seed during the exploitation of the host. Our results show that the *D. basalis* accessory glands are likely to be, at least, one of

TABLE 1. IDENTIFICATION OF THE HYDROCARBONS PRESENT IN THE *D. basalis* DUFOUR'S GLAND SECRETION

No.	R.T.	Compound	C.N.	M.P. ($N = 5$) ± SEM	Name
1	16.05	N.I. 1	–	1.09 ± 0.48	–
2	17.51	N.I. 2	–	1.90 ± 1.01	–
3	17.82	$C_{21}H_{44}$	21	1.28 ± 0.68	Heneicosane
4	18.73	$C_{22}H_{46}$	22	2.39 ± 0.88	Docosane
5	19.61	$C_{23}H_{48}$	23	4.74 ± 2.40	Tricosane
6	20.02	N.I. 3	–	2.68 ± 1.37	–
7	20.14	N.I. 4	–	3.10 ± 1.13	–
8	20.45	$C_{24}H_{50}$	24	6.66 ± 2.54	Tetracosane
9	20.63	N.I. 5	–	0.65 ± 0.34	–
10	21.25	$C_{25}H_{52}$	25	9.58 ± 1.27	Pentacosane
11	21.73	2-$CH_3C_{25}H_{51}$	26	0.43 ± 0.20	2-Methylpentacosane
12	22.02	$C_{26}H_{54}$	26	8.82 ± 0.93	Hexacosane
13	22.42	10-+12-$CH_3C_{26}H_{53}$	27	0.25 ± 0.17	10-+12-Methylhexacosane
14	22.57	$C_{15}H_{31}$–COO–$C_{10}H_{21}$	26	0.96 ± 0.15	Decylpalmitate
15	22.77	$C_{27}H_{56}$	27	9.79 ± 2.92	Heptacosane
16	23.18	3,9-$(CH_3)_2C_{27}H_{54}$	29	0.48 ± 0.12	3,9-Dimethylheptacosane
17	23.28	3-$CH_3C_{27}H_{55}$	28	0.56 ± 0.25	3-Methylheptacosane
18	23.51	$C_{28}H_{58}$	28	8.05 ± 2.73	Octacosane
19	24.06	N.I. 6	–	0.53 ± 0.08	–
20	24.39	$C_{29}H_{60}$	29	7.07 ± 3.14	Nonacosane
21	24.72	unidentified ester	–	0.96 ± 0.52	–
22	24.97	N.I. 7	–	4.50 ± 1.45	–
23	25.15	N.I. 8	–	4.46 ± 1.20	–
24	25.45	$C_{30}H_{62}$	30	7.64 ± 2.85	Triacontane
25	25.64	Unidentified ester	–	1.29 ± 0.50	–
26	26.12	N.I. 9	–	0.48 ± 0.02	–
27	26.70	$C_{31}H_{64}$	31	3.95 ± 1.86	Hentriacontane
28	27.08	11-+13-+15-$CH_3C_{31}H_{63}$	32	0.13 ± 0.07	11-+13-+15-Methylhentriacontane
29	27.59	9,13-$(CH_3)_2C_{31}H_{62}$	33	0.27 ± 0.09	9,13-Dimethylhentriacontane
30	27.76	3-$CH_3C_{31}H_{63}$	32	0.19 ± 0.03	3-Methylhentriacontane
31	28.23	$C_{32}H_{66}$	32	2.90 ± 1.29	Dotriacontane
32	30.24	$C_{33}H_{68}$	33	1.20 ± 0.77	Tritriacontane
33	30.75	11-+13-+15-+17-$CH_3C_{33}H_{67}$	34	0.16 ± 0.04	11-+13-+15-+17-Methyltritriacontane
34	31.46	11,15-+13,17-$(CH_3)_2C_{33}H_{66}$	35	0.09 ± 0.03	11,15-+13,17-Dimethyltritriacontane
35	32.39	$C_{34}H_{70}$	34	0.76 ± 0.29	Tetratriacontane

R.T. = Retention time (min), C.N. = Carbon number, M.P. = Mean percentage, SEM = Standard error of the mean, N.I. = Not identified.

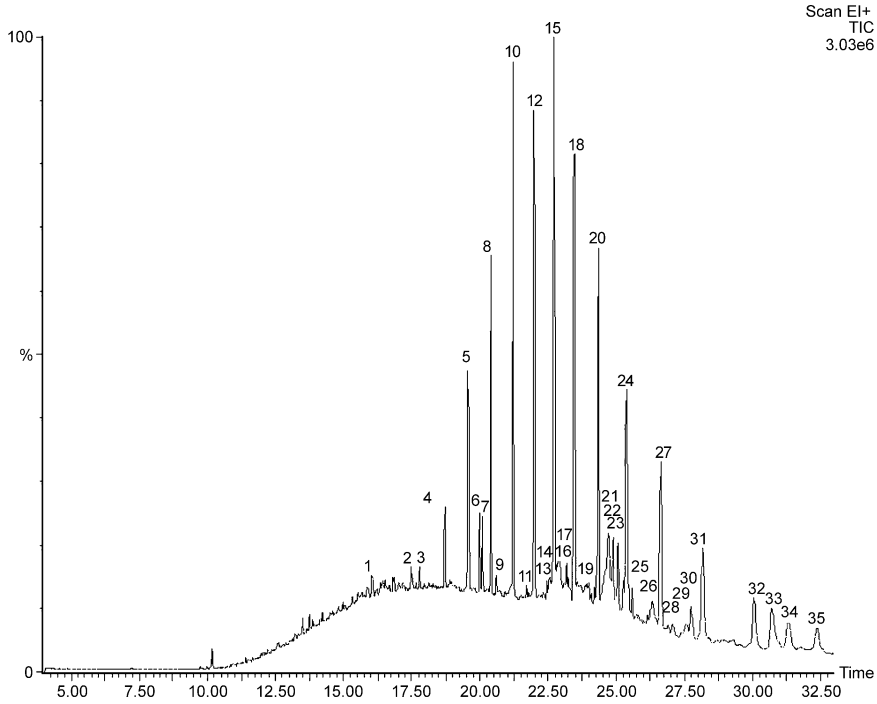

FIG. 4. Gas chromatogram of cuticular secretion of *D. basalis* females. The peak numbers refer to compounds identified in Table 2.

the production sites of the substance(s) involved. The rest of the genital apparatus is not significantly active. The slight preference observed when the rest of the genital apparatus was crushed on the surface of a capsule could be attributable to the contamination of the vagina by accessory gland secretions that naturally pour through a thin duct at the junction of the vagina and the ovipositor. Above the two accessory glands, the Dufour's gland seems to be the main production or storage site of the active substance. The venom gland secretion also induces a preference by *E. vuilleti* females, even though weaker than the Dufour's gland. This activity could be due to contamination during the dissection and the isolation of the two very close glands. It could also be a result of an additional signal (Höller et al., 1991) produced independently by the venom gland.

The deposit of a cuticular extract of *D. basalis* females on the surface of a capsule induces a comparable preference to the one observed with a Dufour's gland extract. The rubbing of the *D. basalis* female's abdomen on the surface of the system could be enough to allow the perception and the recognition by the

TABLE 2. IDENTIFICATION OF THE HYDROCARBONS PRESENT IN THE *D. basalis* CUTICULAR SECRETION

No.	R.T.	Compound	C.N.	M.P. ($N = 5$) ± SEM	Name
1	16.05	N.I. 1	–	0.89 ± 0.34	–
2	17.50	N.I. 2	–	1.26 ± 0.60	–
3	17.80	$C_{21}H_{44}$	21	0.94 ± 0.45	Heneicosane
4	18.71	$C_{22}H_{46}$	22	1.73 ± 0.42	Docosane
5	19.58	$C_{23}H_{48}$	23	4.34 ± 0.88	Tricosane
6	19.99	N.I. 3	–	1.59 ± 0.31	–
7	20.11	N.I. 4	–	1.55 ± 0.28	–
8	20.42	$C_{24}H_{50}$	24	5.85 ± 0.82	–
9	20.61	N.I. 5	–	0.45 ± 0.08	–
10	21.22	$C_{25}H_{52}$	25	8.11 ± 0.66	Pentacosane
11	21.71	2-$CH_3C_{25}H_{51}$	26	0.37 ± 0.16	2-Methylpentacosane
12	21.99	$C_{26}H_{54}$	26	7.40 ± 0.85	Hexacosane
13	22.46	10-+12-$CH_3C_{26}H_{53}$	27	0.33 ± 0.07	10-+12-Methylhexacosane
14	22.55	$C_{15}H_{31}$–COO–$C_{10}H_{21}$	26	0.53 ± 0.11	Decylpalmitate
15	22.74	$C_{27}H_{56}$	27	8.99 ± 0.87	Heptacosane
16	23.19	3,9-$(CH_3)_2C_{27}H_{54}$	29	0.39 ± 0.15	3,9-Dimethylheptacosane
17	23.27	3-$CH_3C_{27}H_{55}$	28	1.38 ± 0.78	3-Methylheptacosane
18	23.48	$C_{28}H_{58}$	28	7.46 ± 0.99	Octacosane
19	24.10	N.I. 6	–	0.24 ± 0.06	–
20	24.36	$C_{29}H_{60}$	29	7.85 ± 0.55	Nonacosane
21	24.70	Unidentified ester	–	1.84 ± 0.52	–
22	24.91	N.I. 7	–	2.36 ± 0.58	–
23	25.08	N.I. 8	–	3.14 ± 0.62	–
24	25.40	$C_{30}H_{62}$	30	6.58 ± 0.92	Triacontane
25	25.61	Unidentified ester	–	0.88 ± 0.14	–
26	26.15	N.I. 9	–	0.46 ± 0.34	–
27	26.66	$C_{31}H_{64}$	31	5.50 ± 0.45	Hentriacontane
28	27.08	11-+13-+15-$CH_3C_{31}H_{63}$	32	1.40 ± 1.02	11-+13-+15-Methylhentriacontane
29	27.58	9,13-$(CH_3)_2C_{31}H_{62}$	33	0.48 ± 0.24	9,13-Dimethylhentriacontane
30	27.77	3-$CH_3C_{31}H_{63}$	32	1.36 ± 0.44	3-Methylhentriacontane
31	28.20	$C_{32}H_{66}$	32	4.02 ± 0.59	Dotriacontane
32	30.07	$C_{33}H_{68}$	33	2.87 ± 0.24	Tritriacontane
33	30.71	11-+13-+15-+17-$CH_3C_{33}H_{67}$	34	3.32 ± 1.12	11-+13-+15-+17-Methyltritriacontane
34	31.33	11,15-+13,17-$(CH_3)_2C_{33}H_{66}$	35	2.41 ± 0.90	11,15-+13,17-Dimethyltritriacontane
35	32.38	$C_{34}H_{70}$	34	1.74 ± 0.15	Tetratriacontane

R.T. = Retention Time (min), C.N. = Carbon number, M.P. = Mean percentage, SEM = Standard error of the mean, N.I. = Not identified.

E. vuilleti female. The identical activity of the cuticular and Dufour's gland secretion is linked to the similarity in their hydrocarbon composition. This similarity seems to be widespread in Hymenoptera (bumblebees, Oldham et al., 1994; polist wasps, Dani et al., 1996; *Apis mellifera*, Gozansky et al., 1997). With hymenoptera parasitoids, the Braconidae *Cardiochiles nigriceps* (Syvertsen et al., 1995), the two Bethylidae *Cephalonomia tarsalis* and *C. waterstoni*, and the two Pteromalidae *Anisopteromalus calandrae* and *Pteromalus cerealellae* (Howard and Baker, 2003), wherever a joint analysis of the cuticular and Dufour's gland secretion has been performed, such similarity has been described. In the Pteromalidae, the composition in hydrocarbons of the two secretions in *D. basalis* is similar, even though slight differences between the relative amounts of some compounds were observed. Some heavy alkanes were present in higher amounts in the cuticular secretions. The production of cuticular hydrocarbons may involve another or several different production sites.

The kind of compounds, their diversity, the carbon number range, and the absence of any unsaturated hydrocarbons in *D. basalis* profiles are quite similar to those of two other Pteromalidae where analysis has been performed, *A. calandrae* and *P. cerealellae* (Howard and Baker, 2003). On the other hand, the presence of even-numbered alkanes in the same amount as odd-numbered alkanes is unusual. In all parasitoid species where the composition of the Dufour's gland secretion has been studied, odd-numbered alkanes are the only ones present (Mudd et al., 1982; Marris et al., 1996) or are in the majority (Syvertsen et al., 1995; Howard and Baker, 2003).

The location on the system and the origin and nature of the stimulus recognized by *E. vuilleti* agree with the marking pheromone characteristics described by Guillot and Vinson (1972). Marking pheromones are used by many phytophagous and parasitoid species to avoid laying their eggs in already exploited hosts or plants, which are of lower quality for the larval development of their offspring (Hofsvang, 1990; Nufio and Papaj, 2001). This pheromone is generally produced by the Dufour's gland and is deposited on the surface of the host after laying during a marking posture. *D. basalis* females are able to avoid laying on hosts already parasitized by a conspecific (Gauthier and Monge, 1999a). However, even though the marking posture is frequently observed, this intraspecific discrimination does not seem to involve the perception of an external mark. Superparasitism avoidance is performed after ovipositor insertion into host lodge, and perception of an internal signal which is transferred from the living egg to the parasitized host (Gauthier and Monge, 1999b). The possible deposit of Dufour's gland secretion on the surface of the seed would not be involved in intraspecific discrimination and, thus, could not be considered a pheromone. Furthermore, the *D. basalis* marking posture does not seem to be efficient: it is not systematic, only 71% of laying is followed by this posture, and no drop or deposit linked to this posture has been observed.

D. basalis marking does not seem to be necessary for the start of the *E. vuilleti* cleptoparasitic behavior. The recognition of the hosts parasitized by *D. basalis* could only involve external cuticular cues left unintentionally by the *D. basalis* female during the exploitation of the host. The *D. basalis* marking posture could constitute a relic behavior of an external marking system, now discarded because of disadvantageous competition conditions with *E. vuilleti* (Gauthier et al., 2002), which uses this mark to locate and destroy *D. basalis* eggs. A more discreet internal marking system would then have been selected for in *D. basalis* females. The similarity between Dufour's gland secretion and cuticular secretion could have allowed the adaptation of *E. vuilleti*, by changing from discrimination based on the external mark to discrimination based on the perception of exploitation cuticular cues.

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CONFIRMATION OF POTENTIAL HERBICIDAL AGENTS IN HULLS OF RICE, *Oryza sativa*

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Abstract—An ethyl acetate extract of *Oryza sativa* (rice) hulls yielded seven compounds: hentriacontane, 1-tetratriacontanol, β-sitosterol, momilactone A, momilactone B, tricin (a flavonoid), and β-sitosterol-3-*O*-β-D-glucoside. The structures of these compounds were elucidated with 500 MHz nuclear magnetic resonance (NMR), using 1D and 2D spectral methods, aided by electron ionization mass spectrometry (EI–MS), fast atom bombardment mass spectrometry (FAB–MS), infrared (IR), and ultraviolet (UV) spectrophotometry. The complete ^{1}H NMR assignments for momilactone A and B and ^{13}C NMR assignments for tricin are discussed. To the best of our knowledge, hentriacontane, 1-tetratriacontanol, and β-sitosterol-3-*O*-β-D-glucoside were identified for the first time in rice hulls. In biological activity tests using these identified compounds, momilactone A and B showed potent inhibitory activity against duckweed (*Lemna paucicostata*). 1-Tetratriacontanol and β-sitosterol-3-*O*-β-D-glucoside also showed about 13–20% inhibitory activity based on chlorophyll reduction. Hentriacontane and β-sitosterol did not show any herbicidal activity. In a germination assay of three weed species (*Leptochloa chinenesis* L., *Amaranthus retroflexus* L., and *Cyperus difformis* L.) in culture tubes both momilactones A and B had high inhibitory effects. Momilactone B completely inhibited germination of all three weed species at 20 ppm. Germination of *L. chinensis* L. was completely inhibited by a 4 ppm solution of momilactone B.

Key Words—*Oryza sativa* L., Gramineae, rice hull composition, hentriacontane, 1-tetratriacontanol, β-sitosterol, momilactone A, momilactone B, tricin, β-sitosterol-3-*O*-β-D-glucoside, herbicidal activity, *Lemna paucicostata* Hegelm 381, germination assay in culture tube.

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INTRODUCTION

Rice (*Oryza sativa* L.) is the principal cereal food in Asia and the major staple of the majority of the population. It generally occurs as two types, with white and colored hulls, although the white hulled variety is more common (85%). The germination of rice seed is of great agricultural importance, and it has long been known to be influenced by compounds present in the seed coat (hull) (Dutta, 1973). The compounds momilactone A and B from rice hulls cause germination and growth inhibition in the roots of rice (Kato et al., 1973, 1977; Takahashi et al., 1976). They were later found in rice leaves and straw as phytoalexins (Cartwright et al., 1981; Kodama et al., 1988). A putative growth inhibitor was isolated from rice root exudates and identified as momilactone B (Kato-Noguchi et al., 2002), and it was recently reported that rice seedlings release momilactone B into the environment (Kato-Noguchi and Ino, 2003). The antioxidant activities of methanol extracts (Ramarathnam et al., 1988) and the compound isovitexin (a *C*-glycosyl flavonoid) from methanol extracts (Ramarathnam et al., 1989) of rice hulls have been described. The growth inhibitor sakuranetin, a flavanone phytoalexin from ultraviolet (UV)-irradiated rice leaves, has also been isolated (Kodama et al., 1992). Little information is available on the influence of rice hull compounds on rice yield. However, it has been suggested that rice hulls, the most abundant agricultural by-product in rice growing areas, possess phytotoxic substances that could serve as natural herbicides by inhibiting seed germination and the growth of weeds. This study has implications for the use of agricultural residues because farmers in Korea have always left rice hulls in the field after harvesting. Such information might indicate the potential of inhibitory substances contained in rice hulls as natural herbicides that could control weeds in an environmentally acceptable and sustainable manner. Because there are few reports in the literature about the chemical constituents of rice hulls, identification of bioactive constituents with growth or germination inhibitory properties is required. Thus, the objectives of our research were: (1) to isolate and identify bioactive constituents from rice hulls, and (2) to examine the bioactivity of these compounds including momilactone A and B. Although use of synthetic herbicides is increasing around the world, little information is available on the development of natural herbicides. The isolation of compounds from rice hulls and their inhibitory activity, based on germination assays in culture tubes, are discussed in this paper.

METHODS AND MATERIALS

Plant Material. The hulls of *O. sativa* were collected from the Konkuk University Experimental Farm, Seoul, South Korea, in October 2002. The

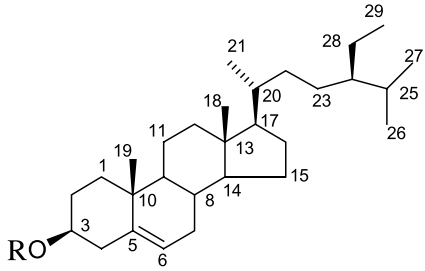
voucher specimen (no. KKU 96, HOCHOKJINDO) has been dried and deposited in the herbarium of our department.

Preparation of Extracts. Dried hulls of *O. sativa* (10 kg) were immersed in methanol (MeOH) for 1 wk at room temperature and concentrated under vacuum to produce an extract (150 g), which was suspended in water and extracted successively with ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH).

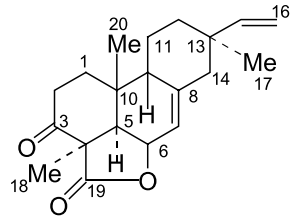
Fractionation of the Ethyl Acetate Extract. The EtOAc extract (35 g) was subjected to normal-phase column chromatography over silica gel and yielded 40 fractions with the following eluants: fraction 1 in hexane, fractions 2–5 in hexane:EtOAc (9:1), fractions 6–11 in hexane:EtOAc (8:2), fractions 12–15 in hexane:EtOAc (7:3), fractions 16–20 in hexane:EtOAc (1:1), fractions 21–22 in EtOAc, fractions 23–28 in EtOAc:MeOH (9.5:0.5), fractions 29–32 in EtOAc:MeOH (9:1), fractions 33–34 in EtOAc:MeOH (7:3), and fractions 35–40 in MeOH.

Isolation and Identification of Compounds from the Fractions. Fractions 1 and 5, with further column chromatography and thin-layer chromatography (TLC) over silica gel with hexane:EtOAc, yielded two pure compounds: hentriacontane (100 mg) and 1-tetratriacontanol (50 mg). Fraction 6 was crystallized and, after purification by column chromatography, yielded β-sitosterol (1 g). This was confirmed by comparison with an authentic sample from Sigma. Fraction 11, which was further purified by column chromatography over silica gel with methylene chloride and methanol, produced two pure compounds: momilactone A (150 mg) and momilactone B (100 mg). Fraction 12, after column chromatography over silica gel and Lichroprep RP-18 [octadecyl silica (ODS)], yielded one yellow compound as a powder. This was identified as tricin (a flavonoid). Fraction 23, after column chromatography over silica gel with chloroform and methanol, yielded one pure compound: β-sitosterol-3-O-β-D-glucoside. All isolated compounds (Figure 1) were identified with different spectroscopic techniques as described in Results and Discussion.

General Experimental Procedures and Instrumentation. Melting points of isolated compounds were determined on an electrochemical engineering apparatus. Thin-layer chromatography (TLC) was carried out on precoated silica gel plates (Merck) with a layer thickness of 0.5 mm, unless otherwise indicated. Spots were detected under UV light (254 and 366 nm) before and after the plates were dipped in a chamber containing 1% vanillin–sulfuric acid (ethanol solution). Column chromatography was carried out on silica gel (70–230 mesh; Merck) and optical rotations were measured on an AA-10 model polarimeter. Both nuclear magnetic resonance spectra—^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz)—were measured with a Bruker Avance (DRX-500) spectrometer using deuterated chloroform ($CDCl_3$), methanol (CD_3OD), and pyridine (C_5D_5N) as solvents. Electron ionization mass spectrometry (EI–MS) spectra were recorded with a JEOL JMS-SX 102A spectrometer and fast atom



3: R=H; **7:** R=β-D-glucose



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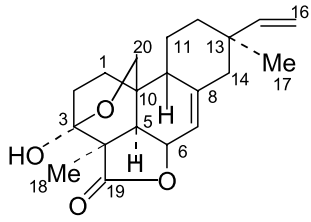

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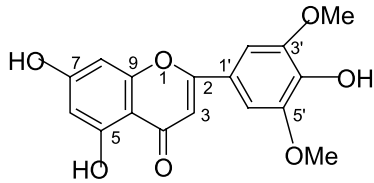

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FIG. 1. Structures of β-sitosterol (**3**), β-sitosterol-3-*O*-β-D-glucoside (**7**), momilactone A (**4**), momilactone B (**5**), and tricin (**6**).

bombardment mass spectrometry (FAB–MS) were recorded with a JEOL JMS-AX 505 WA instrument. Infrared (IR) spectra were recorded with a Thermo-Mattson 60-AR spectrophotometer, and ultraviolet (UV) spectra were measured with a TU-1800_{PC} UV–VIS spectrophotometer.

Chemical Elucidation of the Allelopathic Compounds. The details of the compounds isolated from rice hull are given in Appendix 1 (available online at www.springerlink.com; search for DOI: 10.1007/s10886-005-5290-5; Electronic Supplementary Material can be found at the end of the article).

Bioassay of Phytotoxicity (Lemna Assay). Test compounds were dissolved in acetone, which contained the nonionic surfactant Tween-20, and were then mixed with 1/2 Hunter medium. The final concentrations of acetone and Tween-20 were 1 and 0.01%, respectively.

The technique used to analyze the bioactivity of the identified compounds was based on the method of Hong et al. (2000). The test solution (2 ml) and one duckweed (*Lemna paucicostata* Hegelm 381) frond were added to each well of a 24-well plate. The plate was incubated in a growth chamber (26°C, 14-hr photoperiod, 50 μmol m^{-2} sec^{-1}) for 5 d. Herbicidal activity was then rated by visual scoring and by measuring chlorophyll content. The chlorophyll was extracted with dimethyl sulfoxide (DMSO) and analyzed using a UV–VIS spectrophotometric method (Hiscox and Israelstam, 1979). Visual injury symptoms were recorded on d 5 on an injury symptoms scale of 0 to 100 (0 = no effect, 100 = complete death). Other measurements included:

- Chlorophyll content (μg ml^{-1}) = $2.367 \times (\text{absorbance})^2 + 6.299 \times \text{absorbance} + 0.169$ ($r = 0.999$);
- Inhibition of chlorophyll content (%) = [(control − treatment) / control] × 100

Germination Assay in Culture Tubes. The compounds used in the experiment were isolated from rice hulls, and included hentriacontane, 1-tetratriacontanol, β-sitosterol, β-sitosterol-3-*O*-β-D-glucoside, momilactone A, momilactone B, and tricin. This test was applied to detect any inhibitory activities. Three weed species were used for the test: *Leptochloa chinensis* L., *Amaranthus retroflexus* L., and *Cyperus difformis* L. Both *L. chinensis* and *C. difformis* can be found growing in rice paddies, whereas *A. retroflexus* prefers the area around paddies. Weed seeds were collected in October 2002 and after debris was removed from the seeds by flotation in distilled water, the seeds were stored at −35°C until used for bioassays. The seeds were surface sterilized in a 1:10 (v/v) dilution of commercial hypochlorite bleach for 10 min and rinsed several times with distilled water.

A modified bioassay technique developed by Choi et al. (2002) was applied. Sea sand (650 mg) was placed into disposable culture tubes (12 × 75

mm) with the isolated rice hull compounds, which had been dissolved in acetone to obtain the following concentrations: 4, 20, 100, and 1000 ppm. Weed seeds from the three species (2 mg) were added to the tubes, which were then put in the growth chamber under the following conditions: 25°C, 12 hr daylight, 20 μmol m^{-2} sec^{-1}. Percentage germination was determined after 7 d, and the inhibitory effects of identified compounds on germination and growth were evaluated using the equations:

- Germination inhibition (%) = [(control − treatment) / control] × 100; and
- Growth inhibition (%) = [(control − treatment) / control] × 100.

Statistical Analysis. Analysis of variance for all data was undertaken using the general linear model (GLM) procedure of the SAS program (SAS Institute, 2000). All of the aforementioned experiments were replicated three times using a completely randomized design. The pooled mean values were separated based on least significant difference (LSD) at the 0.05 probability level.

RESULTS

Identity and Phytotoxicity of Compounds From Rice Hulls. Seven constituents of rice hulls were isolated, including hentriacontane (**1**), 1-tetratriacontanol (**2**), β-sitosterol (**3**), momilactone A (**4**), momilactone B (**5**), tricin (a flavonoid) (**6**), and β-sitosterol-3-*O*-β-D-glucoside (**7**). These were identified using 500 MHz NMR with ^{1}H, ^{13}C, ^{1}H–^{1}H correlation spectroscopy (COSY), and ^{1}H–^{13}C heteronuclear chemical shift correlation (HETCOR) spectra, together with EI–MS, FAB–MS, IR, and UV spectrophotometry. In earlier published work, momilactone A, momilactone B (^{1}H NMR), and tricin (^{13}C NMR) values were incompletely described. Hentriacontane, 1-tetratriacontanol, and β-sitosterol-3-*O*-β-D-glucoside are reported for the first time from rice hulls.

Hentriacontane (**1**) was isolated as a colorless white solid from the first fraction of the ethyl acetate extract. It exhibited a peak *m/z* of 436 on EI–MS (calculated for $C_{31}H_{64}$). The FAB–MS data of compound **1** gave a positive-mode $[M + H]^{+}$ ion peak at 437 and a negative-mode $[M - H]^{-}$ ion peak at 435, which suggested a molecular ion peak $[M]^{+}$ of 436. The IR spectrum displayed intense absorption bands at 2920, 1464, 1215, and 760 cm^{-1}. The 500 MHz ^{1}H NMR spectrum in $CDCl_3$ showed protons at δ 0.88 (6H, t, $J = 7.0$ Hz) for two methyls and at δ 1.25–1.31 for all 58 methylene protons. The 125 MHz ^{13}C NMR in $CDCl_3$ gave only six signals for all carbons at δ 14.33 for two methyls and at δ 22.91–32.15 for methylene carbons. The above evidence established the structure of **1** as hentriacontane.

Tetratriacontanol (**2**) was isolated as white granular crystals with a melting point of 83–86°C. It exhibited a peak at *m/z* 476 $[M - H_2O]^+$ on EI–MS, and FAB–MS gave a positive-mode $[M + H]^+$ ion peak of *m/z* 495 and a negative-mode $[M - H]^-$ ion peak of *m/z* 493, suggesting a molecular weight of 494. The IR spectrum displayed intense absorption bands at 3450, 2917, 1465, 1215, and 757 cm^{-1}, with the band at 3450 cm^{-1}, indicating the presence of a hydroxyl group. ^1H NMR of compound **2** in $CDCl_3$ gave signals that appeared as a triplet at δ 0.88 (3H, t, $J = 6.7$ Hz) for methyl protons, a multiplet at δ 1.55 for methylene protons of CH_2CH_2OH, and a triplet at δ 3.64 (CH_2OH). All remaining methylene protons appeared as a broad singlet at δ 1.25–1.33. ^{13}C NMR in $CDCl_3$ produced 15 carbon signals at δ 63.34, 33.04, 32.15, 29.92, 29.88, 29.84, 29.83, 29.66, 29.58, 29.47, 29.30, 25.96, 24.98, 22.91, and 14.33 for methylene attached to hydroxyl group, all methylenes, and one methyl carbon, respectively. Analysis of compound **2** by ^1H, ^{13}C, and 2D NMR (COSY, HETCOR) supported the assigned structure **2** as 1-tetratriacontanol.

β-sitosterol (**3**) was isolated as colorless needles that exhibited an *m/z* of 414 $[M]^+$ (possible $C_{29}H_{50}O$). The IR spectrum showed absorption bands at 3429, 2936, 1644, 1462, 1376, and 1057 cm^{-1}, with the band at 3429 cm^{-1} characteristic of a hydroxyl group. The ^1H NMR of compound **3** displayed a one-proton broad multiplet at δ 3.51, which was assigned to a carbinol 3α-proton. Two three-proton broad signals at δ 0.63 and 0.94 were ascribed to the tertiary C-18 and C-19 protons, respectively. Three doublets at δ 0.92 ($J = 7.4$ Hz), 0.81 ($J = 7.5$ Hz), and 0.91 ($J = 7.15$ Hz), which integrated for three-protons each, were attributed to secondary C-21, C-26, and C-27 methyl groups, respectively. Another triplet at δ 0.88 ($J = 7.5$ Hz) was attributed to the C-29 methyl protons, and a one-proton broad multiplet at δ 5.56 for one vinylic proton was attributed to C-6. The remaining methine and methylene protons resonated between δ 2.29 and 1.13. The ^{13}C NMR spectrum of compound **3** showed the presence of 29 carbon signals. The signals at δ 140.72 and 121.71 were ascribed to vinylic carbons at positions C-5 and C-6. Other carbon signals appeared at δ 37.31 (C-1), 31.57 (C-2), 71.80 (C-3), 42.19 (C-4), 31.87 (C-7, 8), 50.10 (C-9), 36.48 (C-10), 21.11(C-11), 39.81 (C-12), 42.33 (C-13), 56.79 (C-14), 24.32 (C-15), 28.26 (C-16), 56.11 (C-17), 11.87 (C-18), 19.39 (C-19), 36.17 (C-20), 18.82 (C-21), 33.95 (C-22), 26.13 (C-23), 45.85 (C-24), 29.18 (C-25), 19.48 (C-26), 19.07 (C-27), 23.09 (C-28), and 12.32 (C-29). Based on the above results, the structure **3** was determined to be β-sitosterol, and confirmed by comparison with data from the literature.

Momilactone A (**4**) was isolated as colorless crystals that exhibited an *m/z* of 314 on EI–MS. A FAB–MS of compound **4** gave a positive-mode molecular ion peak $[M + H]^+$ at *m/z* 315 and a negative mode at *m/z* 313 $[M - H]^-$, suggesting a molecular weight of 314. The IR spectrum of compound **4** gave absorption bands at 2932, 1765, 1700, 1386, and 1181 cm^{-1}. The bands at 1765

and 1700 cm^{-1} suggested lactone and ketone groups, respectively. The ^{1}H NMR ($CDCl_3$) spectrum of **4** displayed three singlets at δ 0.89, 1.52, and 0.99 for C-17, C-18, and C-20 tertiary methyl groups, respectively, and two multiplets at δ 2.60–2.64 and δ 1.89–1.91 for C-1 and C-2 methylene groups, respectively. Signals at δ 2.31 (d, J = 5.0 Hz), δ 4.85 (t, J = 5.0 Hz), and δ 5.71 (d, J = 5.0 Hz) were assigned to the C-5 to C-7 protons for two methine and one olefinic groups. The multiplets at δ 1.80 and 1.60 were assigned to C-9 and C-12 methine and methylene protons, and the double doublets at δ 1.32, 1.74 and δ 2.06, 2.21 were assigned as the C-11 and C-14 methylene protons. Finally, other signals in the downfield region including double doublets at δ 5.85 (J = 17.5 and 10.7 Hz) for the C-15 proton and δ 4.94 (J = 10.8 and 1.2 Hz) and 4.98 (J = 17 and 1.2 Hz) for the two protons on C-16 were ascribed to olefinic protons. The ^{13}C NMR spectrum of **4** showed resonances for all 20 carbons (δ 35.08, 31.41, 205.37, 53.76, 46.66, 73.36, 114.23, 148.22, 50.39, 32.66, 24.19, 37.44, 40.32, 47.73, 149.15, 110.36, 22.16, 21.66, 174.51, and 21.99), which included three methyl, six methylene, five methine, and six quaternary carbons. The ^{13}C NMR spectrum suggested the presence of both a ketone and lactone group from signals at δ 205.37 and 174.51, respectively. Olefinic carbons were assigned to 149.15, 148.22, 114.23, and 110.36. Analysis of compound **4** by ^{1}H, ^{13}C, and 2D NMR (COSY, HETCOR) confirmed the structure as the known momilactone A.

Momilactone B (**5**) was isolated as colorless crystals with m/z of 330 on EI–MS. A FAB–MS of compound **5** gave a positive-mode molecular ion peak at m/z $[M + H]^+$ of 331 and a negative mode m/z at 329 $[M - H]^-$. This suggested a molecular weight of 330. The IR spectrum gave bands at 2939, 1735, 1670, 1233, 1129, 1037, and 932 cm^{-1}, with the band at 1735 cm^{-1} suggesting a lactone. The ^{1}H NMR ($CDCl_3$) spectrum of **5** displayed 3H singlets at δ 0.86 and 1.40 for the C-17 and C-18 tertiary methyl groups. There were two multiplets at δ 1.52–1.56 and δ 2.09 for the C-1 and C-2 methylene groups, respectively, and a singlet at δ 4.13 for the hydroxyl proton. Signals at δ 2.20 (dd, J = 7.1 and 2.1 Hz), δ 4.94 (dd, J = 7.1 and 4.5 Hz), and δ 5.67 (d, J = 4.5 Hz) were assigned to the C-5, C-6, and C-7 protons (all methine groups), respectively. Other double doublets were at δ 1.44 for C-9 (methine) and 1.22 and 1.66 for C-11 methylene protons, respectively. A multiplet at δ 1.55 was for protons on C-12 (methylene) and a doublet at δ 2.01 (J = 12.0 Hz) and double doublet 2.13 (J = 12.0 and 1.8 Hz) were for the C-14 methylene protons. Double doublets at δ 5.82 (J = 17.6 and 10.7 Hz), δ 4.92 (J = 10.5 and 1.2 Hz), and δ 4.94 (J = 17.6 and 1.2 Hz) were for the C-15 and C-16 olefinic protons. Two more double doublets at δ 3.56 (dd, J = 9.1 and 3.2 Hz) and δ 4.06 (dd, J = 9.1 and 1.9 Hz) were for the C-20 methylene protons. The ^{13}C NMR spectrum of this compound showed resonances for all 20 carbons (δ 29.00, 26.63, 96.78, 50.53, 43.17, 73.93, 114.19, 146.88, 44.87, 30.93, 24.97, 37.41, 40.17, 47.72,

149.02, 110.40, 22.06, 19.16, 180.64, and 72.90), which included two methyl, seven methylene, five methine, and six quaternary carbons. The ^{13}C NMR spectrum confirmed the presence of a ketone at δ 180.00, and a hydroxyl carbon at δ 96.7 shifted downfield due to being attached to another oxygen in the hemiketal. The methylene carbon for C-20 also was shifted downfield due to being attached to an oxygen. Analysis of compound **5** by ^{1}H, ^{13}C, and 2D NMR (COSY, HETCOR) supported confirmation of the structure as momilactone B.

Tricin (**6**) was isolated as a yellow powder with a molecular ion peak on EI–MS at *m*/*z* of 330 $[M]^+$. A FAB–MS gave a positive-mode molecular ion peak at *m*/*z* $[M + H]^+$ of 331 and a negative-mode molecular ion peak $[M - H]^-$ at *m*/*z* of 329. This suggested a molecular weight of *m*/*z* 330. The IR spectrum of compound **6** gave intense absorption bands at 3468, 2928, 1647, 1506, 1359, 1166, and 830 cm^{-1}. The bands at 3468 and 1647 cm^{-1} suggested hydroxyl and ketone groups, respectively. The ^{1}H NMR showed a singlet at δ 7.27, which was assigned to aromatic H-2′ and H-6′. Another two singlets at δ 6.16 and 6.62 were assigned to H-6 and H-8, respectively. Moreover, a singlet at δ 3.95 was assigned to two methoxy groups. The ^{13}C NMR spectrum showed 15 discrete signals for the 17 carbons in the molecule. Analysis of compound **6** by ^{1}H, ^{13}C, and 2D NMR (COSY, HETCOR) confirmed the structure as tricin.

β-Sitosterol-3-*O*-β-D-glucoside (**7**) was isolated as a colorless powder that exhibited a fragmentation ion peak at *m*/*z* 414 $[M - \text{glucose}]^+$. The IR spectrum gave absorption bands at 3429, 2933, 1635, 1376, and 1073 cm^{-1}, with the absorption band at 3429 cm^{-1} characteristic of a hydroxyl group. Compound **7** is a glucoside of compound **3**; other than the glucose protons and carbons, the ^{1}H and ^{13}C NMR values of **7** were almost the same as those of **3**. A one-proton doublet at δ 4.97 (J = 11.1 Hz) was attributed to anomeric H-1. Four one-proton doublets at δ 4.16 (J = 9.3 Hz), δ 3.92 (J = 4.45 Hz), δ 3.85 (J = 4.85 Hz), and δ 3.56 (J = 4.85 Hz), and two one-proton multiplets at δ 3.83 and 3.60, were assigned to the remaining glucose protons. In ^{13}C NMR, the H-1 anomeric carbon appeared at δ 103.09, and other glucose carbons appeared at δ 79.11, 78.80, 75.84, 72.22, and 65.58. Based on the above evidence, compound **7** was confirmed as β-sitosterol-3-*O*-β-D-glucoside.

Inhibitory Effects of Compounds on Plants. The inhibitory activity of the compounds against duckweed, and the results of the germination assays in culture tubes on three weed species, *C. difformis*, *L. chinensis*, and *A. retroflexus*, are shown in Tables 1 and 2. Momilactone A and B had high inhibitory activity against duckweed. The other compounds had lower or no inhibitory activity (Table 1). Momilactone A and B showed reduction of chlorophyll content (85.8, 52.3, and 27.0%, and 98.3, 91.9, and 33.9%, respectively) at the concentrations of 10, 3.3, and 1 ppm. At concentrations of 100 and 33 ppm, momilactone A reduced chlorophyll content by 98.9 and 95.6%, respectively.

TABLE 1. INHIBITORY EFFECTS OF SEVEN IDENTIFIED COMPOUNDS AGAINST *Lemna paucicostata* HEGELM 381

Compounds	Concentration (ppm)	Inhibition of chlorophyll content (%)[a]	Visual injury symptoms (%)
Hentriacontane	100	0	0
	10	0	0
1-Tetratriacontanol	100	14.3 ± 9.5	0
	33	13.1 ± 3.5	0
	10	12.8 ± 4.1	0
	3.3	0	0
	1	0	0
β-Sitosterol	100	0	0
	10	0	0
Momilactone A	100	98.9 ± 1.0	95
	33	95.6 ± 1.2	90
	10	85.8 ± 3.7	70
	3.3	52.3 ± 3.0	50
	1	27.0 ± 4.4	20
Momilactone B	100	100	100
	33	100	100
	10	98.3 ± 2.9	98
	3.3	91.9 ± 2.3	70
	1	33.9 ± 8.5	20
Tricin	100	0	0
	10	0	0
β-Sitosterol-3-*O*-β-D-glucoside	100	21.3 ± 4.91	0
	33	20.5 ± 3.6	0
	10	19.4 ± 7.6	0
	3.3	0	0
	1	0	0

[a] Data represent mean ± SD of three replicates.

Visual injury due to momilactone A and B was 70, 50, and 20%, and 98, 70, and 20%, respectively, at concentrations of 10, 3.3, and 1 ppm. The momilactone A caused visual injury symptoms of 98.9 and 95.6% at concentrations of 100 and 33 ppm, respectively (Table 1). This result is similar to that of Kato et al. (1977), who showed that momilactone A and B and their derivatives inhibited the germination of lettuce seeds.

1-Tetratriacontanol reduced chlorophyll by 12.8, 13.1, and 14.3% at the concentration of 10, 33, and 100 ppm, respectively (Table 1). β-Sitosterol-3-*O*-β-D-glucoside reduced chlorophyll by 19.4, 20.5, and 21.3% at 10, 33, and 100 ppm respectively (Table 1). Hentriacontane, β-sitosterol, and tricin did not show any inhibition of chlorophyll nor any visual injury symptoms at concentrations of 10 and 100 ppm.

TABLE 2. GERMINATION AND GROWTH INHIBITORY EFFECTS OF MOMILACTONES A AND B ON THREE WEED SPECIES[a]

Compounds	Concentration (ppm)	Weed species					
		Germination inhibition (%)			Growth inhibition (%)		
		CYPDI[b]	LEFCH[b]	AMARE[b]	CYPDI[b]	LEFCH[b]	AMARE[b]
Momilactone A	1000	100	100	100	100	100	100
	100	100	70	30	100	48.4 ± 0.7	33.3
	20	97.5 ± 2.9	0	20	100	0	18.6 ± 0.56
	4	20	0	20	0	0	0
Momilactone B	1000	100	100	100	100	100	100
	100	100	100	100	100	100	100
	20	100	100	100	100	100	100
	4	23.3 ± 5.8	100	15 ± 5.8	15	23.3 ± 5.8	12.5 ± 6.29

[a] Data represent mean ± SD of three replicates.
[b] CYPDI = *Cyperus difformis* L., LEFCH = *Leptochloa chinensis* (L.) Ness, AMARE = *Amaranthus retroflexus* L.

Germination Assay in Culture Tube. Momilactones A and B inhibited germination of seeds of three weed species (Table 2), whereas hentriacontane, 1-tetratriacontanol, β-sitosterol, tricin, and β-sitosterol-3-*O*-β-D-glucoside did not inhibit germination or growth of the three weed species at the concentrations of 4, 20, 100, and 1000 ppm tested (data not shown). Increasing concentrations of test compounds appeared to be generally correlated with inhibitory effects on germination and growth and showed 100% inhibition at concentrations equal to or greater than 20 ppm. Momilactone A inhibited germination from 0 to 20% at 4 ppm and between 30 and 100% at a concentration of 100 ppm, depending on weed species. Momilactone B had an inhibitory effect on weed growth (~12.5–23.3%) at 4 ppm, whereas momilactone A had no effect at this concentration. Momilactone B inhibited growth completely for all three species at 20 ppm, whereas momilactone A only showed 100% inhibition at 1000 ppm (Table 2).

DISCUSSION

In a search for growth-regulating substances in higher plants, Kato et al. (1973) identified momilactones A and B from the seed hulls of *O. sativa*, and determined that these compounds inhibited the growth of roots of rice at less than 100 ppm. Kato et al. (1977) further reported that momilactones A and B, especially the latter, inhibit the germination of lettuce seeds and growth of roots of rice. In order to establish the functional groups in the momilactones necessary for activity, several derivatives were prepared and assayed for inhibitory activity. It was found that all derivatives that inhibited lettuce seed germination had activity against rice root growth.

Momilactones A and B also were isolated from UV-irradiated, dark-grown rice coleoptiles (Cartwright et al., 1981). The same compounds were also produced in blast-infected, WL 28325-treated rice leaves. They appear to be the first clearly identified cereal phytoalexins. Furthermore, rice seedlings (Kato-Noguchi et al., 2002) inhibited the growth of cress (*Lepidium sativum*) and lettuce (*Lactuca sativa*) seedlings when the cress and lettuce were grown with rice seedlings. The putative compound causing the inhibitory effect of rice seedlings was isolated from their culture solution and identified as momilactone B.

Momilactone B was found recently in root exudates of rice, and 3-d-old rice seedlings were transferred to hydroponic culture and the level of momilactone B released into the environment from the seedlings was measured (Kato-Noguchi and Ino, 2003). The concentration at which momilactone B is released and its effectiveness as a growth inhibitor suggest that it may play a role in rice allelopathy.

There are no reports in the literature for growth or germination inhibition of 1-tetratriacontanol and β-sitosterol-3-*O*-β-D-glucoside. Song et al. (2004) reported that allelochemicals from root exudates of rice varieties at various growth stages were different and caused growth inhibition in barnyard grass. These compounds may be used to control barnyard grass, i.e., weed management based on allelopathy, to reduce labor cost and use of synthetic agricultural chemicals for weed management.

The inhibitory effects of the compounds varied between weed species. In general, germination and growth of *C. difformis* was more inhibited by momilactone A than the other weeds, whereas germination and growth of *L. chinensis* was the most susceptible to momilactone B. *Amaranthus retroflexus* was the least affected at 100 ppm. Momilactone A inhibited germination and growth of *C. difformis* by 97.5 and 100%, respectively, at 20 ppm, whereas *L. chinensis* was not inhibited at the same concentration (Table 2).

Our results were similar to those of Kato et al. (1977), who reported that momilactone B was more inhibitory than momilactone A on lettuce seed germination at 10, 100, and 1000 ppm. This result might suggest that momilactone B has more potential of being used as a natural herbicide than momilactone A. If these compounds are used to contribute to the control of weed species, they might also be used as genetic markers for identifying allelopathic varieties by analyzing hulls before sowing seeds into the field.

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CHEMICAL PROFILES OF SCENT GLAND SECRETIONS IN THE CYPHOPHTHALMID OPILIONID HARVESTMEN, *Siro duricorius* AND *S. exilis*

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Abstract—Gas chromatographic–mass spectrometric analyses of the scent gland secretions of *Siro duricorius* and *S. exilis* (Opiliones, Cyphophthalmi, Sironidae) revealed a set of 24 components, comprising a series of saturated and unsaturated methyl ketones (C11–C15) and four naphthoquinones. Whereas the scent gland secretions of *S. duricorius*, collected in Austria, and *S. exilis* from USA were qualitatively nearly indistinguishable (with the exception of acetophenone that was specific to *S. duricorius*), they distinctly differed in their relative quantitative compositions: major components of the secretion of *S. duricorius* were 7-tridecen-2-one, tridecan-2-one, undecan-2-one, 1,4-naphthoquinone, 6-methyl-1,4-naphthoquinone (tentatively identified only), and 4-chloro-1,2-naphthoquinone. In contrast, in *S. exilis* a compound tentatively identified as 6-methyl-4-chloro-1,2-naphthoquinone was present in large amounts (in *S. duricorius* a trace component), whereas undecan-2-one only occurred in minor quantities. Secretion profiles of juveniles and adults (both sexes) of each species showed high correspondence.

This is the first report on the chemistry of scent gland secretions of the opilionid suborder Cyphophthalmi. 4-Chloro-1,2-naphthoquinone was identified as a new exocrine product of arthropods, whereas 1,4-naphthoquinone and the tentatively identified 6-methyl-1,4-naphthoquinone are known constituents of exocrine secretions from one species of palpatorid opilionids, *Phalangium opilio*. In contrast, all ketones identified were new for opilionid scent glands, although similar ketones are characteristic of scent gland secretions of palpatorid genera *Leiobunum* and *Hadrobunus*. With regard to the near-basic position of Cyphophthalmi in currently proposed phylogenetic

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trees of Opiliones, naphthoquinones and ketones from *Siro* may represent the condition ancestral to the (derived) naphthoquinone- and ketone-rich secretions in phalangid Palpatores.

Key Words—*Siro*, Cyphophthalmi, Opiliones, scent glands, naphthoquinones, methyl ketones.

INTRODUCTION

The arachnid order Opiliones (also known as harvestmen or daddy longlegs) is estimated to comprise about 5,000 species (Shear, 1982). All possess large scent glands in the prosoma (Juberthie, 1976; Martens, 1978). These glands constitute paired hollow sacs that are surrounded by secretory tissue, each sac opening to the body surface via one single pore at either of the lateral margins of the cephalothorax (Juberthie, 1961; Clawson, 1988). Glandular openings may be developed as oblique slits (Acosta et al., 1993) or, as is the case in the opilionid suborder Cyphophthalmi, they may be located atop conspicuous tubercles, so-called ozophores that dorsolaterally protrude from the carapace (Juberthie, 1961). With regard to biological roles, opilionid scent glands are considered to serve mainly for chemical defense: their secretions, discharged as fine sprays or administered by so-called "leg-dabbing" behavior, have been shown to deter ants and other predatory invertebrates (Juberthie, 1976; Martens, 1978; Holmberg, 1986). In addition, pheromonal roles, such as for territorial marking, have been suggested (Juberthie et al., 1991), and recently, the scent gland secretion of a laniatorid opilionid has been reported to possess alarm pheromonal properties (Machado et al., 2002).

Opilionid scent glands not only represent sources of diverse natural products with interesting biological functions, but also may prove useful in chemosystematic studies. Several authors have already alluded to the chemotaxonomic potential of these secretions (Roach et al., 1980; Ekpa et al., 1985), particularly as the taxonomy of this group is in a state of flux (Shultz and Regier, 2001; Giribet et al., 2002). In fact, in Laniatores, benzoquinones and phenols seem to characterize the scent gland secretions of the superfamily Gonyleptoidea (Eisner et al., 1971, 1977; Roach et al., 1980; Duffield et al., 1981; Gnaspini and Cavalheiro, 1998) whereas from one representative of the laniatorid superfamily Travunioidea, terpenes, bornyl esters, and nitrogen-containing products have been reported (Ekpa et al., 1984). In Palpatores, acyclic compounds have been found in representatives of the genera *Leiobunum* and *Hadrobunus*, and naphthoquinones have been found in scent glands of *Phalangium* (overview in Ekpa et al., 1985). In contrast, nothing is known about the scent gland chemistry of the mitelike representatives of the third classical suborder of Opiliones, the Cyphophthalmi. This small group of Opiliones, so far

comprising about 115 species (Giribet and Boyer, 2002), holds a key position in the phylogenetic systematics of Opiliones, possibly representing the most basal opilionid group (e.g., Giribet et al., 2002).

In the present paper, to address this gap in opilionid scent gland chemistry, we investigated the scent gland secretions of two species of cyphophthalmid opilionids from two continents, *Siro duricorius* (mainly distributed in Southeastern Europe) and *S. exilis* (from the United States).

METHODS AND MATERIALS

Specimen Collection. *S. duricorius* (Joseph, 1868) was collected from soil samples at two different localities in Austria. Collection site I was South Carinthia, near Waidischbach (= first collection: 30 adult and 7 juvenile individuals). Collection site II was South Carinthia, Sattnitz (= second and third collection: 27 adults, 9 juveniles and 38 adults, 3 juveniles, respectively). *Siro exilis* Hoffman (1963) was collected from two soil samples from West Virginia, USA (= fourth and fifth collection: 12 adults, 1 juvenile and 14 adults, 8 juveniles, respectively).

Sample Preparation. Scent gland secretions were either directly collected from ozophores or indirectly by whole-body extraction. For direct sampling from ozophores, a specimen was grasped by a leg with forceps, which usually led to the immediate extrusion of a yellowish or brownish droplet of scent gland secretion. The droplet was absorbed on a small piece of filter paper (2 × 2 mm), then extracted for 5 min in hexane (100 μl). Alternatively, whole specimens were extracted in 100 μl of hexane for about 15 min, expelling their secretions directly into the solvent. Extracts were separated from filter paper pieces or bodies, respectively, and frozen until analysis. Each extract contained the exudate from one individual.

Chemical Analysis. Aliquots of crude extracts, in most cases 1 μl, were analyzed by gas chromatography–mass spectrometry (GC-MS), using a Fisions MD 800 GC-MS (Thermo-Quest, Vienna, Austria). The GC column (ZB-5, 30 m × 0.25 mm × 0.25 μm film thickness, Phenomenex via HPLC Service, Vienna, Austria) was directly connected to the ion source of the MS. The splitless Grob injector was held at 260°C with helium carrier gas (1.5 ml/min). The electron impact (EI) ion source of the MS was kept at 200°C and the transfer line at 310°C. The following temperature program was used: initial temperature 50°C/1 min, 10°C/min to 200°C, then 15°C/min to 300°C and hold for 5 min. Dimethyldisulfide (DMDS) derivatives, to determine the positions of double bonds, were prepared according to Vincenti et al. (1987). Methyloxime derivatives of the DMDS-derivatized alkanones were prepared by concentrating 20 μl of the solution containing the DMDS adducts under a stream of N_2

and then adding 50 μl of *O*-methylhydroxylamine hydrochloride (Pierce Biotechnology Inc., Rockford, IL, USA) in pyridine (2%, w/w). The mixture was heated at 75°C for 1 hr. After cooling, 0.5 ml of water was added and the methyloximes were extracted with hexane (2 ml). The solvent was removed under nitrogen and the residue was dissolved in 100 μl ethyl acetate for GC-MS analysis.

Reference Compounds. Undecan-2-one, dodecan-2-one, tridecan-2-one, 1,4-naphthoquinone, and 2-methyl-1,4-naphthoquinone, were purchased from Aldrich (Vienna, Austria). 4-Chloro-1,2-naphthoquinone was prepared as described by Perumal and Bhatt (1980) and Paquet and Brassard (1989): 800 mg lead tetraacetate were added to a solution of 200 mg of 2,4-dichloro-1-naphthol (Aldrich) in 12 ml benzene. The mixture was stirred under nitrogen for 10 hr at room temperature, then poured into 50 ml of water and extracted with ether. The product was purified using silica gel chromatography and $CHCl_3$:hexane (1:2) as eluent.

Scanning Electron Microscopy. For scanning electron microscopy (SEM), specimens were fixed in glutaraldehyde, dehydrated, and mounted on small dishes before sputtering with gold. Micrographs were prepared at the Research Institute for Electron Microscopy, Technical University of Graz, Austria.

RESULTS

Extraction, Separation, and Identification of Scent Gland Components. GC analyses of scent gland secretions of *S. duricorius* and *S. exilis*, either collected directly after discharge from ozophores (Figure 1) on filter paper pieces or indirectly by the technique of whole-body extraction in hexane, led to the same spectrum of 24 gas chromatographically separable compounds (Figure 2, peaks A–X). Chromatographic and mass spectrometric data for the identification of compounds are summarized in Table 1. Generally, the compounds fell into two chemical groups, ketones and naphthoquinones. Among ketones, a homologous series of 20 saturated to triply unsaturated ketones was present. However, peak A (a minor component exclusively present in the secretion of *S. duricorius*) was identified as acetophenone (M^+ at *m*/*z* 120).

Ten compounds of this series (peaks B, C, D, F, J, K, M, N, Q, and W) appeared to be saturated C11–C15 methyl ketones, showing characteristic mass spectra that generally exhibited a prominent fragment ion at *m*/*z* 58 (arising from McLafferty rearrangement). The compounds displayed molecular ions of weak intensity along with M-15, M-18, M-43, and M-58 fragments (see Table 1). The identities of major components of this series (peak B: undecan-2-one; peak D: dodecan-2-one; peak J: tridecan-2-one) were confirmed by comparison of retention times with those of authentic standards. Peaks C and F, with

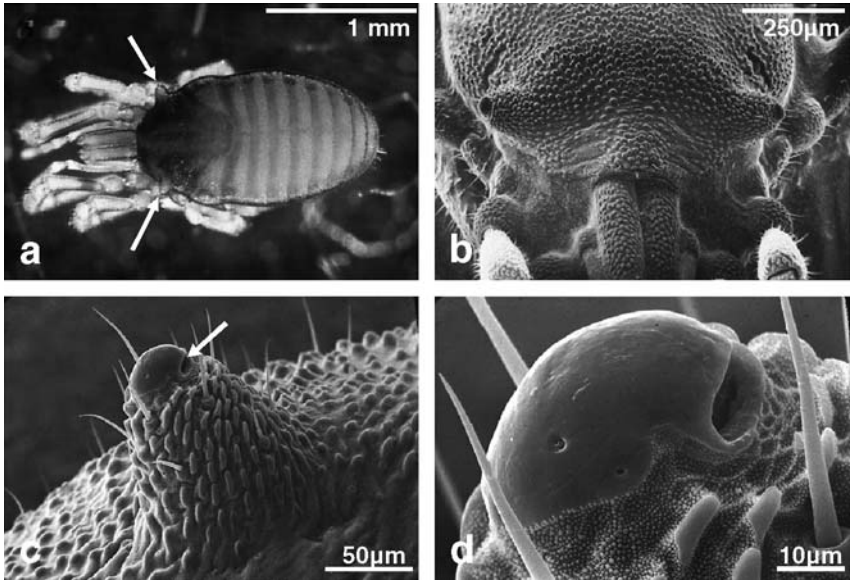

FIG. 1. Topography and external morphology of scent glands in *S. duricorius*. (a) Dorsal view of an adult specimen; arrows point to tubercles of scent glands (ozophores). (b) SEM micrograph of scent gland tubercles; frontal view. (c and d) Details of an ozophore, showing microstructured surface of tubercle and smooth cuticular cap; arrow points to the scent gland orifice.

spectra nearly identical to dodecan-2-one and tridecan-2-one) are tentatively proposed as branched-chain isomers of those compounds, with branch points unknown. All other components of this series (four isomeric tetradecanones: peaks K, M, N, Q, and a pentadecanone: peak W) were of minor abundance and were tentatively identified on the basis of their mass spectra (Table 1).

Five components (peaks G, H, O, P, and U) exhibited mass spectra typical of monounsaturated C13–C15 methyl ketones. Peaks G and H, the major components of this series, were identified as isomeric tridecen-2-ones (M^+ at *m*/*z* 196). The double bond positions were determined by derivatization with dimethyldisulfide. The adducts appeared as one large peak at RT = 20.05–20.15 min. This peak obviously consisted of two components, both with a molecular ion at *m*/*z* 290 as expected, but differing in fragmentation patterns. The larger of the two components (thus derived from larger peak H) showed prominent ions at *m*/*z* 159 and *m*/*z* 131, being consistent with either a 5-tridecen-2-one (fragment at *m*/*z* 131 bearing the carbonyl group) or a 7-tridecen-2-one (fragment at *m*/*z* 159 bearing the carbonyl group). In spectra of the DMDS adduct after *O*-methyl-oximation, adding 29 amu to the carbonyl-group-bearing

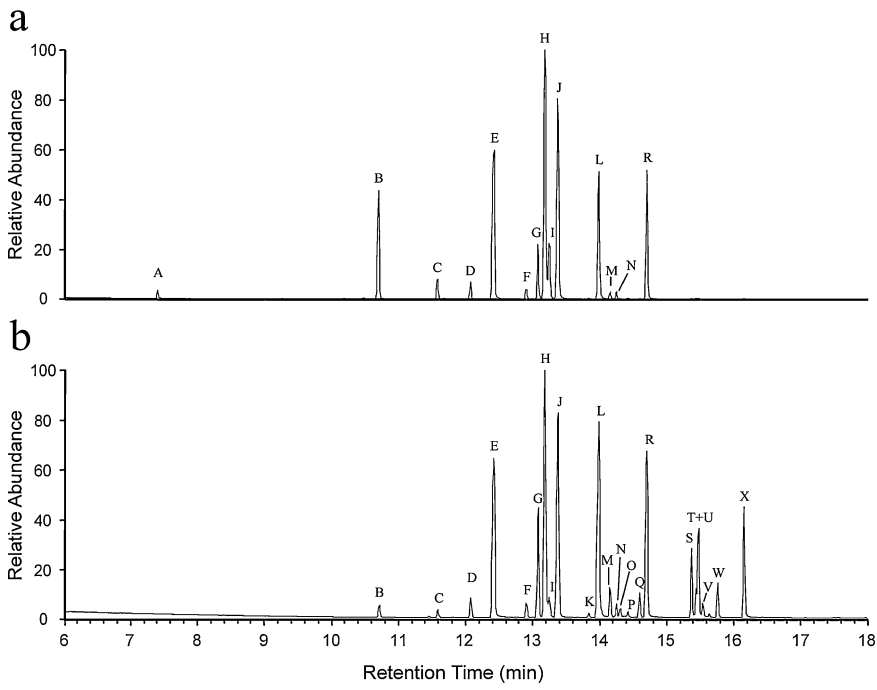

FIG. 2. Gas chromatographic profiles of scent gland secretions of *S. duricorius* (a) and *S. exilis* (b). Peaks marked with an asterisk have been conclusively identified. All other identifications remain tentative. Peak A* (acetophenone), B* (undecan-2-one), C (branched dodecan-2-one), D* (dodecan-2-one), E* (1,4-naphthoquinone), F (branched tridecan-2-one), G* (6-tridecen-2-one), H* (7-tridecen-2-one), I (tridecadienone), J* (tridecan-2-one), K (tetradecan-2-one isomer 1), L (6-methyl-1,4-naphthoquinone), M (tetradecan-2-one isomer 2), N (tetradecan-2-one isomer 3), O (tetradecenone isomer 1), P (tetradecenone isomer 2), Q* (tetradecan-2-one isomer 4), R* (4-chloro-1,2-naphthoquinone), S (pentadecadienone), T (pentadecatrienone), U (pentadecenone), V (unknown), W* (pentadecan-2-one), X (6-methyl-4-chloro-1,2-naphthoquinone).

fragment, the fragment at *m*/*z* 131 remained unaffected whereas the fragment at *m*/*z* 159 disappeared along with the formation of a new fragment at *m*/*z* 188. Thus, the carbonyl group could clearly be classed with the fragment at *m*/*z* 159 in the original DMDS adduct, consistent with the structure of a 7-tridecen-2-one for compound H. The DMDS adduct of the minor compound originating from peak G exhibited a single prominent ion, the base peak, at *m*/*z* 145, indicating a hydrocarbon fragment, and a carbonyl-group-bearing fragment of equal mass. This result is consistent only with the structure of a 6-tridecen-2-one. The remaining monounsaturated ketones (peaks O, P, and U) were present in minor or trace amounts and could only be tentatively identified by their mass spectra

as isomeric tetradecenones and a pentadecenone, respectively (Table 1). Positions of double bonds in these compounds remained undetermined.

Another four components of this series (peaks I, S, T, and V), all of them of minor abundance, were tentatively identified as further homologous C13 and C15 ketones, their spectra indicating double or triple unsaturation. Peak V may possibly represent a mixture of doubly and triply unsaturated C15 ketones (presumed molecular ions at *m*/*z* 220 and *m*/*z* 222).

Naphthoquinones. A second, chemically distinct group of compounds was represented by naphthoquinones (peaks E, L, R, and X). Peak E was identified as 1,4-naphthoquinone by comparisons with an authentic sample. Peak L appeared to be a methylated homolog of 1,4-naphthoquinone, exhibiting a molecular ion at *m*/*z* 172 (base peak), and showing the loss of a methyl group at *m*/*z* 157, together with double neutral loss of CO leading to ions at *m*/*z* 144 and *m*/*z* 116, and loss of CO and COH (fragment at *m*/*z* 115). Prominent ions at *m*/*z* 118, *m*/*z* 90, and *m*/*z* 89 were interpreted to arise from (1) loss of CO and C_2H_2, (2) double loss of CO and C_2H_2, and (3) loss of CO, COH, and C_2H_2, respectively. The spectrum and gas chromatographic retention time, however, were different from authentic 2-methyl-1,4-naphthoquinone (menadione), only leaving the possibilities of positions 5 or 6 for the methyl group. The compound was tentatively identified as 6-methyl-1,4-naphthoquinone by its good correspondence to a literature mass spectrum (from the NIST mass spectral database).

Peak R exhibited the typical isotopic pattern of a monochlorinated compound, indicated by M^+ at *m*/*z* 192 (84%) and an isotopic M+2 peak at *m*/*z* 194 (27%), corresponding to a relative intensity of 32% of the molecular ion. Furthermore, the compound showed sequential loss of two CO fragments from *m*/*z* 192 and 194, leading to twin peaks at *m*/*z* 164 and 166 and to peaks at *m*/*z* 136 and 138, respectively. Also, ions due to loss of Cl (*m*/*z* 157), Cl and CO (*m*/*z* 129, base peak), Cl and double loss of CO (*m*/*z* 101) were observed. The compound was conclusively identified by matches with an authentic standard of 4-chloro-1,2-naphthoquinone.

Peak X also exhibited the isotopic pattern of a mono-chlorinated compound, showing a molecular ion at *m*/*z* 206 (72%) and M+2 at *m*/*z* 208 (22%; corresponding to 31% of M^+). The compound appeared to be a methylated homolog of 4-chloro-1,2-naphthoquinone, exhibiting the loss of a methyl group (twin peaks at *m*/*z* 191 and 193) and again showing repeated decarbonylation and loss of Cl (–CO: *m*/*z* 178 and 180; –CO–CO: *m*/*z* 150 and 152; –Cl: *m*/*z* 171; –Cl and –CO: *m*/*z* 143, base peak). Thus, if peak L is indeed 6-methyl-1,4-naphthoquinone, the compound might be 6-methyl-4-chloro-1,2-naphthoquinone on the basis of mass spectral data.

Secretion Patterns of S. duricorius and S. exilis. In all samples investigated, scent gland secretion profiles of *S. duricorius* and *S. exilis* each showed

TABLE 1. ANALYTICAL DATA TO SCENT GLAND SECRETION COMPONENTS OF *S. duricorius* AND *S. exilis*

Peak no.	Retention time (min)	EI fragmentation (relative intensity)	Identified as[a]
A	7.46	120 (30), 105 (88), 77 (100), 51 (49), 43 (21)	Acetophenone
B	10.72	170 (4), 155 (2), 127 (2), 112 (4), 110 (3), 96 (2), 95 (2), 86 (4), 85 (6), 71 (27), 58 (54), 57 (24), 43 (100), 41 (49)	Undecan-2-one
C	11.59	184 (3), 169 (1), 126 (2), 85 (10), 71 (29), 58 (57), 57 (9), 43 (100), 41 (29)	Dodecan-2-one (branched)
D	12.09	184 (5), 169 (2), 141 (1), 126 (5), 124 (4), 85 (11), 71 (30), 58 (62), 57 (12), 55 (15), 43 (100), 41 (31)	Dodecan-2-one
E	12.43	159 (M+1, 11.5), 158 (M+, 100), 130 (73), 104 (86), 102 (94), 77 (10), 76 (90), 75 (25), 74 (58), 66 (14), 51 (21), 50 (71)	1,4-Naphthoquinone
F	12.95	198 (3), 183 (1), 140 (1), 85 (8), 71 (31), 58 (60), 57 (22), 43 (100), 41 (30)	Tridecan-2-one (branched)
G	13.08	196 (2), 181 (1), 178 (2), 153 (1), 138 (11), 125 (7), 110 (23), 109 (12), 96 (29), 95 (15), 82 (26), 81 (35), 79 (14), 68 (22), 67 (29), 58 (6), 55 (23), 54 (34), 43 (100), 41 (51)	6-Tridecen-2-one
H	13.18	196 (1), 178 (2), 138 (5), 125 (9), 111 (8), 110 (8), 109 (5), 97 (12), 96 (14), 95 (9), 93 (6), 82 (16), 81 (24), 79 (21), 71 (34), 69 (15), 68 (15), 67 (25), 58 (9), 55 (25), 54 (16), 43 (100), 41 (43)	7-Tridecen-2-one
I	13.28	194 (2), 179 (2), 176 (1), 151 (8), 136 (10), 112 (13), 107 (10), 105 (10), 95 (25), 93 (16), 91 (19), 82 (15), 81 (28), 80 (25), 79 (52), 77 (18), 71 (6), 68 (13), 67 (46), 65 (6), 58 (2), 55 (15), 53 (12), 43 (100), 41 (47)	Tridecadienone (tentative)
J	13.38	198 (4), 183 (2), 180 (1), 155 (1), 140 (4), 138 (3), 127 (2), 111 (3), 110 (2), 97 (4), 96 (6), 95 (3), 85 (10), 71 (35), 58 (70), 57 (14), 55 (16), 43 (100), 41 (36)	Tridecan-2-one
K	13.89	212 (1), 197 (1), 169 (1), 154 (3), 152 (1), 125 (2), 111 (3), 109 (3), 96 (8), 95 (5), 86 (12), 85 (18), 71 (13), 67 (12), 58 (15), 57 (53), 56 (35), 43 (100), 41 (88)	Tetradecan-2-one (isomer 1, tentative)
L	14.00	173 (M+1; 13), 172 (M+; 100), 157 (10), 144 (41), 129 (2), 118 (59), 116 (55), 115 (65), 90 (43), 89 (98), 64 (18), 63 (33), 51 (9), 50 (9)	6-Methyl-1,4-naphthoquinone

			(tentative identification)
M	14.15	212 (3), 197 (1), 194 (1), 169 (1), 154 (2), 152 (2), 127 (2), 109 (3), 96 (7), 85 (10), 71 (33), 69 (9), 58 (57), 57 (13), 55 (16), 43 (100), 41 (31)	Tetradecan-2-one (isomer 2, tentative)
N	14.26	212 (3), 197 (1), 194 (1), 183 (1), 154 (1), 125 (6), 109 (4), 96 (11), 85 (10), 83 (10), 71 (37), 58 (57), 57 (18), 55 (21), 43 (100), 41 (39)	Tetradecan-2-one (isomer 3, tentative)
O	14.32	210 (2), 192 (2), 152 (12), 125 (10), 124 (22), 110 (16), 109 (16), 96 (30), 95 (24), 82 (42), 81 (53), 79 (18), 71 (14), 68 (49), 67 (83), 55 (36), 54 (77), 43 (100), 41 (47)	Tetradecenone (isomer 1, tentative)
P	14.44	210 (1), 195 (1), 182 (1), 152 (3), 139 (14), 125 (6), 96 (7) 95 (12), 82 (12), 81 (12), 79 (8), 71 (25), 67 (23), 58 (11), 57 (7), 55 (18), 54 (17), 43 (100), 41 (24)	Tetradecenone (isomer 2, tentative)
Q	14.61	212 (3), 197 (1), 183 (1), 169 (1), 154 (3), 152 (2), 96 (6), 95 (4), 85 (8), 71 (43), 59 (35), 58 (100), 57 (11), 55 (20), 43 (91), 41 (28)	Tetradecan-2-one (isomer 4)
R	14.70	194 (27), 192 (84), 166 (5), 164 (18), 157 (50), 138 (4), 136 (15), 129 (100), 104 (35), 101 (49), 76 (51), 75 (48), 74 (53), 50 (53)	4-Chloro-1,2-naphthoquinone
S	15.38	222 (1), 207 (1), 204 (1), 179 (3), 164 (10), 95 (14), 94 (15), 93 (25), 91 (19), 81 (23), 80 (44), 79 (100), 77 (20), 67 (44), 55 (15), 54 (14), 43 (71), 41 (29)	Pentadecadienone (tentative)
T	15.44	220 (1), 205 (1), 202 (1), 177 (2), 173 (7), 162 (13), 159 (6), 133 (14), 131 (8), 119 (21), 117 (17), 108 (24), 106 (40), 105 (41), 94 (25), 93 (91), 91 (94), 80 (32), 79 (100), 78 (53), 77 (56), 65 (9)	Pentadecatrienone (tentative)
U	15.49	224 (2), 209 (1), 206 (1), 166 (8), 138 (12), 125 (10), 110 (11), 109 (14), 96 (41), 95 (29), 82 (41), 81 (54), 79 (19), 69 (24), 68 (48), 67 (62), 55 (25), 54 (51), 43 (100), 41 (44)	Pentadecenone (tentative)
V	15.55	Data not affirmative	Unknown
W	15.77	226 (2), 211 (1), 208 (1), 168 (2), 96 (6), 95 (4), 85 (11), 71 (38), 59 (32), 58 (100), 55 (17), 43 (71), 41 (22)	Pentadecan-2-one
X	16.15	208 (22), 206 (72), 193 (4), 191 (10), 180 (6), 178 (22), 171 (58), 150 (14), 143 (100), 118 (22), 115 (67), 113 (9), 90 (22), 89 (74), 75 (13), 73 (16), 63 (36)	6-Methyl-4-chloro-1,2-naphthoquinone (tentative)

[a] Compounds B (undecan-2-one), D (dodecan-2-one), E (1,4-naphthoquinone), J (tetradecan-2-one), and R (4-chloro-1,2-naphthoquinone) were identified by comparison of retention times to authentic samples; all other compounds were tentatively identified on the basis of their mass spectral data.

qualitatively and quantitatively consistent compositions (see Table 2). Qualitatively, profiles of the two species were nearly indistinguishable: *S. duricorius* exhibited a maximum of 24 components (some of the trace components were not detectable in certain individuals of two of the three collections), whereas *S. exilis* consistently showed 23 of these compounds, only lacking acetophenone (peak A). In contrast, profiles of *S. duricorius* and *S. exilis* differed significantly in the relative proportions of secretion components, based on the comparison of relative proportions of peak areas from profiles of 114 samples of *S. duricorius* and 35 samples of *S. exilis*. Major components of the *S. duricorius* secretion of adults were (in order of decreasing abundance) tridecan-2-one (about 20%), 7-tridecen-2-one (about 19%), 1,4-naphthoquinone (about 18%), the tentatively identified 6-methyl-1,4-naphthoquinone (about 12%), undecan-2-one (about 10%), 4-chloro-1,2-naphthoquinone (about 7%), and 6-tridecen-2-one (4%). Other components appeared in minor or trace amounts only (Table 2). Secretion patterns did not show large differences between samples of the three different collections, nor between samples of adults and juveniles. Thus, the secretion profile appeared to be consistent within this species. In juveniles, however, the relative quantitative composition of secretion showed higher variation, leading to higher standard deviations, even within samples of the same collection.

Scent gland profiles of *S. exilis* revealed eight major components (five were also major components in *S. duricorius*: see above) including tridecan-2-one (about 20%), 7-tridecen-2-one (about 15%), 1,4-naphthoquinone (about 14%), the tentatively identified 6-methyl-1,4-naphthoqinone (about 13%), 4-chloro-1,2-naphthoquinone (about 12%), pentadecan-2-one (about 5%), the tentatively identified 6-methyl-4-chloro-1,2-naphthoquinone (about 4%), and 6-tridecen-2-one (about 4%). The same components were also major components of juvenile extracts, although in slightly different proportions (Table 2).

DISCUSSION

Cyphophthalmid Scent Gland Chemistry. We present here results from the first chemical investigation into scent gland secretions of cyphophthalmid opilionids for a European and an American species of Sironidae. All components detected belong to the scent gland secretions of these species, as indicated by direct sampling of secretions from ozophores. Profiles appeared to be consistent within each species, only varying slightly between populations and among developmental stages, representing stable and possibly species-specific characters. However, a common ketone- and naphthoquinone-rich chemistry is obvious. Apart from acetophenone, profiles in *S. exilis* seem to be shifted to larger homologous components such as pentadecanone with regard to the ketone series, or the tentatively identified 6-methyl-4-chloro-1,2-naphthoquinone with

regard to the naphthoquinone series, both of which are only trace components in *S. duricorius*. Even though only two species have been studied here, this kind of scent gland chemistry may be representative for a whole group of Cyphophthalmi, at least for the genus *Siro*, considering that the two study species were from different continents.

All methyl ketones as well as the chloronaphthoquinones found in our investigation represent new compounds for scent gland secretions of Opiliones. However, similar ketones, mainly smaller methyl or dimethyl-branched ethyl ketones (Meinwald et al., 1971; Blum and Edgar, 1971; Jones et al., 1976, 1977) are widespread among phalangid Palpatores, and 1,4-naphthoquinone and 6-methyl-1,4-naphthoquinone also have been found in the scent gland secretion of one phalangid species (Wiemer et al., 1978). On the other hand, chlorinated naphthoquinones, to our knowledge, have never been reported previously from exocrine secretions of any arthropod species. Chlorinated exocrine components of arthropods seem to be rare, with the only well-known source for such compounds being the foveal glands of ticks, which produce chlorophenols for sexual communication (Berger, 1972; Yoder et al., 2002; Benoit et al., 2004).

With regard to current concepts of opilionid phylogeny (Martens, 1976; Martens et al., 1981; Shultz and Regier, 2001; Giribet et al., 2002) and the suggested near-basic position of Cyphophthalmi, the *Siro* secretions may represent examples for the ancestral composition of opilionid scent gland chemistry. Interestingly, as mentioned above, phalangid Palpatores, but not Laniatores display characters of the *Siro* secretions. Thus, the ketone-rich secretion profiles of *Leiobunum* and *Hadrobunus* may be derived from ketones analogous to those found in *Siro*. Also, the naphthoquinones detected in *Phalangium opilio* may originate from a cyphophthalmid-like chemistry. In contrast, the lack of ketones and naphthoquinones in scent gland secretions of Laniatores, at least with respect to hitherto known data, would either indicate the complete loss of these compounds and replacement by other compounds in the course of evolution or, alternatively, it might even suggest a distinct evolutionary root for Laniatores, not sharing a common ancestor with Cyphophthalmi. The former case may be supported by preliminary results suggesting the presence of ketones in the scent gland secretion of the laniatorid species, *Parampheres ronae* (Gonzalez et al., 2004), whereas the latter case would confirm the Cyphopalpatores concept of Martens (1976). In this concept, Cyphophthalmi are placed within the classic Palpatores (*sensu* Shear, 1982), and Laniatores represent a separate lineage. However, to begin a comprehensive chemosystematic analysis of Opiliones, chemical data from scent glands of groups hitherto not investigated such as Caddoidea, Ischyropsalidoidea, Troguloidea, and Oncopodoidea must be obtained.

Biology and Roles of Scent Glands of Sironidae. As generally assumed for scent glands of opilionids, also the small, short-legged and mitelike repre-

TABLE 2. RELATIVE ABUNDANCES OF SCENT GLAND SECRETION COMPONENTS OF *S. duricorius* AND *S. exilis*. (CONCLUSIVELY IDENTIFIED COMPOUNDS ARE MARKED WITH AN ASTERISK)

		S. duricorius[a]		*S. exilis*[b]	
Peak no.	Component	Adults	Juveniles	Adults	Juveniles
A	Acetophenone*	0.45 ± 0.25	0.54 ± 0.36	0	0
B	Undecan-2-one*	**9.71 ± 1.59**	**8.84 ± 1.70**	0.57 ± 0.19	0.82 ± 0.30
C	Dodecan-2-one (branched isomer)	1.66 ± 0.50	1.97 ± 0.40	0.34 ± 0.10	0.49 ± 0.13
D	Dodecan-2-one*	2.01 ± 0.61	2.04 ± 0.75	0.89 ± 0.22	0.88 ± 0.17
E	1,4-Naphthoquinone*	**17.61 ± 2.82**	**18.63 ± 7.40**	**14.01 ± 1.90**	**14.58 ± 1.99**
F	Tridecan-2-one (isomer 1, branched)	1.00 ± 0.37	1.09 ± 0.45	0.59 ± 0.20	0.61 ± 0.12
G	6-Tridecen-2-one*	**4.02 ± 1.03**	3.72 ± 1.96	**4.13 ± 0.92**	3.19 ± 1.06
H	7-Tridecen-2-one*	**18.98 ± 2.38**	**19.67 ± 3.56**	**15.47 ± 1.35**	**13.40 ± 2.15**
I	Tridecadienone	3.27 ± 1.56	2.99 ± 0.73	0.65 ± 0.40	0.47 ± 0.26
J	Tridecan-2-one*	**20.21 ± 3.56**	**22.73 ± 2.84**	**20.28 ± 3.79**	**25.98 ± 4.25**
K	Tetradecanone (isomer 1)	0.06 ± 0.08	0.03 ± 0.07	0.17 ± 0.08	0.12 ± 0.04
L	6-Methyl-1,4-naphthoquinone	**12.15 ± 2.03**	9.47 ± **1.63**	**13.08 ± 1.43**	**11.50 ± 2.07**
M	Tetradecanone (isomer 2)	0.52 ± 0.24	0.70 ± 0.30	1.15 ± 0.28	1.29 ± 0.24

N	Tetradecanone (isomer 3)	0.65 ± 0.25	0.67 ± 0.27	0.45 ± 0.23	0.31 ± 0.06
O	Tetradecenone (isomer 1)	0.01 ± 0.01	Trace	0.24 ± 0.14	0.19 ± 0.07
P	Tetradecenone (isomer 2)	0.06 ± 0.09	0.03 ± 0.08	0.19 ± 0.11	0.20 ± 0.05
Q	Tetradecanone* (isomer 4)	0.05 ± 0.08	0.02 ± 0.05	1.10 ± 0.33	1.14 ± 0.17
R	4-Chloro-1,2-* naphthoquinone	**7.09 ± 2.44**	**6.64 ± 2.81**	**11.83 ± 1.68**	**8.62 ± 1.10**
S	Pentadecadienone	0.04 ± 0.06	0.02 ± 0.05	3.00 ± 0.86	2.72 ± 0.75
T	Pentadecatrienone	0.05 ± 0.08	0.02 ± 0.05	0.92 ± 0.35	0.49 ± 0.38
U	Pentadecenone	0.03 ± 0.05	0.01 ± 0.03	**4.57 ± 1.14**	**4.94 ± 0.89**
V	Unknown	0.01 ± 0.01	Trace	0.37 ± 0.19	0.36 ± 0.13
W	Pentadecan-2-one*	0.01 ± 0.02	0.01 ± 0.02	1.68 ± 0.50	2.12 ± 0.35
X	6-Methyl-4-chloro-1, 2-naphthoquinone	0.36 ± 0.51	0.17 ± 0.12	**4.30 ± 1.24**	**5.59 ± 1.68**

[a]Mean values based on 95 adult and 19 juvenile profiles; [b]mean values based on 26 adult and 9 juvenile profiles.
Main components (comprising more than 3% relative peak area) are printed in bold.

sentatives of Sironidae use their secretions for defense. When disturbed, *S. duricorius*, for example, expels a small yellowish to brownish droplet from ozophores and transfers this droplet to the aggressor by leg dabbing. This behavior is also known from other species of Sironidae (Juberthie, 1961). The defensive or toxic potential of the secretions to predatory microarthropods seems to be high: as reported for *S. rubens*, spiders and isopods died immediately when contaminated with the secretion, and when exposed to vapors of secretions, narcosis occurred (Juberthie, 1976). For *S. rubens*, no chemical data are available yet, but a ketone–naphthoquinone-dominated chemistry of scent gland secretions, comparable to our congeneric study species, is to be expected. Thus, similar defensive properties against predatory arthropods may be proposed for secretions of *S. duricorius* and *S. exilis* as well. However, bioassays with single secretion components have not yet been performed. Also, a possible alarm pheromonal role, as recently demonstrated for an opilionid scent gland secretion (Machado et al., 2002), should be investigated. In addition, compounds such as naphthoquinones are known to possess antimicrobial properties. For example, 6-methyl-1,4-naphthoquinone inhibits growth of *Staphylococcus aureus* (Bendz, 1951). Thus, the *Siro* secretion might also have a role in protecting against microorganisms, which would be of particular importance to inhabitants of a humid, fungi- and bacteria-rich environment in soil.

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IDENTIFICATION OF A MALE-PRODUCED AGGREGATION PHEROMONE IN THE WESTERN FLOWER THRIPS *Frankliniella occidentalis*

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Abstract—Two major components have been detected in the headspace volatiles of adult male *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) that are not present in the headspace volatiles of adult females. The compounds were identified as (*R*)-lavandulyl acetate and neryl (*S*)-2-methylbutanoate by comparison with synthetic standards using gas chromatography (GC), GC–mass spectrometry (MS), and chiral GC. Field trials were conducted with synthetic compounds in naturally infested crops of sweet pepper grown in large plastic greenhouses in Spain. The catch of adult females and males on blue sticky traps was increased by neryl (*S*)-2-methylbutanoate alone or by a 1:1 blend of (*R*)-lavandulyl acetate and neryl (*S*)-2-methylbutanoate, but (*R*)-lavandulyl acetate was not active alone. This is the first identification of an aggregation pheromone in the order Thysanoptera. The possible role of (*R*)-lavandulyl acetate is discussed.

Key Words—Western flower thrips, *Frankliniella occidentalis*, Thysanoptera, Thripidae, aggregation pheromone, (*R*)-lavandulyl acetate, neryl (*S*)-2-methylbutanoate.

INTRODUCTION

The adult males of many species of thrips (order Thysanoptera) possess sternal glands or *areae porosae* (Mound et al., 1980), which are structurally consistent with the production of a pheromone (Bode, 1978; Sudo and Tsutsumi, 2002).

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Until now, no sex or aggregation pheromones have been identified in the Thysanoptera, although many defensive anal secretions are known (Suzuki et al., 2004). Recent experiments have provided behavioral evidence for a male-produced sex pheromone in two species in the genus *Frankliniella*, both of which possess sternal glands. Live adult male *Frankliniella schultzei* (Trybom) increased the catch of adult females, but not of adult males, on gray sticky traps (Milne et al., 2002), and the odor of adult male *Frankliniella occidentalis* (Pergande), but not of adult females, attracted adult females in a Y-tube olfactometer (Hamilton and Kirk, 2003; de Kogel and van Deventer, 2003; Kirk and Hamilton, 2004). The male odor of *F. occidentalis* was found by Kirk and Hamilton (2004) to be attractive also to adult males, but this was not found by de Kogel and van Deventer (2003).

F. occidentalis has spread since the 1970s to become a major worldwide pest of horticulture and agriculture (Kirk and Terry, 2003), so an attractive pheromone could have considerable applied value. The males form mating aggregations, within which there are aggressive male–male interactions, and females arrive, mate, and leave immediately (Terry and Gardner, 1990; Terry and Dyreson, 1996). A male-produced pheromone could play several roles in these complex interactions. Kirk and Hamilton (2004) used gas chromatography (GC) analysis to demonstrate that two major odor components and five minor components were present in male odor and not in female odor of adult *F. occidentalis*.

The objectives of this study were to identify the major components of the male volatiles and to test these compounds for attractiveness in the field.

METHODS AND MATERIALS

Thrips Rearing. A colony of *F. occidentalis* from commercial glasshouses in the UK was maintained on potted chrysanthemums (*Dendranthema grandiflora* Tzvelev) at 25 ± 2°C and L8:D6. Lighting was provided by full-spectrum fluorescent tubes (58 W Sylvania Activa 172 Professional). Adult male thrips collected arbitrarily from this culture are described as "mixed age."

Collection of Male Volatiles. Mixed age thrips were knocked from the chrysanthemum flowers onto a white tray. Adult males were collected with a small aspirator, anesthetized with carbon dioxide, and transferred into a clean glass container (1.9 ml) that was then sealed with Teflon tape. The thrips were illuminated from above with a bright light (60 W tungsten filament lamp) to induce patrolling behavior (Kirk and Hamilton, 2004). Patrolling occurs when the males swarm, and this behavior may be necessary for pheromone production. Head space volatiles were collected on a divinylbenzene (DVB)/carboxen/polydimethylsiloxane (PDMS) solid phase microextraction (SPME)

fiber assembly (57348-U, Supelco, Poole, UK) at 27 ± 2°C for 4–5 hr (Kirk and Hamilton, 2004). The numbers of males entrained in this way varied from 30 to 70 per replicate.

Gas Chromatography–Mass Spectrometry (GC–MS). GC–MS analyses were carried out on HP 5890 II+ gas chromatographs coupled to HP 5972A (Keele) or HP 5973 Natural Resources Institute (NRI) mass spectrometers (Agilent Technologies, Ipswich, UK) operated in electron impact (EI) (70 eV, 180°C) or chemical ionization (CI) mode. CI analyses were carried out using isobutane or ammonia as the reagent gas.

SPME-collected headspace volatile samples and synthetic standards were analyzed at Keele with a DB5MS or a DBWax (30 m × 0.25 mm i.d., 0.25-μm film) analytical column (Agilent Technologies, Ipswich, UK). Samples were introduced into the GC via a Merlin Microseal (Thames-Restek, High-Wycombe, UK) septumless heated injector (180°C) fitted with a SPME glass injection sleeve (0.75-mm i.d.; Supelco, UK). SPME samples were desorbed for 8 min before the fiber assembly was withdrawn. The GC was programmed with 2 min at 40°C, then 10°C/min to 250°C (10 min).

Synthesis products were analyzed at NRI using SPB1 (30 m × 0.25 mm i.d., 0.2-μm film; Supelco, Belefonte, PA) or DBWax columns programmed from 60°C for 2 min then 6°C/min to 250°C. Kovats retention indices (KI) were calculated relative to the retention times of saturated hydrocarbons.

Chiral Chromatography. Analysis of enantiomeric composition was carried out by GC on either a Beta-Dex 325 column (30 m × 0.25 mm; 0.25-μm film: Supelco, UK) for lavandulyl acetate, or a CP-Chirasil-Dex CE column (25 m × 0.32 mm; 0.25-μm film: Varian, UK) for neryl 2-methylbutanoate. The injector temperature was set at 200°C, and the oven was run isothermally at 80°C for lavandulyl acetate and at 120°C for neryl 2-methylbutanoate.

Chemicals and Synthesis of Compounds. Racemic lavandulyl acetate (99% purity) was obtained from TCI-America (Portland, OR). The enantiomers of lavandulol were obtained previously (Cross et al., in press) from racemic lavandulol by kinetic resolution using conversion to the acetate by *Pseudomonas fluorescens* lipase (Amano AYS, Nagoya, Japan), isopropenyl acetate, and diisopropyl ether, essentially as described by Zada and Harel (2004). The enantiomerically enriched *R* acetate was hydrolyzed back to the alcohol with potassium carbonate in methanol and recycled twice. GC analysis on the cyclodextrin capillary column showed this material to have enantiomeric excess (ee) of 98.2%. The remaining alcohol was similarly recycled to give (*S*)-lavandulol with ee of 87.0%.

The stereochemistry was confirmed by comparison of the corresponding alcohol with (*R*)-(−)-lavandulol isolated from lavender oil (König et al., 1992). Lavender oils of different origins were screened by GC for lavandulol and the acetate, and the one with the highest levels (Goodebodies, Dublin, Ireland),

containing 4.7%, was selected. A sample (6 g) was hydrolyzed with potassium carbonate in methanol, and the lavandulol was isolated by column chromatography on silica gel eluted with 20% diethyl ether in hexane. Two fractionations gave material (0.18 g) containing 75% (*R*)-(−)-lavandulol, the major impurities being bomeol (13%) and linalool (4%). In GC analysis on the cyclodextrin column, the *S*-enantiomer was undetectable (<0.1%).

Geranyl and neryl acetate were synthesized by acetylation of the corresponding alcohols with acetic anhydride in pyridine. Other monoterpense esters were synthesized from the corresponding alcohol and acid in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 4-*N*,*N*′-dimethylaminopyridine (DMAP) (Neises and Steglich, 1978), as described below for neryl (*S*)-2-methyl-butanoate.

Neryl (S)-2-methylbutanoate. Nero1 (0.77 g, 5 mmol; Sigma-Aldrich, Gillingham, Dorset, UK), (*S*)-2-methylbutanoic acid (0.61 g, 6 mmol; Sigma-Aldrich), and DMAP (61 mg, 0.05 mmol; Sigma-Aldrich) were dissolved in dry dichloromethane (10 ml), and the solution was stirred in an ice bath. DCC (1.24 g, 6 mmol; Sigma-Aldrich) was added portionwise over 30 min, and stirring was continued for another 30 min with cooling and then for 3 hr at room temperature. The *N*,*N*′-dicyclohexylurea was filtered off, and the precipitate was washed with petroleum spirit (b.p. 40–60°C). The filtrate was washed with saturated aqueous sodium bicarbonate solution, dilute hydrochloric acid, and water, dried over magnesium sulfate, and filtered. After concentration, the residue was purified by flash chromatography on silica gel (40 g, 230–400 mesh) eluted with 1.5% diethyl ether in petroleum spirit. The resulting product was distilled in a Kugelrohr apparatus (90°C/0.02 mmHg, 1.05 g, 88%, e.e. 97.8%). ^{1}H NMR ($CDCl_3$, 270 MHz): δ 5.35 (br t, 1H, J = 7 Hz, =C*H*–CH_2O), 5.09 (m, 1H, =C*H*–CH_2), 4.56 (d m, 2H, J = 7 Hz, =CH–CH_2O), 2.36 (hextuplet, 1H, J = 7 Hz, C*H*–COOR), 2.1 (m, 4H, =C–CH_2–CH_2–C=), 1.77 (br s, 3H, CH_3–C=), 1.68 (br s, 3H, CH_3–C=), 1.67 (hextuplet, 1H, J = 6.5 Hz, CH_3–C*H*H–CH(CH_3), 1.61 (br s, 3H, CH_3–C=), 1.45 (hextuplet, 1H, J = 6.5 Hz, CH_3–CH*H*–CH(CH_3), 1.14 (d, 3H, J = 6.5 Hz, CH_3–CH), 0.90 (t, 3H, J = 7 Hz, CH_3–CH_2). ^{13}C NMR ($CDCl_3$, 67.8 MHz) δ 176.83, 142.32, 132.14, 123.63, 119.41, 60.86, 41.10, 32.19, 26.85, 26.69, 25.69, 23.52, 17.66, 16.62, 11.64.

Field Trials. The biological activity of the compounds was tested in May 2004 in commercial crops of sweet pepper (var. Habana) grown in greenhouses at Zamora (37°42.945′N, 0°57.720′W) near Torre Pacheco in the Murcia region of Spain. The crops were about 110 cm high, planted in rows 1 m apart, and were naturally infested with a low level of *F. occidentalis*. Three 2-d experiments were carried out, alternating between two nearby houses, each with an area of about 6500 m^2.

The compounds were released from rubber septa (6.3-mm diameter × 10.8 mm long) (100706; Aldrich, UK) that had been preextracted in hexane–dichloromethane and dried in an oven at 50°C. Septa were loaded with

solutions of the test compounds in hexane or with hexane alone (control). After evaporation of the solvent, the rubber septa were stored in aluminum foil bags. They were placed on traps in the field within 2 hr of preparation.

Blue plastic rectangular takitraps (10 × 25 cm) (Syngenta Bioline, UK), coated on both surfaces with insect glue, were suspended vertically with the base about 10 cm above crop height, by attaching them to crop support strings with a wooden clothes peg. One rubber septum was stuck to the center of the north-facing side of each trap.

Each experiment was laid out in a randomized complete block design with 20 blocks and one replicate per block. Traps were placed about 3.4 m apart down rows of sweet pepper plants with 16 m between each row of traps. The first experiment compared two doses, 30 ng and 30 μg, of neryl (*S*)-2-methylbutanoate with a control. The second experiment compared two doses, 30 ng in total and 30 μg in total, of a 1:1 blend of (*R*)-lavandulyl acetate and neryl (*S*)-2-methylbutanoate with a control. The third experiment compared two doses, 30 ng and 30 μg, of (*R*)-lavandulyl acetate with a control.

Trap Counts and Statistical Analysis. Thrips caught on the blue sticky traps were examined under a stereoscopic microscope to identify their sex and to distinguish *F. occidentalis* from about 2% of the thrips that were other species (*Thrips* spp., aeolothripids, and phlaeothripids). The data were $\log_{10}(x + 1)$ transformed and analyzed by two-way ANOVA for females, males, and both sexes combined. Multiple comparisons with the control used Dunnett's multiple comparison test. Statistical analysis was carried out with Minitab 14 (Minitab Inc., Pennsylvania, USA).

RESULTS

Pheromone Identification. We collected volatiles from the headspace around male thrips with a SPME fiber. Subsequent coupled GC–MS analysis showed the presence of two principal compounds (Kirk and Hamilton, 2004). The earlier eluting compound (peak *a*) gave an EI mass spectrum with no clearly identifiable molecular ion (Figure 1A). However, the presence of a trace ion at *m*/*z* 154 (0.4%) with stronger ions at *m*/*z* 136 (14%), 121 (23%), 93 (73%), 69 (100%), and 41 (47%) suggested a compound with a monoterpenoid substructure. The presence of the ion at *m*/*z* 43 (72%) suggested the presence of a $C_2H_3O^+$ ion derived from an acetate moiety. Comparison with published spectra (SDBS, 2004) revealed close matches with the spectra of lavaudulyl acetate, neryl acetate, and geranyl acetate. Isobutane CI analysis gave a strong ion at *m*/*z* 197 ($[M + H]^+$) and ammonia CI gave strong ions at *m*/*z* 197 and 214 ($[M + H]^+$ and $[M + NH_4]^+$), confirming the molecular weight as 196. The

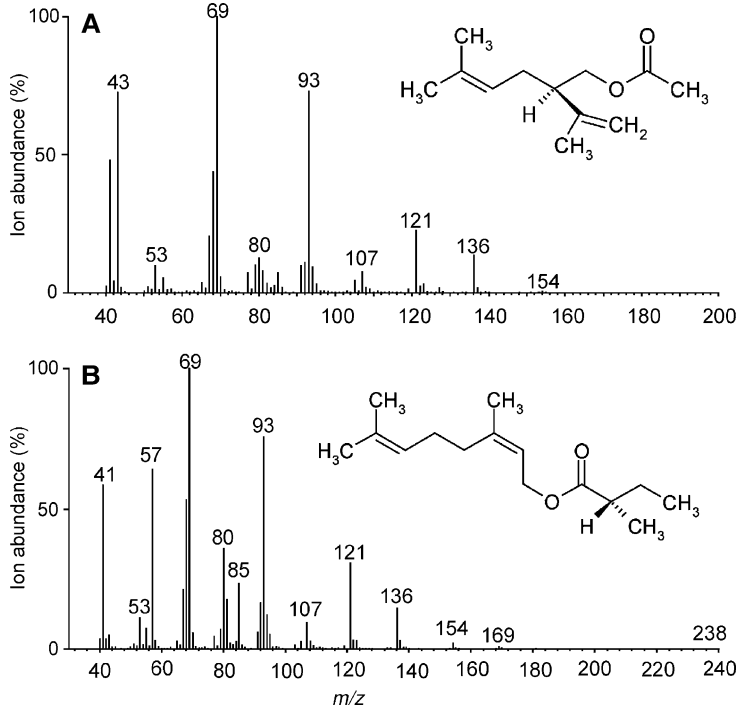

FIG. 1. EI mass spectra (70 eV) of the major components of the headspace volatiles of male western flower thrips *Frankliniella occidentalis*: (A) peak *a*; (B) peak *b*. Insets in (A) and (B) show the structures of the major components (*R*)-lavandulyl acetate and neryl (*S*)-2-methylbutanoate of the headspace volatiles of male western flower thrips *F. occidentalis*.

identity of the thrips-produced compound was confirmed by comparison of the EI and CI mass spectra and relative retention times of the unknown with (±)-lavandulyl acetate on polar and nonpolar GC columns (Table 1). Peak enhancement was achieved on coinjection of the entrained thrips volatiles and (±)-lavandulyl acetate.

The EI mass spectrum of peak *b* (Figure 1B) was similar to that of peak *a*, with ions at *m*/*z* 154 (1.9%), 136 (15%), 121 (31%), 93 (75%), 69 (100%), and 41 (60%), again suggesting a monoterpenoid substructure. An ion at *m*/*z* 238 (0.03%) suggested a molecular weight of 238 and an ion at *m*/*z* 85 (23%) suggested the loss of a $C_5H_9O^+$ fragment derived from a 5-carbon acid moiety. No database matches were found for this spectrum. Isobutane CI analysis gave a strong ion at *m*/*z* 239 ($[M + H]^+$) and *m*/*z* 256 ($[M + NH_4]^+$), confirming the molecular weight as 238.

TABLE 1. RETENTION INDICES (KI) OF NATURAL AND SYNTHETIC COMPOUNDS

	KI			
	DBSMS (Keele)	SPBI (NRI)	DBWax (Keele)	DBWax (NRI)
Natural component (peak *a*)	1292		1593	
Natural component (peak *b*)	1579		1850	
Lavandulyl acetate	1292	1274	1593	1608
Geranyl acetate		1363		1760
Neryl acetate		1346		1728
Lavandulyl pentanoate		1543		1839
Lavandulyl 3-methylbutanoate		1498		1772
Lavandulyl 2-methylbutanoate		1497		1761
Geranyl pentanoate		1636		1994
Geranyl 3-methylbutanoate		1590		1925
Geranyl 2-methylbutanoate		1584		1902
Neryl 3-methylbutanoate		1565		1884
Neryl 2-methylbutanoate	1581	1560	1851	1865

A series of isomeric esters of monoterpene alcohols and 5-carbon acids was synthesized for comparison of GC retention data (Table 1) and EI mass spectra with those of peak *b* in the thrips volatiles. The candidate compounds were selected on the basis of the changes in GC retention times and small changes in the EI mass spectrum that resulted from varying each part of the molecule. Neryl 2-methylbutanoate had identical retention times on nonpolar and polar GC columns to those of natural peak *b* (Table 1), and the mass spectra were superimposable. Coinjection of the thrips volatiles with neryl (*S*)-2-methylbutanoate gave peak enhancement of peak *b*.

Chiral Chromatography. Racemic lavandulyl acetate gave two peaks at 27.91 and 25.09 min, (*R*)-lavandulyl acetate eluted at 27.89 min, and peak *a* of the entrained thrips headspace volatiles eluted at 27.97 min. Coinjection of entrained thrips headspace volatiles (peak *a*) and (*R*)-lavandulyl acetate (1 ng) gave an enhanced single peak at 27.94 min proving the (*R*)-configuration of the thrips-produced compound.

Racemic neryl 2-methylbutanoate (5 ng) gave two peaks at 21.85 and 22.23 min on the chiral column, whereas neryl (*S*)-2-methylbutanoate (5 ng) gave a peak at 22.43 min, and entrained thrips headspace volatile peak *b* gave a peak at 22.51 min. Coinjection of entrained thrips headspace volatiles and neryl (*S*)-2-methylbutanoate (5 ng) gave a single peak at 22.54 min, confirming the (*S*)-configuration for peak *b*.

The ratio of the area of peak *a* to peak *b* varied considerably among entrainments, ranging from 1:0.8 to 1:5. A ratio within this range (1:1) was selected for field trials.

TABLE 2. MEAN CATCH OF ADULT *F. occidentalis* ON BLUE STICKY TRAPS WITH (*R*)-LAVANDULYL ACETATE [(*R*)-LA] OR NERYL (*S*)-2-METHYLBUTANOATE [N(*S*)2MB] SEPARATELY OR AS 1:1 BLEND

Compound	Thrips	Mean catch over 2 days ± SE ($N = 20$)		
		Control	Low dose	High dose
N(*S*)2MB[a,b]	Females	5.9 ± 0.7	6.8 ± 0.7 ns	9.1 ± 1.2*
	Males	905 ± 0.8	11.9 ± 1.2 ns	13.2 ± 1.1**
	Both sexes	15.4 ± 1.2	18.7 ± 1.7 ns	22.2 ± 2.0***
(*R*)-LA[a,b]	Females	8.3 ± 1.2	8.3 ± 0.9 ns	7.8 ± 0.9 ns
	Males	9.0 ± 1.5	7.6 ± 1.0 ns	7.2 ± 1.0 ns
	Both sexes	17.4 ± 2.4	16.0 ± 1.6 ns	15.0 ± 1.8 ns
1:1 blend[b,c]	Females	2.8 ± 0.6	4.4 ± 1.1 ns	5.2 ± 1.1**
	Males	3.9 ± 1.0	5.5 ± 1.7 ns	5.8 ± 1.3*
	Both sexes	6.8 ± 1.6	10.0 ± 2.7 ns	11.1 ± 2.4**

[a]Of the compound, 30 ng is for low dose and 30 μg is for high dose.
[b]The 1:1 blend comprising 15 ng of each compound for low dose (total 30 ng) and 15 μg of each compound for high dose (total 30 μg).
[c]Dunnett's test comparing each dose with corresponding control using $\log_{10}(x + 1)$ transformed data: ns = not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Field Trials. There was no significant effect of (*R*)-lavandulyl acetate on trap catches at a dose of 30 ng or 30 μg (Table 2). However, neryl (*S*)-2-methylbutanoate and the 1:1 blend of (*R*)-lavandulyl acetate and neryl (*S*)-2-methylbutanoate increased trap catches significantly at a dose of 30 μg (Table 2). Both sexes were attracted, and the effect was similar for both females and males. The percentage increases with neryl (*S*)-2-methylbutanoate (54% for females and 38% for males) were broadly similar to those with the 1:1 blend (84% for females and 50% for males). There were no significant effects at 30 ng for the test compounds.

DISCUSSION

This report presents the first identification of an aggregation pheromone in the Thysanoptera. Although our previous studies of this pheromone have referred to it as a sex pheromone (Kirk and Hamilton, 2004) it should have been referred to as an aggregation pheromone because it attracts both sexes. (*R*)-lavandulyl acetate and neryl (*S*)-2-methylbutanoate were identified as the two main male-specific compounds, but only the latter showed activity in field trials. The sternal glands are the most likely source of the two compounds in *F. occidentalis*, but this has not yet been proven. These two compounds do not

appear to have been identified previously as insect semiochemicals. However, lavandulyl acetate is a component of the essential oil of lavender (Lis-Balchin, 2002), occurring as the *R*-enantiomer (unpublished data). It may be relevant that the natural distribution of the lavender genus (*Lavandula*) in parts of Europe, Asia, and Africa (Upson, 2002) does not overlap the original distribution of *F. occidentalis* in western North America (Kirk and Terry, 2003). Other lavandulyl esters have been identified recently as sex pheromone components in mealybugs: (*S*)-lavandulyl senecioate and (*S*)-lavandulyl isovalerate in the vine mealybug *Planococcus ficus* (Millar et al., 2002; Zada et al., 2003) and (*R*)-lavandulyl (*S*)-2-methylbutanoate in the pink hibiscus mealybug *Maconellicoccus hirsutus* (Zhang et al., 2004).

Field trapping trials showed neryl (*S*)-2-methylbutanoate alone was attractive to both female and male *F. occidentalis*, whereas (*R*)-lavandulyl acetate was not attractive. The 1:1 blend gave similar results to neryl (*S*)-2-methylbutanoate alone; thus, there was no strong indication of synergism between the two compounds. The considerable natural variation in the ratio of the two compounds found in headspace volatiles also suggests that synergism is unlikely or at least does not depend on a critical ratio. Thus, the role of (*R*)-lavandulyl acetate remains unclear. It could have a role in the close-range behavioral interactions between males at a mating aggregation, or it could have a role during copulation. The situation may be analogous to that in the cockroach *Nauphoeta cinerea*, in which the three principal active components from the male sternal glands have overlapping roles in attracting females from a distance and in close-range interactions between females and males (Moore, 1997). Alternatively, although our high dose of (*R*)-lavandulyl acetate was 1000 times that of the low dose, the release rates from the rubber septa could still have been outside the active range. Furthermore, the relative release rates of the two compounds were not determined.

Both females and males responded to neryl (*S*)-2-methylbutanoate, which mirrors the walking response shown earlier by adult females and males to the odor of adult males in a Y-tube olfactometer (Kirk and Hamilton, 2004).

Blue traps are widely used for trapping *F. occidentalis* because one shade of blue gives the highest visual attraction (Brødsgaard, 1989; Vernon and Gillespie, 1990). We tested our compounds on these traps to see whether the catch could be increased on the most visually attractive traps available and also because mating aggregations usually occur on highly visible objects, particularly brighter blues (Kirk, 1985; Matteson and Terry, 1992). The trap catches for each sex were increased by 38–84% upon addition of the high doses of the test compounds. Larger percentage increases in trap catches of thrips have been found with other odors, but those experiments used traps of less attractive colors, such as green (Murai et al., 2000) or much higher doses of chemical (Teulon et al., 1993). Other factors that could have reduced the effect in our

experiments were that the traps were close together; thus, the odor could have reached adjacent control traps, and the air was relatively still in the greenhouse. Little is known about whether thrips fly upwind to odors and thus need a flow of air to respond effectively (Teulon et al., 1999). Also, the best release rate is not yet known, and it is possible that minor components in the male odor may play a role. Further experiments to test these possibilities are in progress.

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4,8-DIMETHYLDECANAL, THE AGGREGATION PHEROMONE OF *Tribolium castaneum*, IS BIOSYNTHESIZED THROUGH THE FATTY ACID PATHWAY

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Abstract—4,8-Dimethyldecanal (4,8-DMD) is the aggregation pheromone produced by male red flour beetles (RFB), *Tribolium castaneum*. To elucidate the biosynthetic origin of 4,8-DMD, the following studies were performed: (1) effects of juvenile hormone (JH) III, and pathway inhibitors mevastatin, an inhibitor of the mevalonate pathway, and 2-octynoic acid, an inhibitor of the fatty acid pathway, were tested to determine whether 4,8-DMD is derived from the fatty acid pathway or the mevalonate pathway; (2) incorporation of ^{13}C-labeled acetate, propionate, and mevalonolactone into 4,8-DMD was measured to directly determine the biosynthetic origin of 4,8-DMD; and (3) incorporation of deuterium-labeled precursors, including 2-methylbutanoate (C5D), 4-methylhexanoate (C7D), 2,6-dimethyloctanoate (C10D), and 4,8-dimethyldecanoate (C12D) was tested to determine whether 4,8-DMD is biosynthesized in the sequence Ac–Pr–Ac–Pr–Ac (Ac; acetate, Pr; propionate). JH III was topically applied to males at various doses. Inhibitors and isotopically labeled substrates were administered orally by feeding the beetles flour treated with the substrates of interest, after which volatiles were collected from both sexes of RFBs. The amount of 4,8-DMD produced was significantly reduced with increasing doses of JH III. Also, 2-octynoic acid inhibited the production of 4,8-DMD, but mevastatin did not. Exposure of RFBs to [1-^{13}C]acetate and [1-^{13}C]propionate, but not [2-^{13}C]mevalonolactone, resulted in incorporation of the labeled compounds into 4,8-DMD. RFBs fed flour treated with deuterium-labeled C5D, C10D, and C12D, but not C7D, incorporated these compounds into 4,8-DMD. The findings that the production of 4,8-DMD was inhibited by 2-octynoic acid but unaffected by mevastatin, combined with the high incorporation of [1-^{13}C]acetate and [1-^{13}C]propionate into 4,8-DMD and the incorporation of

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deuterated precursors, unambiguously demonstrated that 4,8-DMD is of fatty acid rather than terpene biosynthetic origin, and that the biosynthesis of 4,8-DMD proceeds in the sequence Ac–Pr–Ac–Pr–Ac.

Key Words—*Tribolium castaneum*, red flour beetle, Tenebrionidae, aggregation pheromone, 4,8-dimethyldecanal, biosynthesis, JH III, mevastatin, 2-octynoic acid, fatty acid pathway.

INTRODUCTION

Males of the flour beetle *Tribolium castaneum*, *T. confusum*, and *T. freemani* (Coleoptera: Tenebrionidae) produce 4,8-dimethyldecanal (4,8-DMD) as a common aggregation pheromone (Suzuki, 1980; Suzuki et al., 1987). 4,8-DMD has since been thought to be biosynthesized through the mevalonate pathway because it has a partial terpene skeleton and is easily obtained by oxidative cleavage of the isopentenyl group of a sesquiterpene farnesol derivative (Vanderwel and Oehlschlager, 1987; Howse et al., 1998). However, it is also possible that 4,8-DMD is biosynthesized via the fatty acid pathway, as in the case of 4-methyl-1-nonanol, the sex pheromone of *Tenebrio molitor* (Coleoptera: Tenebrionidae) (Tanaka et al., 1986, 1989; Islam et al., 1999).

Enzyme activators and inhibitors have been used for investigation of the biosynthetic origins of pheromones in Coleoptera (Tillman et al., 1999; Seybold and Vanderwel, 2003). JH III reportedly activates 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-R) and synthase (HMG-S) (Tittiger, 2003). JH III and its analogs enhanced the production of pheromones derived from the mevalonate pathway in Scolytidae (Chen et al., 1988; Ivarsson and Birgersson, 1995). In contrast, mevastatin blocks the action of HMG-R in the biosynthesis of the terpene precursor mevalonate (Endo et al., 1976; Monger et al., 1982). Ivarsson et al. (1993) provided indirect evidence for *de novo* biosynthesis of ipsdienol in *Ips duplicatus* using mevastatin. 2-Alkynoic acids, such as 2-octynoic acid, have been reported to inhibit the action of fatty acid synthase (FAS) and general acyl-CoA dehydrogenase leading to blocking of chain elongation of fatty acids (Freund et al., 1985; Barkawi et al., 2003; Zhao et al., 2004). Therefore, we reasoned that if 4,8-DMD is derived from the mevalonate pathway, the production of 4,8-DMD would be enhanced by JH III and decreased by mevastatin. Alternatively, if 4,8-DMD is derived from the fatty acid pathway, the production of 4,8-DMD should be decreased by 2-octynoic acid.

Incorporation of isotopically labeled substrates, such as acetate, propionate, and mevalonate, into pheromones has provided direct evidence for the biosynthetic origins of pheromones (Seybold and Vanderwel, 2003). Acetate serves as the building block for both the fatty acid and mevalonate pathways (Lehninger et al., 1993). Propionate is known to be the source of the methyl

branch(es) in long-chained lipids and fatty acids with odd chain lengths (reviewed by Nelson, 1993), whereas mevalonate is the universal precursor of terpenes in the mevalonate pathway. Based on these facts, it was predicted that if 4,8-DMD is of terpene origin, isotopically labeled acetate and mevalonate would be incorporated into 4,8-DMD by RFBs, whereas if 4,8-DMD is of fatty acid origin, isotopically labeled acetate and propionate would be incorporated into 4,8-DMD by RFBs.

The biosyntheses of pheromones originating from fatty acids in insects have been studied and they are characterized as: (1) preparation of the precursor fatty acid by *de novo* synthesis or acquisition from the host; (2) functionalization and change in chain length; and (3) modification of the terminal carboxyl group (reviewed by Jurenka, 2003, 2004). In Tenebrionidae, 4-methyl-1-nonanol was formed by the sequence of Pr–Ac–Pr–Ac (Ac, Acetate; Pr, Propionate) (Islam et al., 1999). Therefore, we hypothesized that the biosynthesis of 4,8-DMD proceeds via the sequence of Ac–Pr–Ac–Pr–Ac (Figure 1).

In the current studies, the effects of JH III and the inhibitors were examined by topical application and oral administration, respectively, and followed by analysis of the amount of 4,8-DMD produced by the treated animals. In addition, the biosynthetic origin and the hypothesized biosynthetic pathway of 4,8-DMD were investigated by feeding RFBs flour treated with isotopically labeled substrates, followed by analysis of substrate incorporation into 4,8-DMD by gas chromatography-mass spectrometry (GC-MS).

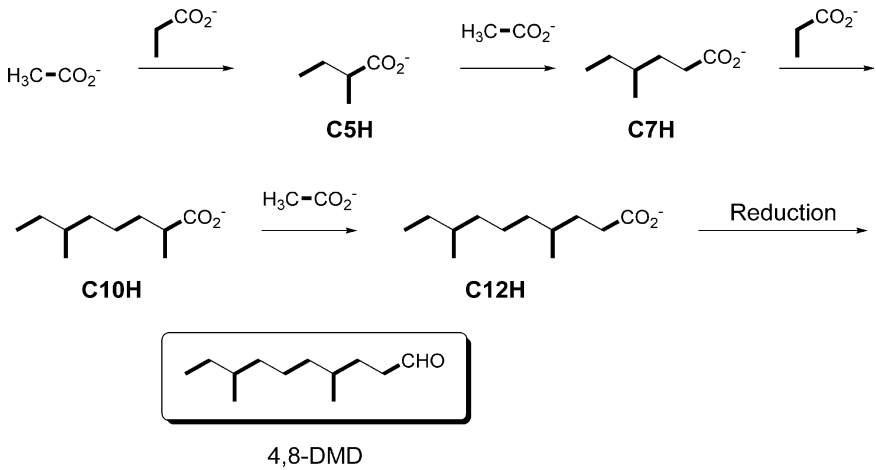

FIG. 1. The hypothesized biosynthetic pathway of 4,8-DMD in *T. castaneum via* a modified fatty acid pathway.

METHODS AND MATERIALS

Insect Cultures. A laboratory colony of *T. castaneum*, established over 20 yr, was used in all experiments. Beetles were reared on a mixture of whole wheat flour and 5% brewer's yeast (Ebios®; Asahi Food & Healthcare Ltd., Tokyo, Japan) at 27 ± 1°C, ca. 70% RH on a L:D 16:8 cycle. Pupae were sexed by the genitalia and after emergence, adults were reared separately for 4 d with flour. In preliminary experiments, aeration of both sexes produced significant levels of 4,8-DMD in males only. Therefore, adults (4 d old) of both sexes were used in all experiments.

Chemicals and Materials. The following chemicals were purchased: mevastatin (EMD Biosciences, Inc., San Diego, CA, USA); mevalonate (Aldrich Chemical Co., Milwaukee, WI, USA); [1-^{13}C]sodium acetate, [2-^{13}C]sodium acetate, and [1-^{13}C]sodium propionate (Cambridge Isotope Laboratories, Andover, MA, USA); methyl decanoate, sodium acetate, sodium propionate, diethyl ether, and Florisil® (Kanto Chemical Co. Inc., Tokyo, Japan); ethanol, hexane, and Wakogel® (C-200) (Wako Pure Chemical Industries Ltd. Osaka, Japan); Super Q, 80/100 mesh (Alltech Associates Inc., Deerfield, IL, USA); Porapak Q, 80/100 mesh (Waters Co., Milford, MA, USA). Hexane and diethyl ether were distilled prior to use.

Syntheses. JH III was prepared as follows: geranyl bromide, prepared from geraniol (Aldrich) following the method of Kim et al. (2004), was chain-extended by acetoacetate synthesis to afford geranylacetone (Nagano et al., 1997, 1999). Ethyl (2*E*/*Z*,6*E*)-farnesoate was obtained by the Wittig–Horner reaction of geranylacetone with triethyl phosphonoacetate (Kim et al. 2004). The resulting ethyl ester was transesterified to the corresponding methyl ester by refluxing in KOH-methanol solution for 4 hr. Epoxidation by the method of Hanzlik (1988) then yielded JH III (chemical purity: 94%, isomeric purity: 73%). 2-Octynoic acid was synthesized according to the procedure of Freund et al. (1985), and its chemical purity was >99.9% as the corresponding methyl ester. Mevalonolactone and [2-^{13}C]mevalonolactone were synthesized according to the synthetic scheme outlined in Figure 2. The final products were purified by Florisil® column chromatography. Spectra of mevalonolactone and [2-^{13}C]mevalonolactone corresponded with those of an authentic standard and the literature (Lawson et al., 1975), respectively (chemical purity of both compounds was >96.0%). The following deuterium-labeled and unlabeled putative precursors (sodium salts) were prepared as described by Kim et al. (2004), and their chemical purity was between 98% and 99.9% as the corresponding ethyl esters: 2-trideuteriomethylbutanoate (C5D), 4-methylhexanoate-*3*,*3*-d_2 (C7D), 2-trideuteriomethyl-6-methyloctanoate (C10D), and 4,8-dimethyldecanoate-*3*,*3*-d_2 (C12D). Deuterated and nonlabeled 4,8-DMDs were similarly prepared (Kim et al., 2004). The structures of the synthetic compounds

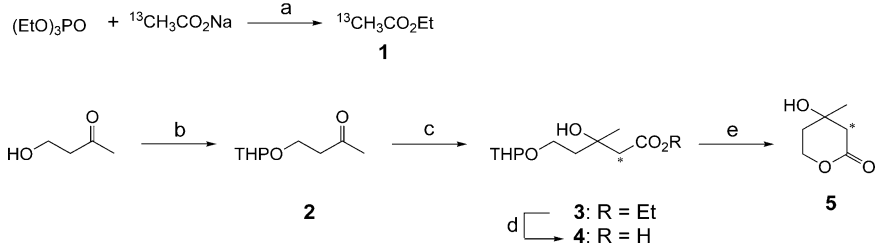

FIG. 2. Synthesis of [2-^{13}C]mevalonolactone. (a) Heating and distillation according to Ropp (1950); (b) dihydropyran, conc. HCl (four drops); (c) **1**, LDA (from *n*-BuLi and diisopropylamine)/THF, −78°C; (d) hydrolysis with NaOH in ethanol; (e) deprotection of THP group using Dowex (50W-X8, H form) in ethanol by modified procedure of Beier and Mundy (1979). Asterisks (*) represent ^{13}C atoms.

were confirmed by GC-MS and ^{1}H and ^{13}C NMR (500 and 125 MHz, respectively) with a Bruker Avance 500 spectrometer using tetramethylsilane (TMS) in $CDCl_3$ as an internal standard.

Effect of JH III on the Production of 4,8-DMD. Male beetles were anesthetized with CO_2 and then attached to double-sided Scotch® tape on a glass slide cooled with ice. After the elytra were slightly lifted with an insect pin, the specified dose of JH III (0, 1, 10, or 100 μg) dissolved in 1 μl acetone was topically applied to the dorsal abdominal cuticle using a 10-μl syringe fitted with a 33-gauge needle (Hamilton Co., Reno, NV, USA). After JH III was applied, beetles were allowed to recover in a Petri dish for 30 min at room temperature. JH III-treated males and untreated females (50 of each) were introduced into a 200-ml Erlenmeyer flask (standard taper, 24/40) containing whole wheat flour (10 g). Each flask was plugged with a joint (standard taper, 24/40), in which two glass tubes (6 mm OD) were embedded. The distance from the surface of the flour to the lower end of the long or short tube was 1 or 8 cm, respectively. A Super Q column (ca. 90 mg, 2 cm in length, 6 mm OD) was connected to the upper end of the long tube for collecting the insects' volatiles, whereas a Porapak Q column (ca. 95 mg, 2 cm in length, 6 mm OD) was connected to the upper end of the short tube to introduce purified air into the chamber. Each Super Q column was connected to an air pump (Takatsuki, Tokyo, Japan) via a metal 10-way distributor (Takatsuki). Each flask was aerated at a rate of 250 ml/min. Volatiles were collected for 4 d. The captured volatiles were eluted with 1.1 ml of hexane and the eluate was concentrated to 100 μl under N_2. After addition of 1 μl of methyl decanoate solution (1000 ppm in hexane) as an internal standard, the solution was subjected to GC-MS analysis. The experiments were replicated three times.

Effects of Mevastatin and 2-Octynoic Acid. The effects of these inhibitors on the production of 4,8-DMD were tested by allowing the RFBs to feed on

flour treated with each inhibitor. Nontreated flour was made as follows: wheat flour (30 g) was added to a round-bottom flask (300 ml) containing ethanol (60 ml), and the ethanol was completely distilled off under reduced pressure at room temperature with a rotary evaporator. Similarly, inhibitor-treated flour was prepared using flour (10 g) and an ethanol solution (20 ml) of each inhibitor (50 mg; 0.5% by weight to media). A glass Petri dish (7 cm ID × 1.5 cm) containing the nontreated flour (10 g) and 50 males and 50 females was placed in a separable flask (500 ml volume; lower part, 12 cm ID × 5 cm) equipped with a Super Q column and a Porapak Q column. Three sets of this apparatus were prepared and each Super Q column was connected to an air pump via a metal 10-way distributor. Each flask was aerated at a rate of 250 ml/min. Volatiles were trapped for 10 d (Aeration I) before the beetles in each set were transferred separately to mevastatin-treated, 2-octynoic acid-treated or non-treated flour (10 g of each). Aeration was continued for an additional 10 d (Aeration II). In preliminary experiments using the same apparatus, when the beetles were fed inhibitor-treated flour during the first 10 d, the amount of 4,8-DMD varied substantially, even after feeding with nontreated flour. Therefore, in order to reduce this variability, beetles were fed nontreated flour for 10 d, and were then fed inhibitor-coated flour. Trapped volatiles were eluted with 1.1 ml hexane every 5 d and pooled in a 2-ml microreaction vessel (Supelco, Bellefonte, PA, USA). The pooled eluates (Eluate I contained volatiles from d 0–5 and 5–10 and Eluate II contained volatiles from d 10–15 and 15–20) were stored in a freezer at −15°C. Each pooled eluate was concentrated to 100 μl and analyzed by GC-MS after addition of methyl decanoate (1 μl of 1000 ppm hexane solution). The experiments were replicated four times.

Incorporation Experiments of ^{13}C-Labeled Substrates into 4,8-DMD. Each sodium salt (500 mg, 5% by weight to media) was dissolved with 1–2 ml of distilled water and diluted to 20 ml with ethanol, whereas ^{13}C-labeled or unlabeled mevalonate was dissolved in ethanol alone (20 ml). To the solution, 10 g of whole wheat flour were added, and the solvent was removed with a rotary evaporator. The resulting lump of flour was crushed with a mortar and pestle, and then sifted with a tea strainer. Nontreated flour was prepared in the same manner as described above without any substrate. Red flour beetles (50 males and 50 females) were fed flour (10 g in each set) coated with ^{13}C-labeled or unlabeled substrate (four sets each), or nontreated flour (two sets). Aeration was performed for 20 d with the same apparatus as that used for the JH III experiments. Volatiles were eluted, pooled, and analyzed as described in the inhibitor experiments (Eluate I and Eluate II).

Incorporation Experiments of Deuterated Putative Precursors into 4,8-DMD. The flour coated with deuterium-labeled or unlabeled putative precursors (500 mg; 5% by weight to media) was prepared as described for the experiments using ^{13}C-labeled substrates. Red flour beetles (50 males and 50 females) were

fed flour (10 g in each set) coated with deuterium-labeled or unlabeled putative precursors (four sets each), or nontreated flour (two sets). Aeration was performed for 20 d with the same apparatus used in the JH III experiments. Volatiles were eluted and pooled as described in the inhibitor experiments (Eluate I and Eluate II). Each pooled eluate was concentrated to ca. 100 μl, then chromatographed on a SiO_2 column (400 mg Wakogel®, Pasteur pipette) using step-wise elution with hexane (1 ml), 5% ether in hexane (2 ml), and ether (1 ml). 4,8-DMD was eluted with 5% ether in hexane solution. This fraction was concentrated to 100 μl and analyzed by GC-MS after addition of methyl decanoate internal standard (1 μl of 1000 ppm hexane solution).

GC-MS Analysis of 4,8-DMD. Mass spectra were obtained using a JEOL MS Router MS-600 (JEOL Ltd., Tokyo, Japan) coupled with an HP 6890N GC equipped with an HP-1MS column (30 m × 0.25 mm ID, 0.25 μm film; J&W Scientific, Folsom, CA, USA). The oven temperature was programmed at 50°C for 1 min, 6°C/min to 200°C, and then 8°C/min to 270°C. Chemical ionization (CI) analysis was carried out using isobutane as reagent gas. 4,8-DMD in the eluate was identified by comparing its retention time and mass spectrum to those of authentic standards. Quantification of 4,8-DMD was performed by comparison to a standard curve constructed for the GC-MS by plotting the ratio of total ion chromatogram (TIC) peak area for various amounts of 4,8-DMD to that of methyl decanoate. The amount of 4,8-DMD was expressed as ng/male-day-equivalents (MDE). Thus, the volatiles obtained in the JH III experiment and the other experiments contained 200 MDE/100 μl (50 males × 4 d) and 500 MDE/100 μl (50 males × 10 d), respectively. To quantify the amount of 4,8-DMD, the internal standard was added to the final concentrated eluate. This method was convenient for comparing the amount of 4,8-DMD in each treatment, but could lead to an underestimate of the absolute amount of 4,8-DMD.

Incorporation of each isotopically labeled substrate into the pheromone was assessed by single ion monitoring (SIM) of the fragments at m/z $(140 + n)$ on EI analysis and at $[M^+H\ (185) + n]$ on CI analysis in the TIC of 4,8-DMD. Incorporation rate of each isotopically labeled substrate was calculated by the following equation reported by Campbell (1974):

$$\text{Incorporation rate } (\%) = \frac{\text{Intensity of } (M^+H + n)}{\text{Sum of intensities of } M^+H \text{ and } (M^+H + n)} \times 100$$

where n represents the different mass unit value(s) between labeled and unlabeled 4,8-DMDs.

CI GC-MS yielded more effective information than EI GC-MS because 4,8-DMD gave the ion $M + H^+$ at m/z 185 as the base peak, whereas its M^+ was not observed in the EI GC-MS spectrum because of the McLafferty rearrangement.

Statistical Analyses. In the JH III experiment, the mean amount of 4,8-DMD was compared between each dose by one-way ANOVA followed by Fisher's LSD test ($P = 0.05$). In the inhibitor experiments, the mean amount of 4,8-DMD between Eluate I and Eluate II for each treatment was compared by *t*-test ($P = 0.05$). Using one-way ANOVA followed by Fisher's LSD test ($P = 0.05$), the mean amounts of 4,8-DMD obtained in Aeration I (Eluate I) among three noncoated treatments were compared, and those in Aeration II (Eluate II) were analyzed similarly. The mean amount of 4,8-DMD were transformed into $\log_{10}(x + 1)$ to assure normality (Sokal and Rohlf, 1995). The incorporation of ^{13}C-labeled acetate, propionate, or mevalonolactone into 4,8-DMD was confirmed by comparing the relative intensities of diagnostic fragments of 4,8-DMD derived from ^{13}C-labeled and nonlabeled substrates using *t*-tests ($P = 0.05$). Diagnostic fragments in the ^{13}C-labeled pheromone were *m*/*z* 112, 138, and 141 in EI mode and $(MH + 1)^+$ and $(MH + 2)^+$ in CI mode. The relative intensities (%) of diagnostic fragments were calculated as follows:

$$\text{Relative intensity of diagnostic fragment}(\%) = \frac{\text{Intensity of } m/z(X+1)}{\text{Intensity of } m/z\ X} \times 100$$

where *m*/*z X* represents 111, 137, and 140 in EI mode, and MH^+ (185) in CI mode. Computer calculations were carried out using S-Plus® 2000 (MathSoft, Seattle, WA, USA).

RESULTS

Effects of JH III and Inhibitors. The production of 4,8-DMD was reduced with increasing doses of JH III ($F_{3,8} = 8.15$; $P < 0.001$) (Table 1). In the in-

TABLE 1. EFFECT OF JH III ON THE PRODUCTION OF 4,8-DMD IN *T. castaneum*

Dose of JH III (μg)	Amount of 4,8-DMD (ng/MDE, mean ± SD)*
0	4.36 ± 0.89[a]
1	3.00 ± 0.39[b]
10	2.65 ± 0.40[b]
100	1.56 ± 0.27[c]

All experiments were replicated three times with 50 JH III-treated males and 50 nontreated females. MDE, male-day-equivalents.
*Values followed by the same letter are not significantly different (ANOVA, $P < 0.05$, means compared by Fisher's LSD).

hibitor experiments, 4,8-DMD was obtained in the range of 4.76–11.68 ng/MDE when RFBs were fed nontreated flour during the first 10 d (Aeration I), and there was no significant difference ($F_{2,9}$ = 1.03; P = 0.40) among the mean amounts of 4,8-DMD in each of the nontreated groups (Figure 3). The RFBs produced larger amounts of 4,8-DMD during d 10–20 (Eluate II) than during d 0–10 (Eluate I) (t-test, $P < 0.01$). However, there were differences among the mean amounts of 4,8-DMD obtained from inhibitor feeding experiments ($F_{2,9}$ = 13.33; P = 0.002) (Figure 3). The amount of 4,8-DMD from mevastatin treatment was 21.00 ± 3.27 ng/MDE (mean ± SD), equivalent to that of the control treatment (22.90 ± 3.27 ng/MDE). In contrast, the production of 4,8-DMD was significantly inhibited (13.13 ± 0.91 ng/MDE) by 2-octynoic acid (Figure 3). These results suggested that the 4,8-DMD produced by *T. castaneum* was derived from the fatty acid pathway rather than from the mevalonate pathway.

Incorporation of ^{13}C-Labeled Substrates into 4,8-DMD. Sufficient amounts of 4,8-DMD were obtained for analysis from both Eluates I (2.7–5.0 ng/MDE) and II (4.3–8.4 ng/MDE) in the incorporation experiments of ^{13}C-labeled substrates. In the mass spectra of 4,8-DMD obtained from RFBs exposed to ^{13}C-labeled acetate and propionate, the relative intensities of all diagnostic fragments were significantly increased compared to RFBs exposed to the unlabeled substrates (Table 2, Figure 4). This indicated that RFBs incorporated [1-^{13}C]acetate and [1-^{13}C]propionate into 4,8-DMDs. When the RFBs were treated with [1-^{13}C]acetate, approximately 9% of the 4,8-DMD was enriched in

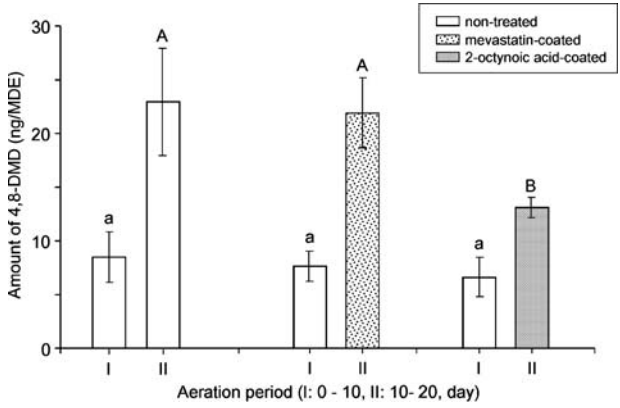

FIG. 3. Effect of inhibitors on the production of 4,8-dimethyldecanal by *T. castaneum.* Values (mean ± SD) followed by the same letter are not significantly different (ANOVA, $P < 0.05$, means compared by Fisher's LSD). All experiments were replicated four times with 50 males and 50 females. MDE, male-day-equivalents.

TABLE 2. INCORPORATION OF ^{13}C-LABELED ACETATE, PROPIONATE, AND MEVALONOLACTONE INTO THE PHEROMONE, 4,8-DMD BY *T. castaneum*: RELATIVE INTENSITIES OF GC-MS (CI MODE) FRAGMENTS OF INTEREST

Aeration period[a]	Treatment	Relative intensities (%, mean ± SD, $N = 4$)		
		MH^+	$(MH + 1)^+$	$(MH + 2)^+$
I	Control (Acetate)	100	13.09 ± 0.51	1.19 ± 0.13
	[1-^{13}C]Acetate	100	23.07 ± 2.03***	2.18 ± 0.78*
II	Control (Acetate)	100	13.47 ± 0.97	1.12 ± 0.24
	[1-^{13}C]Acetate	100	23.76 ± 2.34***	3.33 ± 0.96**
I	Control (Propionate)	100	13.37 ± 1.49	1.32 ± 0.35
	[1-^{13}C]Propionate	100	38.20 ± 11.4**	10.42 ± 3.14***
II	Control (Propionate)	100	12.68 ± 0.90	1.38 ± 0.32
	[1-^{13}C]Propionate	100	29.48 ± 2.70***	6.35 ± 0.78***
I	Control (Mevalonolactone)	100	11.80 ± 0.90	1.23 ± 0.43
	[2-^{13}C]Mevalonolactone	100	14.58 ± 1.36***	1.56 ± 0.09
II	Control (Mevalonolactone)	100	12.63 ± 2.37	1.10 ± 0.27
	[2-^{13}C]Mevalonolactone	100	15.09 ± 1.46	1.74 ± 0.17

[a]Aeration periods I and II correspond to d 0 to 10 and 10 to 20, respectively.
*, **, and *** Significantly different values at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, compared to their corresponding unlabeled compounds (control) by *t*-test (one-tailed).

one ^{13}C atom, and approximately 1–2% was enriched in two ^{13}C atoms. When the insects were treated with [1-^{13}C] propionate, approximately 14–21% of the 4,8-DMD was enriched in one ^{13}C atom, and approximately 4.7–10% was enriched in two ^{13}C atoms (Table 3). When the RFBs were fed flour coated with [2-^{13}C]mevalonolactone, the relative intensities of some diagnostic fragments of 4,8-DMD appeared to be increased in both EI and CI modes (Table 2). However, the mean relative intensity of diagnostic fragment (*m/z* 112) in EI mode was not significantly increased, and the increase in the $[MH + 1]^+$ ion in CI mode was only statistically different when Eluate I of mevalonolactone-treated and [2-^{13}C]mevalonolactone-treated RFBs were compared with 4,8-DMD enriched in one ^{13}C atom at approximately 2.7% by [2-^{13}C]mevalonolactone (Table 3). Thus, at best, RFBs might incorporate small amounts of [2-^{13}C]mevalonolactone into 4,8-DMD only during Aeration I. The much higher and more consistent incorporation of the ^{13}C-labeled acetate and especially propionate provided strong evidence that 4,8-DMD is biosynthesized from these precursors.

Incorporation of Deuterium-Labeled Putative Precursors into 4,8-DMD. The amount of 4,8-DMD obtained from RFBs exposed to each precursor ranged between 0.5–1.4 ng/MDE (in Eluate I) and 1.2–3.5 ng/MDE (in Eluate II). The amount of 4,8-DMD in Eluate I was not sufficient for confirming the incorporation

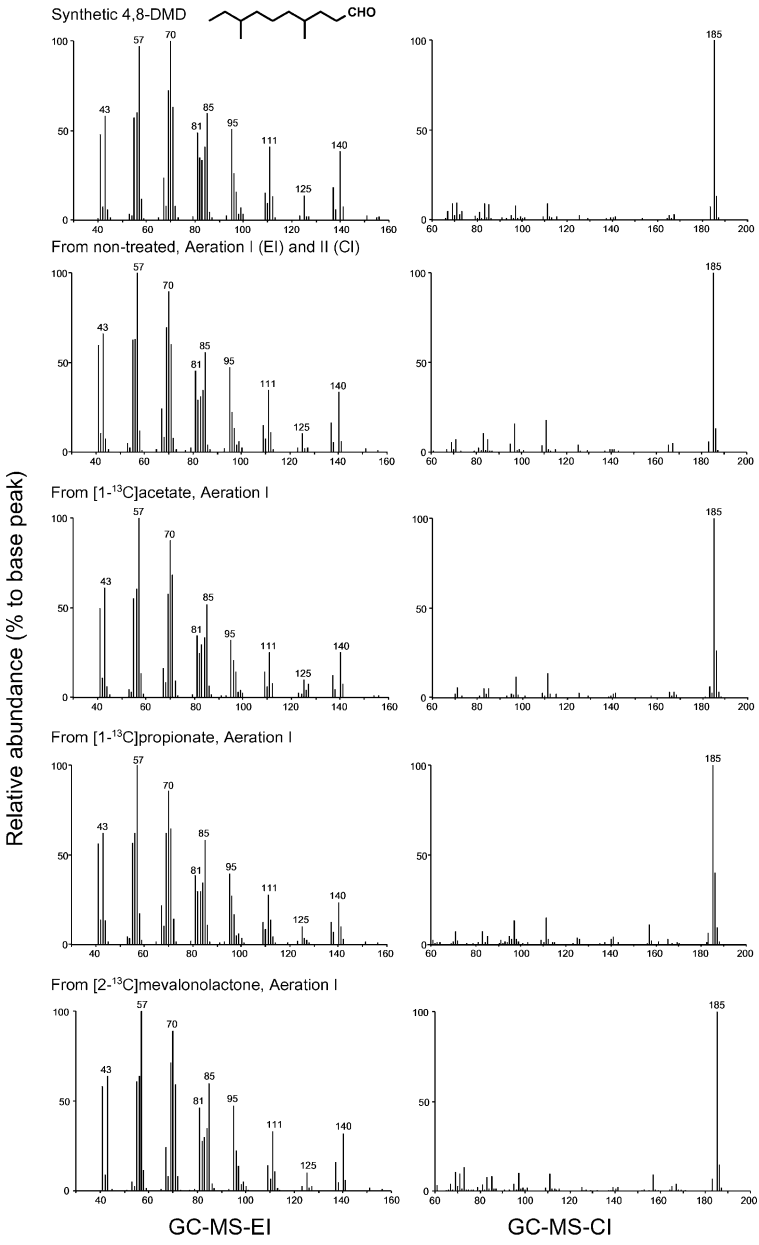

FIG. 4. Mass spectra of 4,8-dimethyldecanal obtained from *T. castaneum* exposed to flour coated with ^{13}C-labeled substrate or to nontreated flour. Also, mass spectra are shown for an authentic standard. (left, EI GC-MS analyses; right, CI GC-MS analyses).

TABLE 3. INCORPORATION RATE OF [1-^{13}C]ACETATE, [1-^{13}C] PROPIONATE AND [2-^{13}C]MEVALONOLACTONE INTO THE PHEROMONE, 4,8-DMD BY *T. castaneum*

Aeration period[a]	Treatment	Incorporation rate (%, mean ± SD, $N = 4$)	
		One ^{13}C atom	2 ^{13}C atoms
I	[1-^{13}C]Acetate	9.05 ± 1.71	0.98 ± 0.65
	[1-^{13}C]Propionate	21.00 ± 7.60	10.06 ± 3.85
	[2-^{13}C]Mevalonolactone	2.68 ± 1.59	–
II	[1-^{13}C]Acetate	9.30 ± 1.90	2.15 ± 0.80
	[1-^{13}C]Propionate	14.34 ± 1.96	4.73 ± 0.45
	[2-^{13}C]Mevalonolactone	–	–

[a]Aeration periods I and II correspond to d 0 to 10 and 10 to 20, respectively.
–: 4,8-DMD was not enriched in ^{13}C.

of the labeled putative precursors into 4,8-DMD because of high background. Therefore, Eluate II was used to determine whether the deuterium-labeled substrates were incorporated into 4,8-DMD. The deuterium-labeled and unlabeled pheromones were separated by GC-MS. Deuterated 4,8-DMDs eluted faster than unlabeled ones (Figures 5 and 6). MS fragmentation patterns of each 4,8-DMD at the maximum peak of m/z (140 + n) were similar to those of the corresponding authentic deuterated standards. The n amu (atom mass unit)-shifted fragment ions were observed in the TIC of 4,8-DMDs obtained from RFBs treated with C5D, C10D, and C12D (Figures 5 and 6). This indicated that RFBs incorporated C5D, C10D, and C12D into 4,8-DMD, and that the deuterated substrates served as precursors. Unlike these deuterated substrates, 4,8-DMDs obtained from C7D treatment did not result in any n amu-shifted fragment ion in any of the treatments (Figure 5). The incorporation rates of these substrates are summarized in Table 4. Based on these experiments, we demonstrated that 4,8-DMD is biosynthesized in the sequence of Ac–Pr–Ac–Pr–Ac via the fatty acid pathway.

DISCUSSION

Our results show that the production of 4,8-DMD by *T. castaneum* is inhibited by 2-octynoic acid but not by mevastatin. These findings, combined with the high incorporation of [1-^{13}C]acetate and [1-^{13}C]propionate into 4,8-DMD and the incorporation of deuterium-labeled putative precursors, provide unambiguous evidence that 4,8-DMD is of fatty acid and not terpene

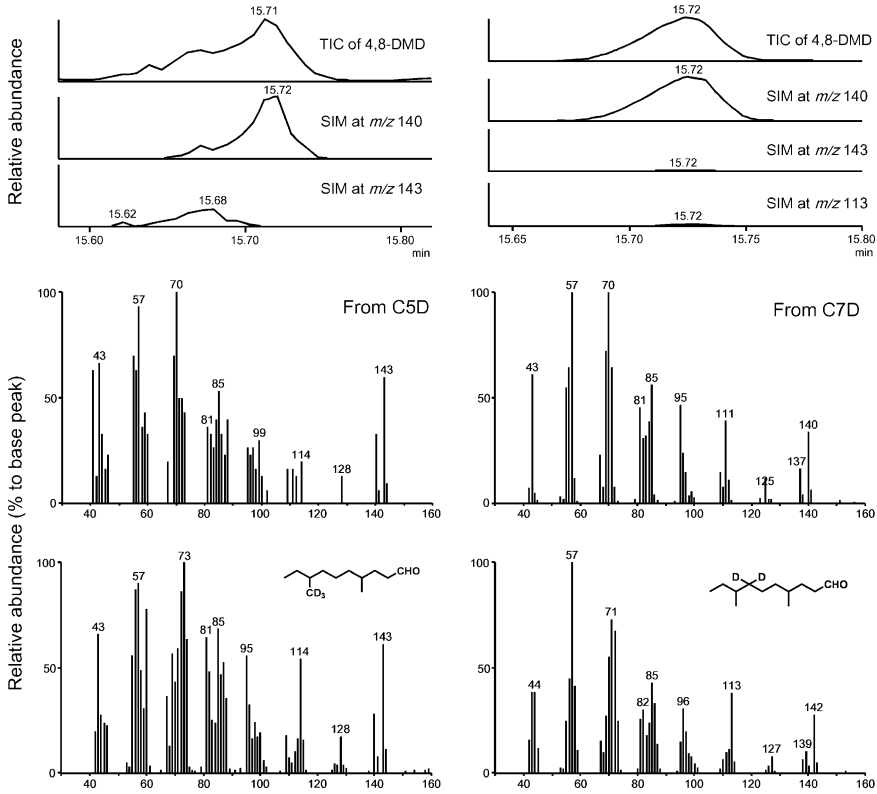

FIG. 5. Mass chromatograms (upper panels) and mass spectra (middle panels) of 4,8-DMD obtained from *T. castaneum* fed on flour coated with deuterium-labeled C5D (left; R_t = 15.68 min) or C7D (right; R_t = 15.72 min). Also shown are the mass chromatograms and spectra of authentic deuterated 4,8-DMD with the precursor incorporated as expected (lower panels). TIC, total ion chromatogram. SIM, single ion monitoring.

biosynthetic origin, and that the biosynthesis of 4,8-DMD proceeds in the sequence Ac–Pr–Ac–Pr–Ac.

Topical application of JH III caused a reduction in the level of 4,8-DMD. To our knowledge, an inhibitory effect of JH III on fatty acid biosynthesis has not been reported. Ivarsson and Birgersson (1995) reported that higher doses of methoprene suppressed the production of pheromones in *I. duplicatus*. A similar suppression mechanism seems to account for the effect of the higher doses of JH III observed here. However, it is surprising that even the lowest dose (1 μg/male) of JH III reduced the production of 4,8-DMD. One possible interpretation is that even 1 μg of JH III is too much for a young male RFB (4 d old). In

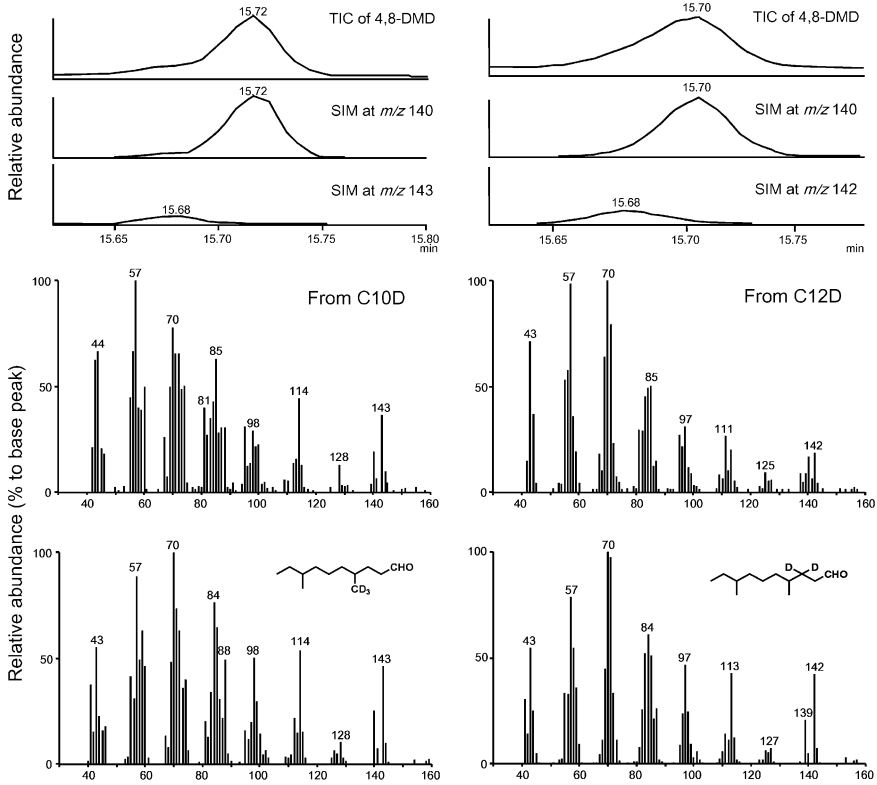

FIG. 6. Mass chromatogram (upper panels) and mass spectra (middle panels) of 4,8-DMD obtained from *T. castaneum* fed on flour coated with deuterium-labeled precursors C10D (left; R_t = 15.68 min) and C12D (right; R_t = 15.68 min). Also shown are the mass chromatograms and spectra of authentic deuterated 4,8-DMD with the precursor incorporated as expected (lower panels). TIC, total ion chromatogram. SIM, single ion monitoring.

contrast to our results, Pierce et al. (1986) reported that the production of both 4,8-DMD and nonmethyl branched cucujolides, which are aggregation pheromones of *Oryzaephilus mercator*, *O. surinamensis*, and *Cryptolestes ferrugineus* (Cucujidae), were enhanced when these species were fed methoprene-treated oats for 20 d. However, the nonmethyl branched cucujolides were later demonstrated to be of fatty acid origin (Vanderwel et al., 1990, 1992). Considering the results of Vanderwel et al. and our present results, the enhanced production of 4,8-DMD reported by Pierce et al. (1986) appears to be different from that of the terpene pheromones observed in *I. duplicatus* and *I. pini*, which

TABLE 4. INCORPORATION RATE OF DEUTERIUM-LABELED PRECURSORS INTO 4,8-DIMETHYLDECANAL BY *T. castaneum* DURING AERATION PERIOD DAYS 10 TO 20

Precursor	Incorporation rate (%, mean ± SD, $N = 4$)
2-Trideuteriomethylbutanoate (C5D)[a]	26.23 ± 2.76
4-Methylhexanoate-3,3-d_2 (C7D)	0.00
2-Trideuteriomethyl-6-methyloctanoate (C10D)[b]	13.03 ± 1.33
4,8-Dimethyldecanoate-3,3-d_2 (C12D)	22.05 ± 3.81

[a] $N = 3$ (4,8-DMD obtained from one of replicates was lost during fractionation).
[b] The value was determined by GC-MS EI analysis.

occurred by activation of the enzymes (HMG-R and HMG-S) involved in the mevalonate pathway (Tittiger, 2003).

The fact that the production of 4,8-DMD was not inhibited by mevastatin but was inhibited by 2-octynoic acid provides further evidence that 4,8-DMD produced by RFB is derived from the fatty acid pathway rather than from the mevalonate pathway. These inhibitors have been used previously to provide indirect information on the biosyntheses of pheromones, and they have been administered by microinjection or topical application to the pheromone gland (Seybold and Vanderwel, 2003; Ivarsson et al., 1993; Barkawi et al., 2003; Zhao et al., 2004). However, oral administration was conducted in our study, because the pheromone gland for 4,8-DMD remains unknown. The femoral setiferous glands has been reported to be the major site of production of 4,8-DMD (Faustini et al., 1981), but it was also reported that they are not the sole source of 4,8-DMD (Hussain, 1993; Qazi et al., 1998). Oral administration was also used because of technical difficulties in microinjection; specifically, there was high mortality following injection of the RFBs with either glass capillaries or a microsyringe fitted with a 33-gauge needle.

The results of the ^{13}C-labeled substrate incorporation experiments provided direct evidence that 4,8-DMD is of fatty acid pathway origin. Both ^{13}C-labeled acetate and possibly lesser amounts of mevalonolactone were incorporated into 4,8-DMD; the incorporation rate of [1-^{13}C]acetate was much higher than that of [2-^{13}C]mevalonolactone. Based on this result, it is unlikely that 4,8-DMD is produced via the mevalonate pathway. If 4,8-DMD was produced by the mevalonate pathway, a higher rate of incorporation of [2-^{13}C]mevalonolactone than [1-^{13}C]acetate into 4,8-DMD would have been expected because acetate would be incorporated into mevalonate before it was incorporated into 4,8-DMD. In addition, loss of a ^{13}C atom in [1-^{13}C]acetate and dilution of [1-^{13}C] acetate by unlabeled acetate would occur during mevalonate formation

(Lehninger et al., 1993). Barkawi et al. (2003) reported that in *Dendroctonus jeffreyi* (Scolytidae), both ^{14}C-labeled acetate and mevalonolactone were incorporated into frontalin derived from the mevalonate pathway, and that the incorporation rate of mevalonolactone was two or three times higher than that of acetate. In addition, when RFBs were fed flours coated with other deuterated terpenoids (geraniol, farnesol, and 2,6,10-trimethyl-2-dodecene), they did not incorporate these substrates into 4,8-DMD (J. Kim, personal communication). The low incorporation of [2-^{13}C]mevalonolactone into 4,8-DMD may be a result of the degradation of [2-^{13}C]mevalonolactone during digestion by the beetles or preparation of the media.

The incorporation of [1-^{13}C]propionate into 4,8-DMD provided further evidence that 4,8-DMD was of fatty acid origin. Propionate is well known to be a source of methyl groups in methyl-branched insect lipids (Nelson, 1993). In tenebrionid pheromones, Islam et al. (1999) demonstrated that 4-methyl-1-nonanol, the sex pheromone of *T. molitor*, was biosynthesized through a modified fatty acid pathway and that [1-^{13}C]propionate was incorporated into the pheromone and responsible for the methyl branch. Propionate could be converted into acetyl-CoA from propionyl-CoA (Chase et al., 1992; Nelson, 1993), and be subsequently incorporated into 4,8-DMD. However, Dillwith et al. (1982) proposed that the ^{13}C atom in [1-^{13}C]propionate would be lost during conversion to acetyl-CoA. Therefore, the enrichment of 4,8-DMD in ^{13}C indicated that propionate was directly involved in the biosynthesis of 4,8-DMD. However, there is no previous evidence that propionate is involved in the mevalonate pathway.

The 4,8-DMD obtained from ^{13}C-labeled substrate incorporation experiments was analyzed by conventional GC-MS. This analysis provided information on the position of ^{13}C atoms in the pheromone and should allow determination of the biosynthetic route (Bartelt and Weisleder, 1996; Islam et al., 1999). Unfortunately, because of the complex MS fragmentation pattern of 4,8-DMD, incorporation studies using ^{13}C-labeled substrates could not identify the exact position of the ^{13}C atom, and, therefore, we were unable to use it to determine the biosynthetic route to 4,8-DMD. However, the results of the experiments using deuterium-labeled precursors provide evidence that 4,8-DMD was formed according to the sequence shown in Figure 1. The deuterated precursors C5D, C10D, and C12D were incorporated into 4,8-DMD, and their MS patterns were similar to those of the corresponding authentic deuterated standards. In contrast to our expectations, C7D, one of the hypothesized precursors, was not incorporated into 4,8-DMD in all replicates. Considering that C5D and C10D were incorporated into 4,8-DMD and that the chain elongation reaction involves reduction of double bonds, it is possible that there is a more complex biosynthetic pathway that is distinct from the pathway producing lepidopteran pheromones from fatty acids. For example, as shown in Figure 7, 2,6-dimethyloctanoate (C10H)

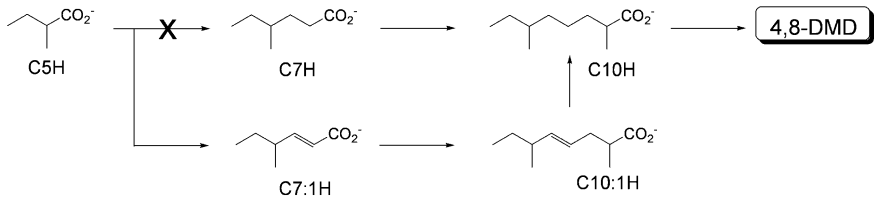

FIG. 7. A plausible alternate chain elongation reaction in biosynthesis of 4,8-DMD.

could originate from 2,6-dimethyl-4-octenoate (C10:1H) via 4-methyl-2-hexenoate (C7:1H), rather than from 4-methylhexanoate (C7H). This detoured pathway could be tested by examining the incorporation of deuterated C7:1H and C10:1H into the pheromone.

Figure 8 shows the structural and biosynthetic similarities between 4,8-DMD and 4-methyl-1-nonanol. The only difference in the biosynthetic sequence between them is whether or not it initiates with Ac (Ac–Pr–Ac–Pr–Ac for 4,8-DMD and Pr–Ac–Pr–Ac for 4-methyl-1-nonanol). Moreover, the absolute configuration at the C-4 position of the natural forms of both pheromones is (4*R*) (Suzuki and Mori, 1983; Tanaka et al., 1989). This information suggests that the pheromones of *T. castaneum* and *T. molitor* are derived from a common biosynthetic pathway.

On the other hand, the male-produced aggregation pheromones of *Gnatocerus cornutus*, a terebrionid beetle, are the sesquiterpenes, (+)-acoradiene and α-cedren-14-al (Tebayashi et al., 1998a,b; Tashiro et al., 2004). Although tenebrionid pheromones are known from only three genera, their pheromones are rather complex. Studies of other tenebrionid pheromones and their biosyntheses would provide useful information for understanding the evolution of chemical communication in this taxonomically complex group.

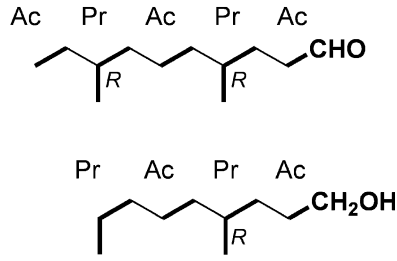

FIG. 8. Structural and biosynthetic analogy of pheromones of *Tribolium castaneum* (upper) and *Tenebrio molitor* (lower).

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ATTRACTION OF MALE EUROPEAN TARNISHED PLANT BUG, *Lygus rugulipennis* TO COMPONENTS OF THE FEMALE SEX PHEROMONE IN THE FIELD

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Abstract—Previous work showed that females of the European tarnished plant bug, *Lygus rugulipennis* Poppius (Heteroptera: Miridae), produced three chemicals, hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal, and that these were suspected to be components of the female sex pheromone. In field experiments, traps baited with blends of these chemicals dispensed from polyethylene vials and sachets failed to catch significant numbers of males. Here, we report more recent field experiments in which the chemicals were released from glass microcapillary tubes. A blend of hexyl butyrate and (*E*)-4-oxo-2-hexenal was significantly attractive to male *L. rugulipennis*. In addition, whereas the mixture of all three components attracted fewer *L. rugulipennis* males, this tertiary blend captured significantly greater numbers of males of the congeneric species *Lygus pratensis* than the binary mixture. The possible reasons for the success of the microcapillaries compared with other dispensers are discussed.

Key Words—*Lygus rugulipennis*, *Lygus pratensis*, Heteroptera, Miridae, tarnished plant bug, sex pheromone, hexyl butyrate, (*E*)-2-hexenyl butyrate, (*E*)-4-oxo-2-hexenal.

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INTRODUCTION

The use of pheromones for detecting, monitoring, and controlling lepidopteran and coleopteran pests has been well explored (Ridgway et al., 1990; Copping, 1998). In contrast, the identification and application of pheromones within the Heteroptera is in its infancy, especially in the Mirid family (Smith et al., 1991; Millar et al., 1997; Millar and Rice, 1998; Kakizaki and Sugie, 2001; Downham et al., 2002; Zhang and Aldrich, 2003a). The genus *Lygus* contains some of the most damaging of all bugs to a range of crops (McBrien and Millar, 1999), and consequently, they are the subject of intensive scientific research, and novel methods of control are constantly being sought. To date, numerous compounds have been reported as potential female sex pheromone components of *Lygus* sp., but synthetic blends do not attract males to traps in the field (e.g., Ho and Millar, 2002).

Our previous work on the European tarnished plant bug, *Lygus rugulipennis* (Heteroptera: Miridae) Poppius, showed that virgin females attracted males to traps (Innocenzi et al., 1998). The male antennae were stimulated by three components released by females, and these were identified as hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal (Innocenzi et al., 2004). All three chemicals were found in body extracts of both virgin males and females, but virgin males did not release detectable amounts of any of them under undisturbed conditions. Despite this circumstantial evidence indicating a possible pheromonal role for one or more of these chemicals, field trials with various mixtures dispensed from different combinations of polyethylene vial and polyethylene sachet dispensers failed to show any significant attraction of male *L. rugulipennis*. Significant repulsion of females by blends containing hexyl butyrate was observed (Innocenzi et al., 2004). Here, we report the results of more recent field trapping experiments in which the same chemicals were released from glass microcapillary tubes, as described by Kakizaki and Sugie (2001) for the pheromone components of another mirid species. Attraction of male *L. rugulipennis* to a two-component blend was demonstrated, and a blend of all three components was found to attract the congener, *Lygus pratensis* Linnaeus.

METHODS AND MATERIALS

Gas Chromatography. Gas chromatography (GC) analyses were performed using a Carlo Erba Mega 5300 instrument equipped with fused silica capillary columns (25 m $\times$ 0.32 mm i.d.) coated with polar CPWax52CB (Carbowax 20 M equivalent; Chrompak, London, UK) or nonpolar CPSil5CB (methyl silicone; Chrompak). The carrier gas was helium at 50 kPa, and the

oven temperature was held at 50°C for 2 min then programmed at 6°C/min to 240°C. Injector and detector temperatures were 200 and 240°C, respectively. Injection was splitless, and detection was by flame ionization detection (FID). Data were captured and processed using EzChrom 6.1 software (Aston Scientific, Stoke-Mandeville, Bucks, UK).

Chemicals. Butyrate esters were synthesized by reaction of butyryl chloride with the corresponding alcohol in dichloromethane in the presence of pyridine at 0°C followed by aqueous workup and distillation: hexyl butyrate (98% purity, b.p. 76°C/7 mm), (*E*)-2-hexenyl butyrate (99% purity, b.p. 120°C/10 mm). (*E*)-4-Oxo-2-hexenal (99% purity, b.p. 100°C/5 mm) was synthesized according to Ward and van Dorp (1969) or by a route similar to that subsequently described by Marques et al. (2000).

Dispensers. Dispensers were glass microcapillaries (vol. 5 μl, cat. no. TLC-940-040X, Fisher Scientific, UK) or polyethylene vials (22 × 8 × 1.5 mm thickness, Just Plastics Ltd., London). The microcapillaries were loaded with a solution (5 μl) of the chemicals in dichloromethane (10 mg/ml). Hexyl butyrate and (*E*)-2-hexenyl butyrate were combined in 3:2 ratio, respectively, in a single solution (total 10 mg/ml), matching that produced by a single female *L. rugulipennis* (Innocenzi et al., 2004). The (*E*)-4-oxo-2-hexenal was loaded into a separate microcapillary. The polyethylene vials were loaded with the 3:2 mixture of butyrates (10 mg total) dissolved in an involatile, chlorinated hydrocarbon, "Cereclor" (100 mg; Shell Chemicals Ltd.). Dilution of volatile materials in an involatile solvent reduces the release rate relative to that of the neat material (Torr et al., 1997). The (*E*)-4-oxo-2-hexenal (2 mg) in Cereclor (20 mg) was dispensed from a separate vial. The butyrates and (*E*)-4-oxo-2-hexenal were placed in separate dispensers because chemical interaction between (*E*)-2-hexenyl butyrate and (*E*)-4-oxo-2-hexenal had been observed previously (Innocenzi et al., 2004).

Release rates were measured from duplicate dispensers maintained in a laboratory wind tunnel (27°C, 8 km/hr wind speed). Volatiles were collected by placing each dispenser in a separate glass chamber (10 × 3 cm diam) and air drawn (2 l/min) into the chamber through an activated charcoal filter (20 × 2 cm, 6–18 mesh) and out through a collection filter containing Porapak Q (200 mg, 50–80 mesh, Waters Corp., MA, USA), held between plugs of silanized glass wool in a Pasteur pipette. The Porapak was purified by soxhlet extraction with chloroform for 8 hr, and filters were washed well with dichloromethane immediately before use. After 1 hr, adsorbed volatiles were removed from the filters with dichloromethane (pesticide grade; 1 × 0.5 ml, 1 × 1 ml), decyl acetate (5 μg) added as internal standard, and the solution analyzed by GC on the polar column. Volatiles from the microcapillaries were collected and analyzed immediately after evaporation of the solvent (2 hr after loading) and then after 24 and 48 hr. Volatiles from polyethylene vials were collected 1, 2, 3,

5, 7, and 9 d after loading. The release rates from microcapillaries were also measured using residual analysis of pheromones 1, 2, 3, 13, and 24 hr after loading. The microcapillaries were extracted with dichloromethane (1 ml) containing undecane (10 μg) as internal standard, and the resulting residue in solution was analyzed by GC on the nonpolar column.

Traps. Traps were constructed from a wooden stake (2 × 2 × 50 cm long) with a band of viscous polybutene sticker (10 cm wide, 2 cm from the top; Oecotac, Oecos, Kimpton, Herts, UK) and a small corrugated plastic board (5 × 5 cm; Correx) on top to act as a rain shield. Lures were suspended from the corner of the latter with sticky tape (microcapillaries) or a wire hook (polyethylene vials).

Field Trials. A field at East Malling Research Station (1.4 ha) was sown with a mixture of the host plants, fathen, *Matricaria recutita*, and scented mayweed, *Chenopodium album* (both at 1 kg/ha), in March 2002 and 2003 to ensure large resident populations of *L. rugulipennis*. Randomized complete block designs were used for all experiments with traps placed 5–10 m apart within a block and blocks separated by at least 10 m.

In 2002, two replicated field experiments were carried out from 29 July to 14 August and from 14 August to 3 September to evaluate the glass microcapillary lures containing all possible binary combinations of the three candidate pheromone components and the three-component blend, previously tested by Innocenzi et al. (2004). Thus, treatments were unbaited, hexyl butyrate + (*E*)-2-hexenyl butyrate, hexyl butyrate + (*E*)-4-oxo-2-hexenal, (*E*)-2-hexenyl butyrate + (*E*)-4-oxo-2-hexenal, and all three chemicals. Traps were examined, and the number of insects was recorded every 48–72 hr, after which the microcapillaries were replaced.

The same experiment was repeated in 0.8 ha of the field between 21 July and 3 August 2003. In a second experiment from 19 to 21 August 2003, treatments were unbaited, microcapillaries containing hexyl butyrate, (*E*)-4-oxo-2-hexenal or hexyl butyrate + (*E*)-4-oxo-2-hexenal, and polyethylene vials containing hexyl butyrate + (*E*)-4-oxo-2-hexenal in Cereclor. Trials began between 1100 and 1400 hr. Traps were checked every hour for the first 3 hr, and again after 24 hr, after which the microcapillaries were replaced.

Insect Identification. Because of the morphological similarity of *Lygus* spp., the diverse coloration of *L. rugulipennis* (Southwood and Leston, 1959), and the damaging effect of the polybutene adhesive on insect structure, *L. rugulipennis* and *L. pratensis* were not distinguished in 2002. Therefore statistical analysis was conducted on the combined *Lygus* spp. catch. However, the catches were saved in 70% ethanol, and retrospective examination of these insects was possible, although statistical testing could not be applied for the separate species as the individual trap catches for each treatment had been combined. In 2003, as trap counts were conducted hourly, it was possible to identify both *L. rugulipennis* and *L. pratensis*, the latter being longer with a

reddish wing coloration and possessing distinct markings on the pronotum and scutellum (Southwood and Leston, 1959). Species identifications were confirmed by the Natural History Museum, London.

Statistical Analysis. After square root transformation, data from the field experiments were analyzed using a general linear model (SPSS v.10, SPSS Inc., Chicago, IL 60606, USA). *Post hoc* pairwise comparisons were computed using Tukey highest significant differences (HSD). A significance level of 5% was used for all statistics.

RESULTS

Release Rate Studies. Estimates from the analysis of residual pheromone (Figure 1) indicate that hexyl butyrate and (*E*)-4-oxo-2-hexenal were released from microcapillaries at approximately 5 and 10 μg/hr, in the first 2 hr. Then, after the solvent had completely evaporated, the release rate decreased to approximately 1 and 2 μg/hr, respectively. Release rate for the 3:2 mixture of hexyl butyrate and (*E*)-2-hexenyl butyrate combined was 0.7 μg/hr and for (*E*)-4-oxo-2-hexenal approximately 2 μg/hr (Table 1), in reasonable agreement with the above figures. Collection of volatiles showed that release of the butyrates was complete within 48 hr and that of the (*E*)-4-oxo-2-hexenal within 24 hr at 27°C.

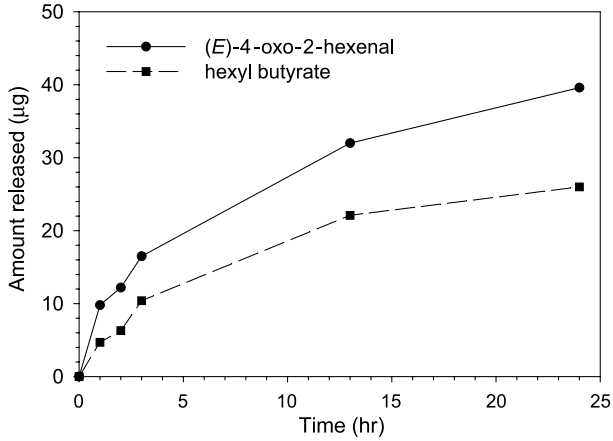

FIG. 1. Release of hexyl butyrate and (*E*)-4-oxo-2-hexenal from separate microcapillary tubes in laboratory wind tunnel determined by analysis of residual material at intervals (27°C, 8 km/hr wind speed; $N = 2$).

TABLE 1. RELEASE RATES OF HEXYL BUTYRATE, (*E*)-2-HEXENYL BUTYRATE, AND (*E*)-4-OXO-2-HEXENAL FROM MICROCAPILLARY TUBES AND POLYETHYLENE VIALS MEASURED BY COLLECTION OF VOLATILES AT 27°C

Dispenser/loading	Duration	Release rate ± SE (μg/hr)		
		Hexyl butyrate	(*E*)-2-Hexenyl butyrate	(*E*)-4-Oxo-2-hexenal
Microcapillary				
50 μg 3:2 hexyl butyrate + (*E*)-2-hexenyl butyrate	<48 hr (n = 8)	0.38 ± 0.016	0.25 ± 0.014	
50 μg (*E*)-4-oxo-2-hexenal	<24 hr (n = 4)			2.07 ± 0.20
Polyethylene vial				
10 mg 3:2 hexyl butyrate + (*E*)-2-hexenyl butyrate in 100 mg Cereclor	9 d (n = 10)	4.49 ± 0.34	3.12 ± 0.24	
2 mg (*E*)-4-oxo-2-hexenal in 20 mg Cereclor	9 d (n = 10)			7.04 ± 0.49

Release of the butyrates from a 10% solution in Cereclor in the polyethylene vial was at least an order of magnitude higher than that from the microcapillary tubes, and for (*E*)-4-oxo-2-hexenal, it was over three times greater. Release was relatively constant over at least 9 d (Table 1).

Field Trials. During the first experiment in 2002, significantly more *Lygus* spp. ($P < 0.05$) were caught in traps baited with all three components dispensed from microcapillary tubes, i.e., a 3:2 mixture of hexyl butyrate and (*E*)-2-hexenyl butyrate in one capillary and (*E*)-4-oxo-2-hexenal in a separate tube, than in traps baited with binary mixtures or unbaited (Figure 2A). Subsequent examination of the catches showed that 70% of the total male catch was *L. pratensis*, and 93% of the catch with the most attractive tertiary blend was of this species (Figure 2A). In the second experiment, significantly more *Lygus* spp. ($P < 0.05$) were caught in traps baited with hexyl butyrate and (*E*)-4-oxo-2-hexenal dispensed from separate microcapillaries (Figure 2B). In this case, 84% of the males captured were *L. rugulipennis* and constituted 97% of the catch with the most attractive blend of hexyl butyrate and (*E*)-4-oxo-2-hexenal (Figure 2B). Small numbers of female *Lygus* spp. were caught in all traps. Similar results were obtained in 2003. In the first 3 hr of trapping, catches of male *L. rugulipennis* were significantly ($P < 0.05$) greater in traps baited with the blend of hexyl butyrate and (*E*)-4-oxo-2-hexenal, whereas *L. pratensis* catches (although at lower densities) were significantly greater in traps baited with the tertiary blend (Figure 3A). Again, catches of female *Lygus* spp. were low. Catches of male *L. rugulipennis* during the period 3–24 hr after

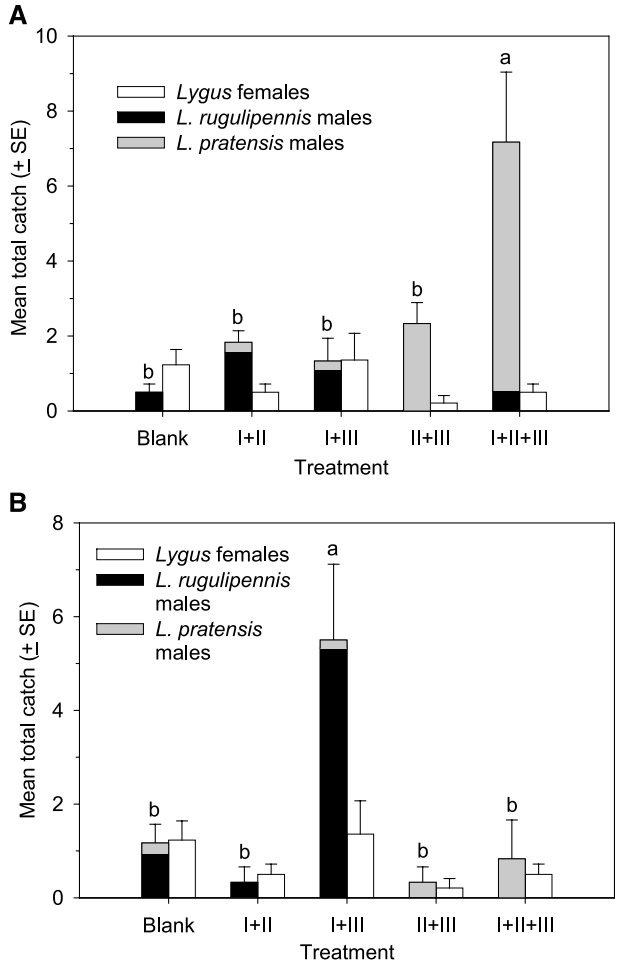

FIG. 2. Mean total catches ($N = 6$) of *L. rugulipennis* and *L. pratensis* males and *Lygus* spp. females in 2002 field experiments: (A) Experiment 1 from 29 July to 14 August; (B) experiment 2 from 15 August to 3 September [blends of hexyl butyrate **(I)**, (*E*)-2-hexenyl butyrate **(II)**, and (*E*)-4-oxo-2-hexenal **(III)** dispensed from microcapillaries; means for each species with different letters are significantly different ($P < 0.05$) by Tukey HSD]. Columns followed by the same letter are not significantly different.

deployment of lures were lower, and there were no significant differences between catches in unbaited or baited traps (0–3 hr, $P < 0.005$; 3–24 hr, $P > 0.05$) (Figure 3B).

In the second experiment, the combination of hexyl butyrate and (*E*)-4-oxo-2-hexenal in microcapillary dispensers attracted significantly more male *L. rugulipennis* than the individual components, although more males were caught in traps baited with (*E*)-4-oxo-2-hexenal alone than in the unbaited traps ($P < 0.001$) (Figure 4). Some male *L. rugulipennis* were caught in traps baited with the binary blend dispensed from a polyethylene vial, but numbers were not significantly different from those caught in unbaited traps or the single components alone in capillary tubes.

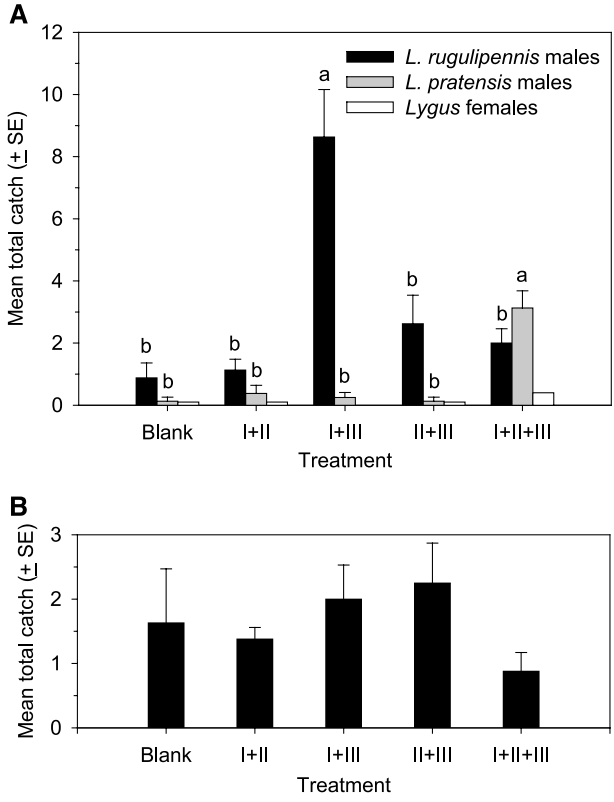

FIG. 3. Mean total catches (±SE; $N = 8$) of *L. rugulipennis* males, *L. pratensis* males, and *Lygus* spp. females in 2003 field experiments: (A) over the first 3 hr and (B) 3–24 hr from 21 July to 3 August [blends of hexyl butyrate **(I)**, (*E*)-2-hexenyl butyrate **(II)**, and (*E*)-4-oxo-2-hexenal **(III)** dispensed from microcapillaries; means for each species with different letters are significantly different ($P < 0.05$) by Tukey HSD]. Columns followed by the same letter are not significantly different.

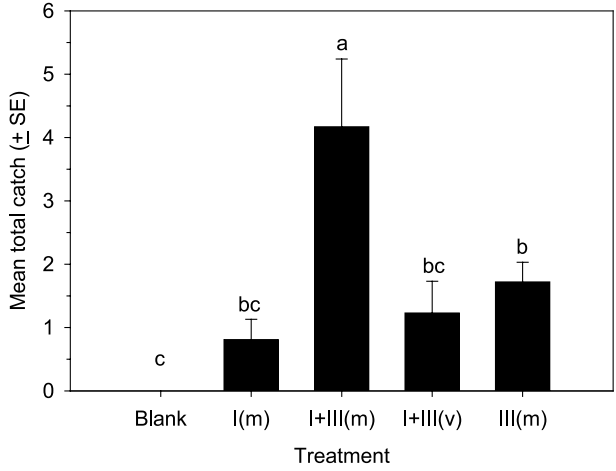

FIG. 4. Mean trap catches (±SE; $N = 6$) of *L. rugulipennis* males over first 3 hr from 19 to 21 August 2003 [blends of hexyl butyrate **(I)** and (*E*)-4-oxo-2-hexenal **(III)** dispensed from microcapillaries (m) or as 10% solution in Cereclor in polyethylene vial (v); means with different letters are significantly different ($P < 0.05$) by Tukey HSD]. Columns followed by the same letter are not significantly different.

DISCUSSION

In the present study, male *L. rugulipennis* were attracted to traps baited with (*E*)-4-oxo-2-hexenal alone, and the efficacy increased with the addition of hexyl butyrate when glass microcapillary tubes were used as the release system. These results differ significantly from those of our previous study (Innocenzi et al., 2004), when the same compounds released from either polythene vials or sachets were not attractive. Hexyl butyrate and (*E*)-4-oxo-2-hexenal are commonly found as defensive chemicals in Heteroptera (Staddon, 1979; Aldrich, 1988), although intraspecific communication functions of compounds traditionally regarded as defensive compounds are becoming increasingly evident, especially within the Miridae (e.g., Blum, 1996; Zhang and Aldrich, 2003b). Zhang and Aldrich (2004) also recently reported use of these types of compound as kairomones by predators of mirid species. The identification of an ester, hexyl butyrate, as a pheromone component is in keeping with other identified mirid pheromones (e.g., Smith et al., 1991; Millar et al., 1997). However, (*E*)-4-oxo-2-hexenal has, to our knowledge, never been previously reported as a sex pheromone component.

Attraction of male *L. rugulipennis* when the blends of these chemicals are dispensed from microcapillary tubes but not from polyethylene vials could be a result of one or more of several factors.

Pheromone Release Rate. Under laboratory conditions (27°C, 8 k/hr wind speed), release rates of the butyrate esters or (*E*)-4-oxo-2-hexenal from polyethylene sachet dispensers were approximately 1,500 and 140 μg/hr, respectively, whereas from polyethylene vials, the values were 158 and 7.5 μg/hr (Innocenzi et al., 2004). Dilution of these compounds as a 10% solution in the involatile, chlorinated hydrocarbon, Cereclor, reduced these release rates from vials by an order of magnitude (Table 1) compared with our previously published results (Innocenzi et al., 2004). Similarly, release rates for the butyrate esters from the microcapillary tubes were a further order of magnitude less and of the (*E*)-4-oxo-2-hexenal more than three times less under the same conditions (Table 1). Single female *L. rugulipennis* released hexyl butyrate at 0.04 μg/hr, whereas groups of four did so at 0.4 μg/hr/female under laboratory conditions (Innocenzi et al., 2004), the latter rate being similar to that of microcapillary tubes. This would suggest that the release rate is a critical factor in attraction of *L. rugulipennis* and that those in our previous experiments were too high. This suggestion is supported by the observation that significantly fewer *L. rugulipennis* females were caught in traps baited with blends containing hexyl butyrate than in unbaited traps (Innocenzi et al., 2004). Similarly, Wardle et al. (2003) found that *Lygus lineloaris* bugs were repelled by high doses of the same chemicals in a laboratory bioassay, although they could not demonstrate this effect in the field.

In 2003, *Lygus* males were no longer attracted to traps baited with the attractive blends after 3 hr. This was probably because of very rapid release of the chemicals, as temperatures in sunlight reached 37°C, and it had previously been shown that microcapillaries loaded with (*E*)-4-oxo-2-hexenal contained little material after 3 hr in the field (Aldrich et al., 1993).

Pheromone Plume Structure. The importance of pheromone plume structure in source location by Lepidoptera has been well established (e.g., Mafra-Neto and Carde, 1997). Thus, the greater success using microcapillary tubes (with an i.d. 0.6 of mm) may result from them producing more effective point source emissions than the relatively diffuse plumes created by sachets and vials. This may be particularly important where two or more dispensers are used for different components of the blend. Todd and Baker (1999) have shown that plumes from different dispensers can remain separate for distances of several meters downwind, so adjacent capillaries may provide a better approximation to a single plume than adjacent vials or sachets.

However, if this point-source emission is important, then it is necessary to reconsider release rate in terms of the concentration of material in the turbulent plume (Murlis and Jones, 1981) as this will influence the insect's behavioral responses (Mayer et al., 1987). The glass microcapillaries have an emission surface area of approximately 0.25 mm^2 at each end, compared with 650 mm^2 for the polyethylene vials and 1250 mm^2 for the polyethylene sachets used

previously (Innocenzi et al., 2004). Assuming pheromone is released uniformly over the dispenser surface, then using the data from Innocenzi et al. (2004), the surface release rates of butyrate esters and (*E*)-4-oxo-2-hexenal would have been 0.24 and 0.01 μg/hr/mm^2, respectively, from the polyethylene vials and 1.2 μg/hr/mm^2 each from the polyethylene sachet. Initial release rates of the butyrate esters and (*E*)-4-oxo-2-hexenal from the microcapillary tubes were up to 5 and 10 μg/hr, respectively, equating to 10 and 20 μg/hr/mm^2, respectively. Thus, attraction of *L. rugulipennis* males to the microcapillary dispensers may be the result of point source emission with a high concentration of the pheromone components, which more closely resembles the female insect.

Pheromone Blend. The blend of hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal released by female *L. rugulipennis* was found to be 1.5:1:0.08 from a single insect and 35:1:2 from four insects (Innocenzi et al., 2004). Release of (*E*)-4-oxo-2-hexenal from the microcapillary tubes was faster than that of the butyrate esters (Figure 1 and Table 1), so the blends used in these experiments would have been different from that produced by the insects. Furthermore, various combinations of vial and sachet dispensers were used in the previous experiments (Innocenzi et al., 2004) so that blends rich in (*E*)-4-oxo-2-hexenal quite similar to those used here would have been tested. Thus, it is unlikely that blend composition accounts for the attractiveness of the microcapillary dispensing system to male *L. rugulipennis*, but clearly, there is considerable scope for optimization of the synthetic blend in relation to that produced by the female.

Microcapillary tubes were used as pheromone dispensers by Kakizaki and Sugie (2001) to attract another mirid species, the rice leaf bug, *Trigonotylus caelestialium*. Release rates were not reported, and dispensers were used for up to 2 wk in the field, although our results show they would have been exhausted within a few days. Rubber septa have also been used as dispensers for the pheromones of other mirid bugs (e.g., Millar et al., 1997; Millar and Rice, 1998; Zhang and Aldrich, 2003a). Interestingly, release of hexyl butyrate (5 mg) from these septa under laboratory conditions was shown to be extremely rapid at first, but dropped to less than 1 μg/hr within 3 d (Innocenzi et al., 2004), comparable to the release rate from the microcapillary tubes.

Whereas (*E*)-2-hexenyl butyrate is produced by *L. rugulipennis*, adding it to the blend of hexyl butyrate and (*E*)-4-oxo-2-hexenal suppressed the attraction of male *L. rugulipennis*. However, this three-component mixture was significantly more attractive to males of the congeneric species, *L. pratensis*. Catching such high numbers of *L. pratensis* was unexpected as this species had not been recorded at East Malling in several years prior to the trials (Easterbrook, 1997, personal communication). Nothing is known about pheromone components produced by female *L. pratensis*, but trapping results suggest an interspecific role for (*E*)-2-hexenyl butyrate between *L. pratensis*

and *L. rugulipennis* that merits further investigation, especially as Glinwood et al. (2003) reported that (*E*)-2-hexenyl butyrate alone attracted *L. rugulipennis* males in an olfactometer.

The above results indicate that hexyl butyrate and (*E*)-4-oxo-2-hexenal are components of the female sex pheromone of *L. rugulipennis*, and the synthetic compounds attract significant numbers of males when dispensed from glass microcapillary tubes. To the best of our knowledge, this is the first report of males of a *Lygus* species being attracted to synthetic components of the female sex pheromone in the field. However, female-baited traps were not used in these preliminary trials, and only a limited range of blends was tested. Thus, it is not possible to say whether these are the only components of the pheromone or what is the optimum blend. Future work will determine the optimal ratio of hexyl butyrate and (*E*)-4-oxo-2-hexenal for attraction of *L. rugulipennis* males, and the role of (*E*)-2-hexenyl butyrate produced by the females will be investigated. The criteria for attractive dispensing systems will also be defined and more practical dispensers will be developed which have a longer field life than the microcapillary tubes. In addition, the trap design must be improved as we often observed that males alighting were able to escape from the sticky surface.

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SEX ATTRACTANT PHEROMONE FROM THE NEOTROPICAL RED-SHOULDERED STINK BUG, *Thyanta perditor* (F.)

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Abstract—Olfactometer bioassays showed that odors from mature *Thyanta perditor* males attracted females but not males. Furthermore, odors from females did not attract either sex, indicating that like other phytophagous pentatomid bugs, the males produce a sex pheromone. Attraction appeared to peak in late afternoon to evening. The headspace volatiles collected from male and female *T. perditor* were analyzed by GC-MS and HPLC. A male-specific compound, methyl (2*E*,4*Z*,6*Z*)-decatrienoate (2*E*,4*Z*,6*Z*-10:COOMe), was identified along with a number of other compounds found in extracts from both sexes. Bioassays carried out with 2*E*,4*Z*,6*Z*-10:COOMe showed it was as attractive to females as the crude extract of male volatiles, suggesting that the male-produced sex pheromone consists of 2*E*,4*Z*,6*Z*-10:COOMe as a single component. Consecutive volatiles collections from males showed that 2*E*,4*Z*,6*Z*-10:COOMe began appearing in extracts from males about 9 d after the final molt, as the males became sexually mature.

Key Words—Sex pheromone, methyl (2*E*,4*Z*,6*Z*)-decatrienoate, defensive compounds.

INTRODUCTION

Soybean, *Glycine max* (L.) Merrill, an important food and forage crop, is susceptible to attack by a variety of phytophagous Hemiptera (Turnipseed and Kogan, 1976; Kogan and Turnipseed, 1987; Jackai et al., 1990; Sediyama et al.,

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1993). Soybean is a relatively new crop in the tropics, but the endemic insect fauna have adapted rapidly to feeding on this plant (Panizzi and Corrêa-Ferreira, 1997). In Brazil, more than 30 species of hemipterans are associated with the soybean crop, with several reaching significant pest status (Panizzi and Corrêa-Ferreira, 1997). The most damaging include *Nezara viridula* (L.), *Euschistus heros* (F.), and *Piezodorus guildinii* (Westwood) (Panizzi and Slansky, 1985; Panizzi and Rossi, 1991), whereas others such as *Acrosternum aseadum* (Rolston) and *Thyanta perditor* (F.) are of lesser importance (Panizzi and Slansky, 1985). More than 4×10^6 l of insecticides are used annually to control stink bugs on soybean in Brazil (Corrêa-Ferreira and Moscardi, 1996). Alternatives, such as the exploitation of semiochemicals in the monitoring and control of these pests, might contribute to a more ecologically benign approach to their management.

Male-produced sex or aggregation pheromones have been identified for several species in the soybean stink bug complex in the last two decades (Aldrich et al., 1987, 1991, 1993, 1994; Baker et al., 1987; Borges et al., 1987, 1998a,b; Borges and Aldrich, 1994; Borges, 1995; Zhang et al., 2003). Two nearctic *Thyanta* species, *T. pallidovirens* and *T. custator accerra* (Millar, 1997; McBrien et al., 2002), use methyl (2*E*, 4*Z*, 6*Z*)-decatrienoate (2*E*,4*Z*,6*Z*-10:COOMe) and the three sesquiterpenes (+)-α-curcumene, (−)-zingiberene, and (−)-β-sesquiphellandrene as a male-produced sex pheromone blend. The congeneric neotropical species, *T. perditor*, is part of the stink bug complex attacking soybeans in Brazil, as well as other crops such as wheat and sorghum (Gomez, 1980; Grazia et al., 1982; Busoli et al., 1984; Panizzi and Corrêa-Ferreira, 1997). As part of our ongoing studies of semiochemicals of economically important stink bugs in Brazil, we initiated an investigation of the pheromone of this species. Our specific objectives were (1) to determine whether the stink bug *T. perditor* produces a sex pheromone, similar to those of its two nearctic congeners, *T. custator accerra* and *T. pallidovirens*, and if so, (2) to identify the chemical structure(s) of the biologically active compound(s). During these investigations, we also identified several defensive compounds from adult bugs of both sexes.

METHODS AND MATERIALS

Insects. T. perditor individuals were obtained from a laboratory colony started from adults collected from soybean fields during 2002/2003 near Embrapa Genetic Resources and Biotechnology Laboratory in Brasilia, DF, Brazil (15°47′S, 47°55′W). They were reared in 5-l containers on raw peanuts (*Arachis hypogaea*) and soybean (*Glycine max*) seeds, fresh green beans (*Phaseolus vulgaris*), boldo (*Plectranthus barbatus*) stalks, and water, at 26 ±

1.0°C and 65% ± 10% relative humidity under a 14:10 light/dark photoperiod (from 0600 to 2000 hr). The food supply was renewed three times per week. A square of plastic mesh (~40 mesh, 15-cm^2) was placed against an inner wall of each container as an oviposition substrate and shelter for the bugs. Egg masses were collected daily and incubated in 9-cm ID plastic Petri dishes until they hatched. When the resulting nymphs molted into second instars, they were transferred to plastic containers and reared as above.

To prevent interactions between the sexes, males were separated from females after cuticular hardening, ca. 24 hr after their imaginal molt. For all experimental bioassays, sexually mature adults, 9 d after the final molt, were used.

Collection of Volatiles. Volatiles were collected from groups of 20 male or female *T. perditor* (N = 13) in 1-l glass containers (Zhang et al., 1994) by drawing air through 6-14-mesh activated charcoal (Fisher Scientific, Pittsburgh, PA, USA), and out through two traps (15 cm × 1.5-cm OD) containing Super Q (200 mg each; Alltech Associates Inc., Deerfield, IL, USA) by vacuum (~1 l/min). Two traps in series were used to check for breakthrough of the volatiles being collected. Collection of volatiles from male and female bugs was started when they were 5 d old and bugs were aerated continuously for 15 d, changing adsorbent traps daily. This provided a continuous series of extracts reflecting the volatiles produced by adult bugs both before and after sexual maturation. Bugs in aeration chambers were fed with fresh green beans (replaced weekly). The adsorbent traps were eluted with hexane (4 × 0.5 ml), and the four resulting aliquots were combined and stored at −30°C until needed for analysis or bioassays.

Additional material for bioassays and analysis were obtained from aerations of groups of approximately 80 sexually mature, virgin individuals of each sex (N = 20) for periods of 15 consecutive d. The resulting daily extracts were concentrated under N_2 to yield a concentration of approximately 0.1 bug day equivalent (B.E.)/μl of solution for bioassays. For quantitative analysis, the volume of the extract was reduced to ~200 μl and an internal standard [40 ng (*E*)-2-decenyl acetate in 40 μl of hexane] was added to the solution.

Analysis and Fractionation of Extracts. The total and fractionated extracts were analyzed by gas chromatography (Perkin-Elmer GC; DB-1 column, 30 m × 0.25 mm ID, 0.25 μm film; J&W Scientific, Folsom, CA, USA), using a temperature program of 50°C/2 min, 15°C/min to 250°C. Data were collected with Autosystem Software in ASCII format and processed using Origin 5.0 (Microcal Software). Mass spectra were obtained with a Varian Saturn 2000 mass spectrometer interfaced to a Varian 3800 GC (DB-5 column, 30 m × 0.25 mm ID, 0.25 μm film; J&W Scientific). Crude extracts were also analyzed using a DB-5 column (30 m × 0.25 mm ID, 0.25 μm film thickness) at the University of California, Riverside, on a Hewlett-Packard 6890 GC interfaced to an H-P

5973 mass selective detector, using electron impact ionization (70 eV). The temperature program was 40°C/1 min, 10°/min to 250°C, injector temperature 250°C, and transfer line 280°C.

An extract of 80 mature virgin males in 1 ml of hexane was fractionated as described by McBrien et al. (2002). A column (0.5 cm ID × 6.0 cm long) with 500 mg of silica gel (80/100 mesh, Merck, Germany) was conditioned by eluting with 6 ml 25% ether in hexane followed by 6 ml hexane. The extract was loaded onto the column, which was then eluted sequentially with 3 × 2 ml hexane, and 3 × 2 ml ether, collecting each aliquot as a separate fraction. Hydrocarbons eluted in the first hexane fraction, whereas more polar esters, alcohols, and other compounds eluted in the first ether fraction.

A thermally unstable compound was previously found in the pheromone blend of the nearctic species *T. pallidovirens* (Millar, 1997), and so a crude extract from aeration of male *T. perditor* was analyzed by high-pressure liquid chromatography (HPLC) (Shimadzu) using a reverse-phase column (Lichrosorb RP-18, 250 × 4.6 mm, 5 μm particle size) eluted with 1:1:1 CH_3CN:CH_3OH:H_2O. The eluent was monitored at 300 nm with a diode array detector.

To determine the number of double bonds in the thermally unstable male-specific compound, a 20-μl aliquot of the crude extract in hexane was reduced in an H_2 atmosphere with 10% Pd on activated charcoal catalyst in a conical vial, stirring for 1 hr. The resulting mixture was filtered through a 1-cm plug of cellite, rinsing with pentane. The filtrate was analyzed by GC-MS.

Some of the insect-produced components from both sexes of *T. perditor* were tentatively identified by comparison of their GC retention times and mass spectral data with library data (NIST-Wiley database, 2000) with those of authentic standards. The only exception was α-ocimene, for which a standard was not available. All standards were obtained from Aldrich Chemical Co., with the exception of undecane (Matheson, Coleman, and Bell, Cincinatti, OH, USA), and tridecane and pentadecane (K&K Laboratories, Cleveland, OH, USA). The identification of the *T. perditor* male-specific ester was confirmed by comparison with authentic standards of 2*E*,4*Z*,6*Z*-10:COOMe and its isomers, available from previous studies on *T. pallidovirens* (Millar, 1997; McBrien et al., 2002).

Olfactometer Bioassays. Bioassays were carried out using a Y-shaped olfactometer, modified from Collaza et al. (1999) and consisting of an acrylic block (27.5 × 21.2 × 1.5 cm) in which a 2.0-cm-wide Y-shaped slot had been milled. The two arms of the Y were 10.3 cm long with a 45° angle between them. A 4.0-cm-diameter circle was cut out at the end of the 11.5-cm-long third leg of the olfactometer as a release chamber for the bug to be tested. A hole (0.5 cm ID) was drilled through the end of the slab into each arm to allow attachment of air lines. The end of the release chamber was perforated with two

holes, one of 0.5-cm diameter as an air outlet, and another in a rectangular shape (1.7 × 1.2 cm) to introduce test insects into the release chamber. The olfactometer block was placed on top of a translucent glass plate, and the upper surface was covered with transparent glass, with the glass plates held in place with large paper clips. Charcoal-filtered, humidified air was drawn through the system at a flow rate of 800 ml/min (regulated by flow meters, Accura Flow Products Div., Advanced Controls Engineering Corp., Hatboro, PA, USA). The olfactometer was surrounded with a black curtain to exclude external light and was illuminated from below by infrared lamps (homogenous emission of light at 950 nm provided by a panel of 108 light-emitting diodes) and from above with four 40-W fluorescent lamps. The temperature in the bioassay room was maintained at 26.0 ± 1.0°C. The insect behavior was monitored from above using a monochrome CCD video camera (Sony SPT M324CE) fitted with a 12.5–75 mm/F1.8 zoom lens interfaced to a personal computer. A video frame grabber digitized analog video signals from the camera, and "Xbug" software (Colazza et al., 1999) was used to process the data.

A single *T. perditor* was gently introduced into the release chamber of the Y tube with the aid of a artist's paint brush (Camel Hair, number 1) and allowed to acclimatize for a short period (ca. 3 min). Then it's pattern of movement (response) was recorded for 15 min/replicate. The first choice of the insect was recorded, i.e., the arm of the olfactometer into which a bug walked up at least 5 cm past the Y junction and remained there for at least 100 sec. The positions of control and treatment arms were alternated every five replicates. The chambers with the treatments consisted of two 10-ml syringes, one each for treatment and control, and were connected to the upwind side of the treatment and control arms, respectively. The apparatus was cleaned with fragrance-free liquid soap, rinsed thoroughly with water, and dried after each five replicates.

Determination of the Pheromone-Producing Sex. The attraction of mature adults of both sexes to odors emitted by five live males or females was tested against a clean air control. The live insects used as an odor source were changed each 10 replicates.

Determination of Period of Maximum Activity. Bioassays were carried out to determine the period of maximum pheromone-mediated activity, using five live male bugs as a pheromone source, with individual, sexually mature, female bugs as the test subjects. The males were replaced every 10 replicates. For this experiment, the following periods were designated: morning (M, 0800–1100 hr), early afternoon (EA, 1200–1500 hr), late afternoon (LA, 1600–1800 hr), and evening (E, 1900–2100 hr). A minimum of 74 individuals were tested during each time period and the number attracted to the pheromone source was recorded.

Bioassays with Insect Extracts and Synthetic Compounds. The Y-tube bioassay was used to compare the biological activity of crude male extracts,

fractions of extracts, and synthesized standards, with the solution of test stimulus [one bug day equivalent (BE) of extract or fraction] being spotted on a strip of filter paper (1.5 × 0.5 cm). Controls consisted of filter papers treated with hexane. Only sexually mature virgin females (>9 d old) were tested in these bioassays, which were carried out during the late afternoon and evening period.

Thirty bioassays were carried out with a crude extract obtained from a 24-hr aeration of 20 male bugs. The extract contained approximately 4 μg of 2*E*,4*Z*,6*Z*-10:COOMe. Hexane and ether fractions of an extract from males were tested vs. a hexane control ($N = 16$), and 20 and 14 replicates of the ether and hexane fractions, respectively, were assayed vs. the crude male extract. The ether fraction was also compared to the hexane fraction ($N = 34$). Finally, bioassays tested the responses of *T. perditor* females to synthetic 2*E*,4*Z*,6*Z*-10:COOMe vs. a hexane control ($N = 45$).

Statistical Analyses. Data from experiments comparing females' responses during different times of the day were analyzed using Kruskal–Wallis ANOVA and Dunn's test. Data on the number of responses of males and females to different treatments (live insects, aeration extracts and fractions thereof, and synthetic compounds, vs. controls) were analyzed using chi-square tests. Statistical analyses were carried out using MathCAD 2001.

RESULTS

Bioassays with Live Insects. Female bugs were attracted to odors from live mature males ($\chi^2 = 18$, $N = 32$, $P < 0.001$) but not to those of mature females ($\chi^2 = 0.25$, $N = 20$, $P = 0.62$) (Figure 1). Males were not attracted to odors of either sex (male: $\chi^2 = 0.29$, $N = 20$, $P = 0.58$; female $\chi^2 = 1.32$, $N = 20$, $P = 0.25$) (Figure 1). The attraction of females to males was highest in the afternoon/evening ($H = 8.60$, $df = 3$, $P = 0.035$; Dunn's test, $P < 0.05$) (Figure 2), indicating that females were most responsive and/or that production of pheromone by males was highest during this period.

Chemical Analyses of Insect Extracts. Comparisons of extracts from both sexes by GC and GC-MS analyses showed one large male-specific compound that was only seen in sexually mature individuals and was produced at a rate of about 200 ng/d, in addition to several compounds that were common to both sexes (Figure 3). Most of the components in the extracts were identified as α-pinene (**1**), β-pinene (**2**), α-ocimene (**3**), (*E*)-2-octenal (**4**), (*E*)-2-octen-1-ol (**5**), undecane (**6**), nonanal (**7**), dodecane (**8**), (*E*)-2-decenal (**9**), tridecane (**10**), and pentadecane (**12**). Most of these compounds are typical components of stink bug defensive secretions (e.g., Borges and Aldrich, 1992).

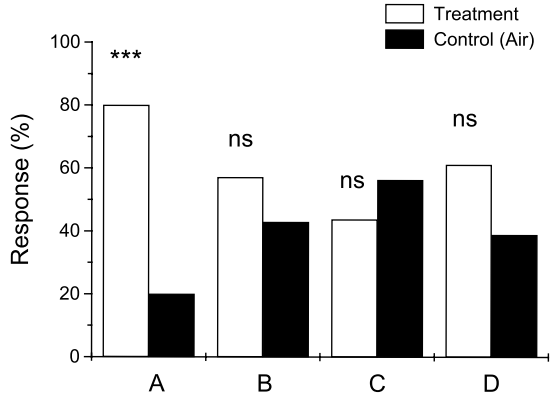

FIG. 1. Proportion of mature *T. perditor* males and females responding in a Y-olfactometer to different treatments vs. solvent controls: (A) Females' response to odors of live males. (B) Females' response to odors of live females. (C) Males' response to odors of live males. (D) Males' response to odors of live females. ***$P < 0.001$; ns, not significantly different (chi-square test).

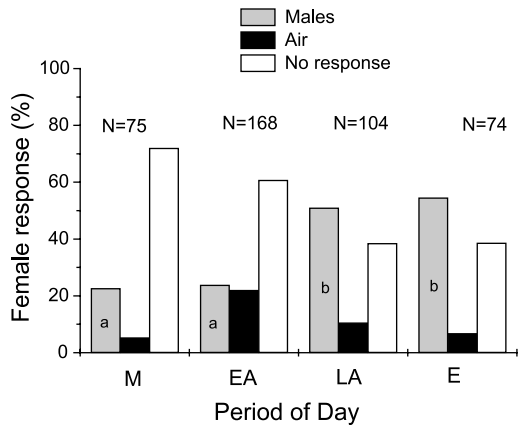

FIG. 2. Response of mature *T. perditor* females to odors of live males during different periods of the day in laboratory bioassays. The photophase in the laboratory was divided into four periods: "morning" (M), from 0800 to 1100 hr; "early afternoon" (EA), from 1200 to 1500 hr; "late afternoon" (LA), from 1600 to 1800 hr; and "evening" (E), from 1900 to 2100 hr. Response bars followed by the same letters are not statistically different (Kruskal–Wallis ANOVA and Dunn's test, $P < 0.05$).

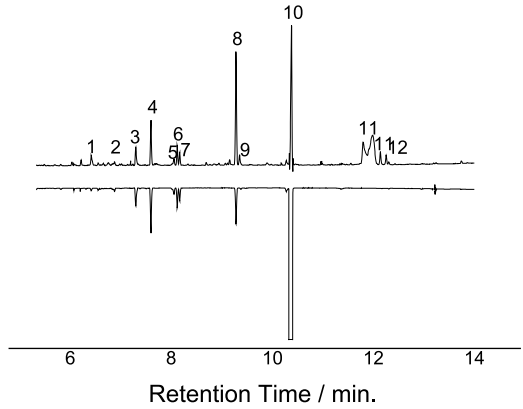

FIG. 3. Gas chromatograms of volatiles collected from live male (top) and female (bottom, inverted) *T. perditor*: **1**, α-pinene; **2**, β-pinene; **3**, α-ocimene; **4**, (*E*)-2-octenal; **5**, (*E*)-2-octen-1-ol; **6**, undecane; **7**, nonanal; **8**, dodecane; **9**, (*E*)-2-decenal; **10**, tridecane; **11**, distorted peak from thermal decomposition of methyl (2*E*,4*Z*,6*Z*)-decatrienoate; **12**, pentadecane.

The male-specific compound (**11**) gave a broad, irregularly shaped peak, suggestive of a thermally unstable compound (Figure 3). The mass spectrum of the unknown showed a distinct molecular ion of *m*/*z* 180 (M^+) and fragments at [*m*/*z* (abundance)] 149 (12), 133 (5), 120 (20), 105 (70), 91 (100), 79 (60), 74 (16), 65 (16), 39 (28) (Figure 4). The presence of fragments at *m*/*z* 105 and 74 suggested McLafferty rearrangement of a methyl ester and the fragments at *m*/*z* 148 and 149 (M^+-32, loss of CH_3OH, and M^+-31, loss of CH_3O, respectively) also indicated a methyl ester (Figure 4). The relatively low intensity of the *m*/*z* 74 ion suggested that the compound might be unsaturated at C_α and C_β or C_γ and C_δ. The molecular weight of 180 amu and the presence of a methyl ester resulted in a possible molecular formula of $C_{11}H_{16}O_2$, corresponding to three double bond equivalents once the ester carbonyl was accounted for. Experiments were carried out to determine the number, position, and stereochemistry of the double bonds. First, an aliquot of the crude extract was hydrogenated, resulting in the disappearance of the irregularly shaped, male-specific peak, and the appearance of a new peak, readily identified as methyl decanoate (*m*/*z* 186 amu) by comparison with an authentic standard, confirming that the insect-produced compound consisted of an unbranched chain with three carbon–carbon double bonds. HPLC analysis of the crude extract using a diode array detector showed that the unknown peak with *m*/*z* 180 amu comprised a single component with a UV absorption maximum at 300 nm, with the position of the absorbance proving that all three double bonds had to be conjugated with the ester carbonyl

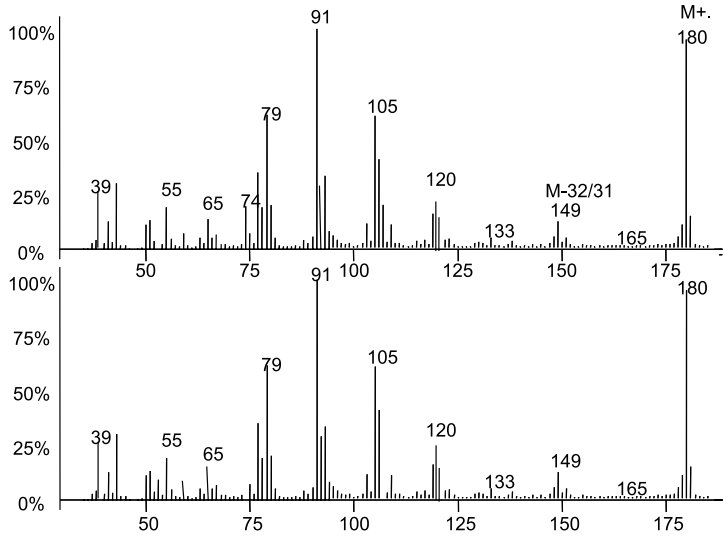

FIG. 4. Mass spectra of the naturally occurring ester in the male volatiles (top) and a synthetic standard of methyl (2*E*,4*Z*,6*Z*)-decatrienoate (bottom).

(Millar, 1997), that is, a methyl 2,4,6-decatrienoate isomer. Comparison of the GC retention time and peak shape, and the mass spectrum (Figure 4) of the unknown peak with those of the (2*E*,4*E*,6*E*)-, (2*E*,4*Z*,6*E*)-, (2*E*,4*E*,6*Z*)-, and (2*E*,4*Z*,6*Z*)-isomers of methyl (2,4,6)-decatrienoate resulted in matches only with the last compound. Furthermore, 2*E*,4*Z*,6*Z*-10:COOMe was the only isomer that was thermally unstable, apparently rearranging upon heating via a 1,7 sigmatropic hydrogen shift (Millar, 1997). Thus, the male-produced compound was identified as 2*E*,4*Z*,6*Z*-10:COOMe, identical to the compound produced by the congeneric *T. pallidovirens* (Millar, 1997; McBrien et al., 2002).

Bioassays with Insect Extracts. T. perditor females chose the treatment arm with the crude extract of males significantly more often than the hexane control arm ($\chi^2 = 6.13$, $N = 30$, $P = 0.013$) (Figure 5A). Whereas the hexane fraction was no more attractive than the hexane control ($\chi^2 = 0.11$, $N = 16$, $P = 0.74$) (Figure 5B), the ether fraction containing 2*E*,4*Z*,6*Z*-10:COOMe was ($\chi^2 = 3.84$, $N = 16$, $P = 0.045$) (Figure 5C). The ether fraction was more attractive than the hexane one ($\chi^2 = 5.76$, $N = 34$, $P = 0.016$) (Figure 5D) and equivalent to the crude extract ($\chi^2 = 0.053$, $N = 20$, $P = 0.82$) (Figure 5E), whereas the crude extract was more attractive than the hexane fraction ($\chi^2 = 0.807$, $N = 14$, $P = 0.005$) (Figure 5F). In a final bioassay, synthetic 2*E*,4*Z*,6*Z*-10:COOMe proved to be highly attractive, with 90% of the females choosing the arm with this stimulus ($\chi^2 = 15.51$, $N = 45$, $P < 0.001$) (Figure 5G).

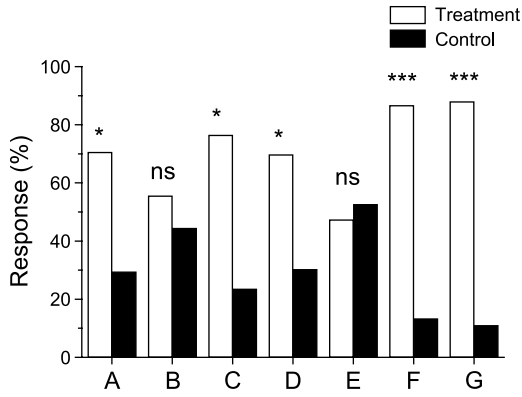

FIG. 5. Response of mature *T. perditor* females in the Y-tube olfactometer to different treatments and controls. In all cases, the first treatment mentioned is the open bar, and the second treatment (control) is the solid bar. (A) Crude aeration extract of male-produced volatiles vs. hexane. (B) Hexane fraction of a crude extract vs. hexane. (C) Ether fraction of a crude extract vs. ether. (D) Ether fraction of a crude extract vs. hexane fraction of the extract. (E) Crude aeration extract of male-produced volatiles vs. the ether fraction of the extract. (F) Crude aeration extract of male-produced volatiles vs. the hexane fraction of the extract. (G) 4 μg of synthetic methyl (2*E*,4*Z*,6*Z*)-decatrienoate in hexane vs. hexane (*$P < 0.05$, ***$P < 0.001$); ns, not significantly different.

DISCUSSION

Bioassays using all combinations of live insects as odor sources and responders showed that females were attracted to odors of male bugs, but that no other combination of odor and test subject produced significant attraction. These results indicated that male *T. perditor* produce a sex pheromone, analogous to the congeneric *T. pallidovirens* and *T. custator accerra* (McBrien et al., 2002).

Maximum attraction of males to females occurred during the afternoon/evening, similar to the peak response period of the stink bug *E. heros* (Borges et al., 1998b). In contrast, other pentatomids such as *N. viridula* (Borges et al., 1987) and *E. obscurus* (Borges and Aldrich, 1994) appear to exhibit no periodicity of response throughout the photophase. The reason for the differences in activity patterns between these species is unclear, but because they often share hosts and habitats temporal separation may play a role in maintaining interspecific reproductive isolation.

Our results suggest that the sex pheromone of *T. perditor* consists of only 2*E*,4*Z*,6*Z*-10:COOMe, one component of the sex pheromones of the nearctic congeners *T. pallidovirens* and *T. custator accerra,* (McBrien et al., 2002).

2*E*,4*Z*,6*Z*-10:COOMe alone attracted neither of these species and required at least one of the three sesquiterpenes, (+)-α-curcumene, (−)-zingiberene, and (−)-β-sesquiphellandrene, produced by the males (McBrien et al., 2002), but none of these sesquiterpenes were present in aeration extracts from *T. perditor.* The phenomenon of shared pheromone components appears to be common in the family Pentatomidae. For example, Aldrich et al. (1991) reported that methyl (2*E*,4*Z*)-decadienoate was a major male-specific component of volatiles produced by several *Euschistus* species. Pheromonal parsimony has also been seen with *N. viridula* and several *Acrosternum* species, all of which produce bisabolene epoxide pheromone components (Aldrich et al., 1993). *P. guildinii* and *E. heros* (Borges et al., 1999) also have methyl-2,6,10-trimethyltridecanoate as a shared component in their pheromones.

The reasons for the comparative simplicity of the pheromone of *T. perditor* relative to its congeners are not immediately apparent and merit further attention. Although they share many host plants, such as soybeans, this may be a relatively recent and, thus, a misleading "ecological" artefact resulting from man's introduction of exotic crop plants into new habitats rather than the evolution of species-specific communication systems. Furthermore, although *T. perditor* is now sympatric with *T. pallidovirens* and *T. curstator accerra* in Florida (Panizzi et al., 2000), it is of tropical origin (Panizzi et al., 2000), whereas the two neartic congeners are restricted to the western and southern United States (Schotzko and O'Keefe, 1990).

The defensive compounds identified in the headspace volatiles from adults of both sexes of *T. perditor*, such as (*E*)-2-octenal, (*E*)-2-decenal, nonanal, tridecane, and undecane, have been reported previously from other stink bug species (Borges and Aldrich, 1992), although (*E*)-2-decenal and nonanal were only observed in the male volatiles. In addition, both genders released some compounds that have been less commonly reported from stink bug airborne collections, such as α-pinene and β-pinene. These compounds are clearly insect-produced and not from the diet, because extracts of exuvia and metathoracic glands of both sexes contain these compounds. The specific functions of these compounds remain to be determined.

In summary, our results have shown that female *T. perditor* are attracted to 2*E*,4*Z*,6*Z*-10:COOMe, a pheromone released by sexually mature males of this species Because female bugs were strongly attracted to synthetic 2*E*,4*Z*,6*Z*-10:COOMe in laboratory bioassays, future efforts will focus on determining whether this compound can be exploited as an effective and selective trap bait for monitoring this stink bug species.

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MATING COMMUNICATION SYSTEMS OF FOUR PLUSIINAE SPECIES DISTRIBUTED IN JAPAN: IDENTIFICATION OF THE SEX PHEROMONES AND FIELD EVALUATION

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Abstract—The sex pheromones of four Plusiinae species in the family Noctuidae, *Ctenoplusia albostriata* (CA), *Macdunnoughia purissima* (MP), *Syngrapha ain* (SA), and *Diachrysia stenochrysis* (DS) were identified by GC-EAD and GC-MS analyses. These were subsequently evaluated by a series of field trials in different ecological habitats. CA females produced (*Z*)-5-decenyl acetate (**I**), (*Z*)-7-dodecenyl acetate (**II**), and (*Z*)-7-dodecen-1-ol (**III**) in a ratio of 2:100:13. While the antennae of CA males responded to all three components, neither **I** nor **III** was essential to capture males. The MP females produced **II**, **III**, and (*Z*)-5-dodecenyl acetate (**IV**) in a ratio of 100:80:20, and this blend captured the most MP males. Compounds **II** and **III** were also identified from SA females, and mixtures ranging from 4:1 to 2:3 were equally attractive to males. DS females only produced one active compound, (*Z*)-7-decenyl acetate (**V**), and DS is the first Plusiinae species identified as using only the ω3-compound and none of ω5-compounds, such as **II** and **III**, which are common components of Plusiinae pheromones. Using previously published chemical and biosynthetic data, together with the finding from this study, we propose a classification of the Plusiinae sex pheromones.

Key Words—Sex pheromones, Lepidoptera, Noctuidae, Plusiinae, male attractants, monoenyl acetate, GC-EAD, GC-MS.

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INTRODUCTION

The Plusiinae is a major subfamily in Noctuidae, comprising more than 400 species worldwide (Nomura, 1998). They are found from the tropics to the arctic, with many occurring in grasslands. The first sex pheromone reported in the Plusiinae was (*Z*)-7-dodecenyl acetate (Z7-12:OAc) from *Trichoplusia ni* females (Berger, 1966). Subsequently, 18 other species have been investigated (Ando, 2005; El-Sayed, 2005; Witzgall et al., 2004), and Z7-12:OAc is a pheromone component in 60% of species in Plusiinae subfamily studied to date (Ando et al., 2004).

There are 59 species from this subfamily found in Japan, one third of which are considered as pests of crops (Ichinose, 1962). Our research group has examined the pheromones of several important grassland species, i.e., *Plusia festucae* (Ando et al., 1995), *Thysanoplusia intermixta* and *Thysanoplusia orichalcea* (Ando et al., 1998), *Anadevidia peponis* (AP) and *Macdunnoughia confuse* (Inomata et al., 2000), and while they produce species-specific blends, Z7-12:OAc is a pheromone component in the latter four species. However, a pheromone blend of AP also attracted males of two other species, *Ctenoplusia albostriata* (CA) and *Macdunnoughia purissima* (MP) that occur in the same habitat (Inomata et al., 2000). In the present study, we examined the sex pheromones of CA and MP, as well as those produced by *Syngrapha ain* (SA) and *Diachrysia stenochrysis* (DS), two Plusiinae that occur in mountainous habitats, as little attention has been given to the pheromones of non-pestiferous species. Using the results of this study, together with previously published information, we discuss the diversity of Plusiinae pheromone chemistry and classify the Japanese species according to the biosynthetic pathways used to produce their key pheromone components.

METHODS AND MATERIALS

Insects and Pheromone Extracts. Laboratory colonies of CA and MP were established using adults on flowers of *Abelia grandiflora* near the vegetable fields of the Tokyo University of Agriculture and Technology (Fuchu, Tokyo). Larvae of CA were reared on fresh leaves of *Erigeron annuus*, and those of MP on an artificial diet [Insecta LF(S), Nippon Nosan Kogyo Co., Yokohama, Japan] mixed with dry powder of *Artemisia princeps* (5% w/w). Colonies of DS were set up, using adults captured in a black light trap run in a highland forest (Yunomaru-kogen, Nagano Prefecture) and larvae maintained on the same diet for MP. Colonies were reared at 25°C under a 16L:8D photoperiodic regime, and an extract for each species was obtained by soaking >100 pheromone glands, excised from 2- to 3-d-old female moths in the middle of the scotophase,

in hexane for 15 min. In the case of SA, a laboratory colony was not established and the extracts were obtained from 15 females captured with a black light trap at the same highland forest site as DS. Their glands were excised at the same time in the scotophase as the other species.

Chemicals. Monoenyl compounds with purity levels greater than 98%, supplied by Shin-Etsu Chemical Co. (Tokyo, Japan), were used as authentic standards for the analyses of natural pheromones and as lures for the field attraction of male moths. The chemical structures of pheromone components and related compounds are abbreviated in the same manner as in databases published on internet web sites (Ando, 2005; El-Sayed, 2005; Witzgall et al., 2004).

GC-EAD Analyses. Responses of male antennae to conspecific pheromone gland extracts were measured by GC-EAD (Struble and Arn, 1984) using an HP-6890 gas chromatograph (Hewlett-Packard, Wilmington, DE, USA) with a DB-23 capillary column (0.25 mm × 30 m, 0.25 μm; J & W Scientific, Folsom, CA, USA). The carrier gas was helium. The oven temperature was maintained at 80°C for 1 min, then heated at rate of 8°C/min up to 210°C, where it was maintained for 10 min. The effluent from the column was split equally into two lines, one leading to a flame ionization detector (FID) and the other to the EAD. An antenna was excised at the base from the corresponding male, and a few distal segments were cut off. *Bombyx mori* saline was used for the electrodes, and airflow (7.8 cm/min) carried eluted compounds to the antenna. Each extract was analyzed with at least four antennae cut from different 2- to 3-d-old males from the laboratory colonies. In the case of SA, antennae were obtained from wild males of unknown age, captured in a black light trap.

GC-MS Analyses. Electron impact GC-MS was carried out on a Hewlett-Packard HP5973 mass spectrometer system equipped with a DB-23 capillary column operating with a mass range of *m*/*z* 50–500. The ionization voltage was 70 eV. The ion source temperature was 230°C, and the column temperature program was the same as the GC-EAD analysis of crude pheromone extracts. A crude extract of 30 female equivalents (FE) from each species except for SA was derivatized at 40°C overnight in a mixture of dimethyl disulfide (DMDS) (50 μl) and diethyl ether solution of iodine (60 mg/ml, 5 μl) (Buser et al., 1983; Inomata et al., 2000). DMDS adducts were then analyzed using the following program: 50°C for 2 min, heated at a rate of 10°C/min to 160°C and 4°C/min to 220°C, where it was maintained for an additional 10 min.

Field Tests. Lures were prepared by applying 100 μl of single or multicomponent hexane solution to individual rubber septa (8 mm OD, white rubber; Aldrich), and tested by using sticky traps (Takeda Chemical Ind., Osaka, Japan) set at 1.5 m above ground. The evaluation of CA and MP lures was carried out on a University farm cultivating vegetables in a suburb of Tokyo,

while those for SA and DS were tested at the Nagano Prefecture forest site where adults had originally been collected. The traps were emptied every 2 wk, while the duration of trials and the number of replicates (two or three) varied depending on the species.

RESULTS

Pheromone Components Produced by CA Females. There were three EAG-active components in the CA female gland extract (1 FE, **I**, **II**, and **III** in Figure 1a): 60 μV at retention time (R_t) 9.7 min [Kováts relative index (RI) 1710], 300 μV at R_t = 12.0 min (RI 1920), and 10 μV at R_t = 12.2 min (RI

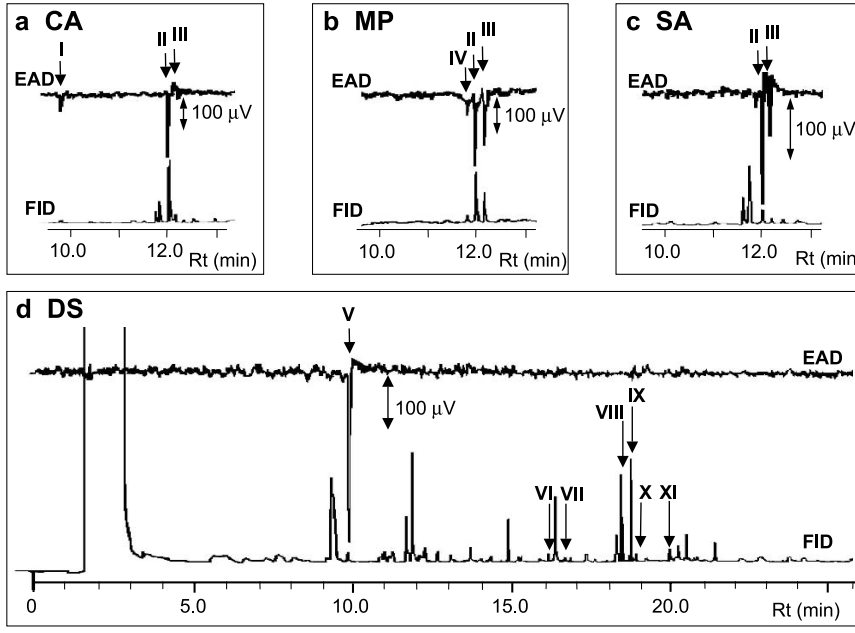

FIG. 1. GC-EAD analysis of the pheromone extract (1 FE) prepared from sex pheromone glands of (a) *Ctenoplusia albostriata* (CA), (b) *Macdunnoughia purissima* (MP), (c) *Syngrapha ain* (SA), and (d) *Diachrysia stenochrysis* (DS). Component **I** = Z5-10:OAc, **II** = Z7-12:OAc, **III** = Z7-12:OH, **IV** = Z5-12:OAc, and **V** = Z7-10:OAc. While the extract of DS females included a C_{10} acetate (**V**) and C_{16}–C_{20} acetates (component **VI** = 16:OAc, **VII** = Z11-16:OAc, **VIII** = 18:OAc, **IX** = Z11-18:OAc, **X** = Z13-18:OAc, and **XI** = 20:OAc), antennae of DS males responded to only **V**.

1930). These values coincided with the R_ts of synthetic Z5-10:OAc, Z7-12:OAc, and Z7-12:OH on the same column and not with the geometrical isomers E5-10:OAc at R_t = 9.5 min (RI 1690), E7-12:OAc at R_t = 11.8 min (RI 1900), and E7-12:OH at R_t = 12.0 min (RI 1920). The identifications were confirmed by comparing the mass spectra values of the components in the crude pheromone gland extract with synthetic standards. The mass spectrum of **I** at R_t = 8.70 min (RI 1706), which shows the largest ion at *m*/*z* 138 indicating $[M\text{-}60]^+$ of decenyl acetate, was almost the same as that of synthetic Z5-10:OAc. The spectrum of **II** at R_t = 11.16 min (RI 1917) with the largest ion at *m*/*z* 166, $[M\text{-}60]^+$ of dodecenyl acetate, and that of **III** at R_t = 11.30 min (RI 1928) with the largest ion at *m*/*z* 166, $[M\text{-}18]^+$ of dodecen-1-ol, coincided with the spectra of synthetic Z7-12:OAc and Z7-12:OH, respectively. A total ion chromatogram (TIC) gave an estimated ratio of 2:100:13 for the three components in the pheromone gland extract (Figure 2a). In addition, the double-bond position of

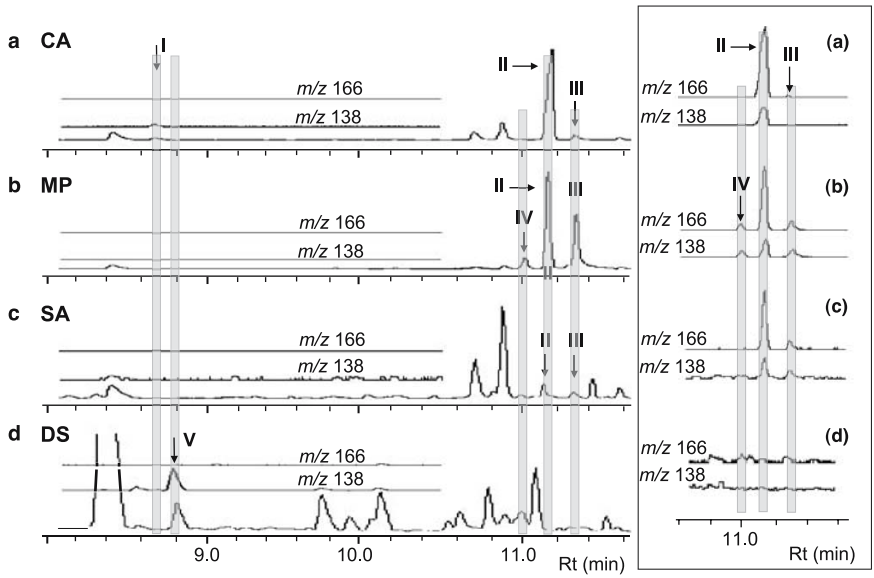

FIG. 2. TIC and mass chromatograms from GC-MS analyses of extracts (1 FE) prepared from sex pheromone glands of (a) *Ctenoplusia albostriata* (CA), (b) *Macdunnoughia purissima* (MP), (c) *Syngrapha ain* (SA), and (d) *Diachrysia stenochrysis* (DS). Component **I** = Z5-10:OAc, **II** = Z7-12:OAc, **III** = Z7-12:OH, **IV** = Z5-12:OAc, and **V** = Z7-10:OAc. Diagnostic fragment ions of the acetates ($[M\text{-}60]^+$) and alcohol ($[M\text{-}18]^+$) were monitored at *m*/*z* 166 for the C_{12} monoenyl compounds and *m*/*z* 138 for the C_{10} monoenyl compound. The mass chromatograms from R_t = 10.6 to 11.6 min are shown separately in the box to the right.

each component was confirmed by a GC-MS analysis of the DMDS derivatized crude pheromone with adducts derived from **I**–**III** showing their diagnostic ions.

Pheromone Components Produced by MP Females. The MP female gland extract (1 FE) showed three EAG-active components (**II**, **III**, and **IV** in Figure 1b) 200 μV at R_t = 12.0 min, 140 μV at R_t = 12.2 min, and 45 μV at R_t = 11.8 min (RI 1900). The first two proved to be Z7-12:OAc and Z7-12:OH, the same as in CA females. However, the third component (**IV**) was a C_{12} straight chain compound with identical chromatographic behavior as Z5-12:OAc but not the geometrical isomer, E5-12:OAc at R_t=11.6 min (RI 1890). GC-MS analysis of the MP extract supported the identification of the three components, which occurred in an estimated ratio of 100:80:20 for Z7-12:OAc, Z7-12:OH, and Z5-12:OAc, respectively (Figure 2b). Figure 3 shows TIC and mass spectra of DMDS adducts of the three C_{12} chain compounds. This measurement revealed the existence of the adducts derived from Z7-12:OAc showing ions at *m*/*z* 117, 203, and 320 [M^+] (Figure 3b), from Z7-12:OH showing ions at *m*/*z* 117, 161, and 278 [M^+] (Figure 3c), and from Z5-12:OAc showing ions at *m*/*z* 145, 175, and 320 [M^+] (Figure 3d).

Pheromone Components Produced by SA Females. GC-EAD analysis of the SA pheromone extract (1 FE) revealed two active components (**II** and **III** in

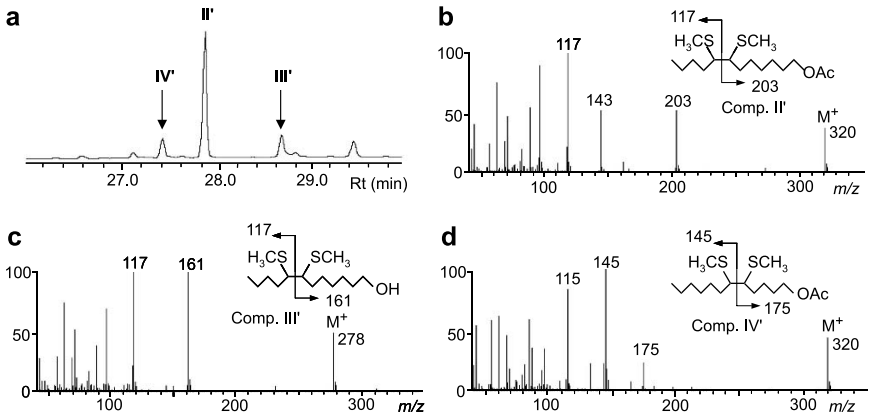

FIG. 3. GC-MS analysis of the pheromone extract of *Macdunnoughia purissima* (MP, 30 FE) treated with DMDS; TIC (a) and mass spectra (b–d) of DMDS adducts; (b) **II′** derived from Z7-12:OAc (R_t = 27.83 min), (c) **III′** derived from Z7-12:OH (R_t = 28.64 min), and (d) **IV′** derived from Z5-12:OAc (R_t = 27.41 min). Each spectrum shows M^+ and some diagnostic fragment ions produced by cleavage of the bond between the sulfur-substituted carbons, indicating the original position of the double bond.

Figure 1c): 160 μV at R_t=12.0 min and 80 μV at R_t=12.2 min. Their R_t and RI values were the same as authentic Z7-12:OAc and Z7-12:OH, and a GC-MS analysis confirmed this identification. The TIC analysis gave an estimated ca. 3:2 ratio of the two components (Figure 2c) but as the gland extract was prepared with a limited number of females of unknown age or mating status, this ratio may have little biological significance.

Pheromone Components Produced by DS Females. Only one compound in the pheromone gland of DS stimulated male antennae (**V** in Figure 1d): 400 μV at an R_t of 9.9 min (RI 1720). The R_t was the same as that of synthetic Z7-10:OAc but different from E7-10:OAc at R_t = 9.7 min (RI 1700). GC-MS analysis of the crude extract gave a mass spectrum at 8.81 min (RI 1720) with the largest ion at *m*/*z* 138, $[M\text{-}60]^+$ of decenyl acetate, that was the same as the synthetic standard (Figure 2d). The DMDS adduct showed the ions at *m*/*z* 89, 203, and 292 $[M^+]$, indicating the double bond at 7-position, which confirmed the identification of the compound as Z7-10:OAc. In addition to this component, GC-MS measurements also indicated the occurrences of six other longer chain (C_{16}–C_{20}) saturated or monoenyl acetates: 16:OAc, Z11-16:OAc, 18:OAc, Z11-18:OAc, Z13-18:OAc, and 20:OAc, with 18:OAc and Z11-18:OAc at very high levels. The ratio of Z7-10:OAc and the six acetates detected in the extract was

TABLE 1. ATTRACTIVENESS OF SYNTHETIC LURES COMPOSED OF THE SEX PHEROMONE COMPONENTS IDENTIFIED FROM *Ctenoplusia albostriata* (CA) FEMALE GLAND EXTRACTS TO MALES OF THREE SYMPATRIC SPECIES OF PLUSIINAE MOTHS IN A SUBURB OF TOKYO (FUCHU-SHI), JAPAN[a]

Lure components (mg/rubber septum)				Total males captured/trap (mean ± SE)[b]		
Z5-10: OAc (**I**)	Z7-12: OAc (**II**)	Z7-12: OH (**III**)	Ratio (**I**:**II**:**III**)	CA	MP	AP
0.02	0.87	0.11	2:100:13	147 ± 23 a	0 ± 0	0.3 ± 0.3 d
0.5	0.87	0.11	57:100:13	7 ± 4 bc	21 ± 0.3 b	31 ± 4 b
1.00	0.87	0.11	115:100:13	2 ± 0.3 bcd	11 ± 4 a	36 ± 8 b
0	0.87	0.11	0:100:13	180 ± 14 a	0 ± 0	0 ± 0
0.02	0.87	0	2:100:0	199 ± 6 a	0 ± 0	4 ± 0.3 c
0.50	0.87	0	57:100:0	10 ± 4 b	0.3 ± 0.3 b	92 ± 25 a
1.00	0.87	0	115:100:0	1 ± 1 cd	0.3 ± 0.3 b	45 ± 4 ab
0	1.00	0		169 ± 13 a	0 ± 0	0 ± 0
0	0	1.00		0 ± 0	0 ± 0	0.3 ± 0.3 d
1.00	0	0		0 ± 0	0 ± 0	0 ± 0
0	0	0		0 ± 0	0 ± 0	0 ± 0

MP = *Macdunnoughia purissima*, AP = *Anadevidia peponis*.

[a] Trials, with three traps for each type of lure, were run from October 20 to November 13, 2003 in a field where several vegetable crops were cultivated.

[b] Values within each column followed by a different letter are significantly different at $P < 0.05$ using a Tukey–Kramer test.

2:2:1:15:25:2:3 (Figure 2d). The antennae of DS males, however, scarcely responded to the longer-chain acetates (Figure 1d).

Field Tests in a Vegetable Field. When the three EAG active components isolated from CA females were tested in the field, lures with the major component Z7-12:OAc alone were as attractive to CA males as either the binary (Z7-12:OAc and Z7-12:OH) or tertiary (Z5-10:OAc, Z7-12:OAc, and Z7-12:OH) blends with relative proportions similar to those found in the pheromone gland extracts. Thus, there is no evidence of synergy by the minor components. However, when the concentration of Z5-10:OAc was increased in either the binary or tertiary blend, the numbers of CA males significantly decreased while those of *A. peponis* (AP) increased. In addition, some MP males were attracted to the tertiary blend with the highest concentration of Z5-10:OAc (Table 1), which was not detected in the MP pheromone extract.

TABLE 2. ATTRACTIVENESS OF SYNTHETIC LURES COMPOSED OF THE SEX PHEROMONE COMPONENTS IDENTIFIED FROM *Macdunnoughia purissima* (MP) FEMALE GLAND EXTRACTS TO MALES OF THREE SYMPATRIC SPECIES OF PLUSIINAE MOTHS IN A SUBURB OF TOKYO (FUCHU-SHI), JAPAN[a]

Lure components (mg/rubber septum)				Total males captured/trap (mean ± SE)[b]		
Z7-12: OAc (**II**)	Z7-12: OH (**III**)	Z5-12: OAc (**IV**)	Ratio (**II**:**III**:**IV**)	MP	CA	AP
(A) 1999						
1.0	0.8	0.2	100:80:20	14 ± 4	0 ± 0	0 ± 0
1.0	0.8	0	100:80:0	0 ± 0	6 ± 2	1 ± 1
1.0	0	0.2	100:0:20	0 ± 0	0 ± 0	0 ± 0
0	0	0		0 ± 0	0 ± 0	0 ± 0
(B) 2003						
0.5	0.4	0.1	100:80:20	4 ± 3	0.3 ± 0.3 c	0 ± 0
0.5	0.05	0.05[c]	100:10:10	4 ± 1	0.3 ± 0.3 c	72 ± 29 a
0.5	0.4	0	100:80:0	0 ± 0	47 ± 11 a	0 ± 0
0.5	0	0.1	100:0:20	2 ± 1	0 ± 0	1 ± 1 b
0	0.4	0.1	0:80:20	0 ± 0	0 ± 0	0 ± 0
1.0	0	0		0 ± 0	23 ± 6 b	3 ± 3 b
0	1.0	0		0 ± 0	0 ± 0	0 ± 0
0	0	1.0		0 ± 0	0 ± 0	0 ± 0
0	0	0		0 ± 0	0.7 ± 0.7 c	0 ± 0

CA = *Ctenoplusia albostriata*, AP = *Anadevidia peponis*.

[a] Trials, with three traps for each type of lure, were run from (A) October 6 to November 9,1999, or (B) November 13 to December 18, 2003 in a field where several vegetable crops were cultivated.

[b] Values within each column followed by a different letter are significantly different at $P < 0.05$ using a Tukey–Kramer test.

[c] Lure for AP, which is additionally baited with fourth and fifth components (0.25 mg of Z5-10:OAc and 0.15 mg of Z5-10:OH).

In the 1999 field trial with the three EAG active components (Z7-12:OAc, Z7-12:OH, and Z5-12:OAc) isolated from MP females, MP males were only captured by the lure including the components at the same 100:80:20 ratio found in the pheromone gland extract (Table 2A). This suggests that all components are essential to attract the males. However, while the highest catches were also seen with the tertiary blend in the 2003 field trail, some MP males were caught in traps baited with the binary blend of Z7-12:OAc and Z5-12:OAc (Table 2B). It is of interest to note that the number of MP males captured in traps baited with a five-component lure for AP (Z7-12:OAc, Z7-12:OH, Z5-12:OAc, Z5-10:OAc, and Z5-10:OH) did not differ from those baited with the MP tertiary blend. Thus, Z5-10:OAc and Z5-10:OH are not inhibitory for MP males. These field trials also indicate that significant greater numbers of CA males were captured by lures containing Z7-12:OAc alone or with Z7-12:OH than any of the other lures, with Z5-12:OAc clearly being an inhibitor (Table 2).

Field Tests in a Highland Forest. Significantly more SA males were captured in traps baited with either Z7-12:OAc or Z7-12:OH than in blank traps, but the binary blend was significantly better than either of the single components alone. However, the ratio of the two components in the lure did not significantly influence trap catches (Table 3). None of the lures tested for SA captured DS males. Z7-10:OAc, the only compound isolated from the DS pheromone gland that gave an EAG response, was significantly more effective at catching DS males than other acetates present in the gland extract (Table 4A). The inclusion of Z5-10:OAc, a pheromone component of two other species in the genus *Diachrysia* (Löfstedt et al., 1994), significantly reduced the number

TABLE 3. ATTRACTIVENESS OF SYNTHETIC LURES COMPOSED OF THE SEX PHEROMONE COMPONENTS IDENTIFIED FROM *Syngrapha ain* (SA) FEMALE GLAND EXTRACTS TO SA MALES IN A FOREST IN THE NAGANO PREFECTURE (YUNOMARU-KOGEN), JAPAN[a]

Lure components (mg/rubber septum)			Total males captured/trap[b]
Z7-12:OAc (**II**)	Z7-12:OH (**III**)	Ratio (**II**:**III**)	(Mean ± SE)
1.0	0	1:0 (100:0)	2 ± 1 c
0.8	0.2	4:1 (100:25)	44 ± 14 a
0.6	0.4	3:2 (100:67)	43 ± 11 a
0.4	0.6	2:3 (100:150)	40 ± 14 a
0.2	0.8	1:4 (100:400)	21 ± 6 ab
0	1.0		8 ± 4 b
0	0		0 ± 0

[a] Trials, with three traps for each type of lure, were run from July 23 to August 21, 2003.
[b] Values within each column followed by a different letter are significantly different at $P < 0.05$ using a Tukey–Kramer test.

TABLE 4. ATTRACTIVENESS OF SYNTHETIC LURES COMPOSED OF COMPOUNDS ISOLATED FROM *Diachrysia stenochrysis* (DS) FEMALE GLAND EXTRACTS TO DS MALES IN A FOREST IN THE NAGANO PREFECTURE (YUNOMARU-KOGEN), JAPAN[a]

(A)				(B)		
Lure (mg/rubber septum)				Lure (mg/rubber septum)		
Z7-10: OAc (**V**)	Z11-18: OAc (**IX**)	Others[b]	Total males/trap[c] (mean ± SE)	Z7-10: OAc (**V**)	Z5-10:OAc (unnatural)	Total males/trap[c] (mean ± SE)
0.04	0.5	0.46	16 ± 3 b	1.0	0	68 ± 25 a
1.0	0	0	40 ± 11 a	0.9	0.1	5 ± 1 b
0	0.5	0.5	0.7 ± 0.3 c	0.7	0.3	5 ± 2 bc
0	1.0	0	0.7 ± 0.7 c	0.5	0.5	2 ± 1 c
0	0	0	0.3 ± 0.3 c	0.3	0.7	0 ± 0
				0.1	0.9	0 ± 0
				0	1.0	0 ± 0
				0	0	0 ± 0

[a] Trials, (A) with three traps for each type of lure, were run from July 18 to August 20, 2002 and (B) with two traps from August 1 to 23, 2004.
[b] Mixture of 16:OAc, Z11-16:OAc, 18:OAc, Z13-18:OAc, and 20:OAc in a ratio of 2:1:15:2:3.
[c] Values within each column followed by a different letter are significantly different at $P < 0.05$ using a Tukey–Kramer test.

of DS males captured (Table 4B). None of the lures tested for DS captured SA males.

DISCUSSION

Analyses of the pheromone gland extracts (Figures 1–3) and field evaluations. of the components (Tables 1–4) identified the sex pheromones of four more Plusiinae species, *C. albostriata* (CA), *M. purissima* (MP), *S. ain* (SA), and *D. stenochrysis* (DS). Previous trials in a vegetable field had shown male attraction of CA and MP by the synthetic pheromone of *A. peponis* (AP) composed of Z5-10:OAc, Z7-12:OAc, and Z7-12:OH (50:100:10) (Inomata et al., 2000), but this study revealed that their actual pheromones differed from the AP pheromone. The CA pheromone is composed of the same three compounds but is produced at a quite different ratio (2:100:13), while MP females produced another tertiary blend with Z5-12:OAc instead of Z5-10:OAc. SA females from a highland forest produced a binary mixture composed of Z7-12:OAc and Z7-12:OH (3:2). This species is a defoliator of larch (*Larex* spp.), and this identification is the first pheromone from a nonpest Plusiinae species in Japan feeding on woody plants. The pheromone of DS, another species inhabiting the same mountainous habitats, consisted of one component, Z7-

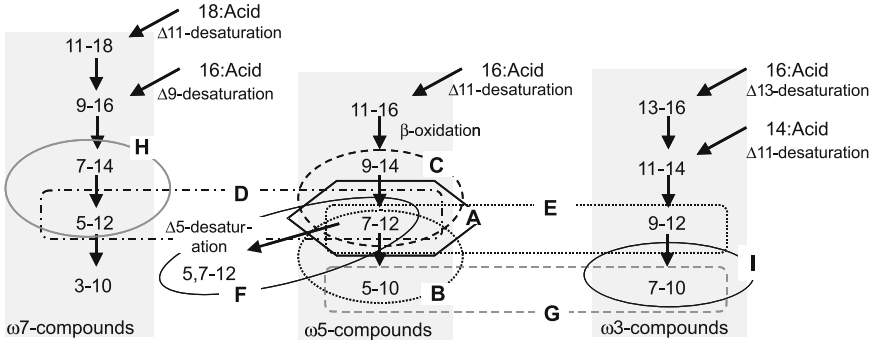

FIG. 4. A proposed classification of Plusiinae pheromones based on their biosynthetic pathways. The species of each group inhabiting Japan are as follows: (A) *Trichoplusia ni* (Berger, 1966), *Autographa gamma* (Dunkelblum and Gothilf, 1983), *Ctenoplusia albostriata* and *Syngrapha ain* (this work), (B) *Anadevidia peponis* (Inomata et al., 2000), (C) *Macdunnoughia confusa* (Inomata et al., 2000), (D) *Autographa nigrisigna* (Sugie et al., 1991) and *Macdunnoughia purissima* (this work), (E) *Chrysodeixis eriosoma* (Benn et al., 1982) and *Ctenoplusia agnata* (Sugie et al., 1990), (F) *Thysanopluia intermixta* and *Thysanopluia orichalcea* (Ando et al., 1998), (G) *Diachrysia chrysitis* (Löfstedt et al., 1994), (H) *Plusia festucae* (Ando et al., 1995), and (I) *Diachrysia stenochrysis* (this work).

10:OAc. Although six other saturated or monoenyl acetates were detected in the pheromone glands, they do not appear to be components of the sex pheromone.

Z7-12:OAc and Z7-12:OH, typical components of Plusiinae pheromones, were found in the pheromone blends of three of the four species, and there is evidence of redundancy for certain components, as reported for other Plusiinae species (Benn et al., 1982; Linn et al., 1984). However, species-specific aspects of the blends would still ensure reproductive isolation between sympatric species, such as CA, MP, and AP that all occur in cultivated fields near Tokyo. CA males respond equally well to lures made up of Z7-12:OAc alone as they do to those with Z7-12:OAc and Z7-12:OH, or the three-component blend Z5-10:OAc, Z7-12:OAc, and Z7-12:OH found in the female gland extract. However, while CA and MP share Z7-12:OAc and Z7-12:OH, the presence of Z5-12:OAc, an essential component for the attraction of MP males, inhibits attraction of CA males (Table 2). It has been shown that Z5-10:OAc may be substituted for Z5-12:OAc in MP lures and still attract males (Inomata et al., 2000). However, Z5-10:OAc must be present at much higher concentrations and at different ratios from those found in CA pheromone glands, so MP males would not respond to the real CA blend (Table 1). Similarly, while males of another sympatric species, AP, are attracted to a blend of the three components produced by the CA females, they respond to a different ratio (Table 1). In

addition, the AP males would not be attracted to MP females, as their pheromone blend lacks Z5-10:OAc.

The two-component Z7-12:OAc and Z7-12:OH blend found in the pheromone gland extract of SA might also attract CA males, although the ratios of compounds in the pheromone glands of the two species are quite different (Tables 1 and 3). There is, however, little likelihood of interbreeding, even if CA males responded to an off ratio, as the two species are not sympatric. DS and SA are sympatric in the forested area in the Nagano Prefecture, but as they share no common pheromone components (Figures 1 and 2), there would be no interspecific attraction (Tables 3 and 4).

The biosynthetic pathways of pheromone production in the Plusiinae have been studied using labeled precursors (Bjostad and Roelofs, 1983; Komoda et al., 2000) and at the molecular level (Knipple et al., 1998; Jurenka, 2004). The major component of many Plusiinae pheromones (Z7-12:OAc), as well as other acetates (Z9-14:OAc and Z5-10:OAc) with a double bond at the ω5-position, are biosynthesized *via* a (*Z*)-11-hexadecenoic intermediate, which is produced by Δ11-desaturation of palmitic acid (16:Acid). This C_{16} monoenyl compound is shortened to C_{14}–C_{10} acyl intermediates by β-oxidation and then converted into pheromone components by acetylation after the reduction of the acyl group into a hydroxyl group. In light of these findings, one could postulate that: (1) 7-tetradecyl (7-14) and 5-dodecyl (5-12) compounds (acetates and alcohols) unsaturated at the ω7-position result from Δ11-desaturation of 18:Acid or Δ9-desaturation of 16:Acid, and (2) other monoenyl components unsaturated at the ω3-double bond, 9-dodecyl (9-12), and 7-decyl (7-10) compounds arise *via* Δ13-desaturation of 16:Acid or Δ11-desaturation of 14:Acid. In fact, biosynthesis of E5,Z7-12:OAc *via* Δ5-desaturation of the 7–12 acyl intermediate has been documented (Ono et al., 2002).

Based on these proposed biosynthetic pathways and the known chemical components, we have classified the Plusiinae pheromones into the following eight groups (Figure 4): (A) 7-12 compounds only, (B) 7-12 and 5-10 compounds, (C) 7-12 and 9-14 compounds, (D) 7-12 and 5-12 compounds, (E) 7-12 and 9-12 compounds, (F) 7-12 and 5, 7-12 compounds, (G) 5-10 and 7-10 compounds, and (H) 5-12 and 7-14 compounds. Species with pheromone components in groups A–C would be biosynthesized *via* Δ11-desaturation of 16:Acid, whereas additional pathways would be required for the biosynthesis of certain compounds found in the pheromones of groups D–H. If one assumes that primitive species of Plusiinae utilized Z7-12:OAc for sexual communication, then one could postulate that the changes in the β-oxidation enzyme and the acquisition of new desaturases enabled the more recently evolved species to biosynthesize new pheromone components.

Z7-10:OAc has been reported as a component in five lepidopteran species: a gelechiid (Arn et al., 1997), a tortricid (McNair et al., 1999), and a Noctuiinae

(Wakamura, 1978), as well as two other species in the genus *Diachrysia*, *D. chrysitis*, and *D. tutti* (Löfstedt et al., 1994). However, DS, a species taxonomically remote from the primitive Plusiinae is the first to have a 7-10 compound as its sole pheromone component. This represents a ninth class of Plusiinae pheromones (group I in Figure 4), while the pheromones of the above two species in the *Diachrysia* genus fall in the group G. We are currently interested in studying the pheromones of several other *Diachrysia* species that inhabit the mountainous regions of Japan as this information could provide additional insight into the taxonomy and evolution of the subfamily Plusiinae.

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ERRATUM

THE ROLE OF CONTACT PHEROMONES IN MATE LOCATION AND RECOGNITION IN *Xylotrechus colonus*

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We regret that Table 1 of this article on page 540 contained several errors. A corrected version of the table is as follows:

TABLE 1. CUTICULAR HYDROCARBONS OF FEMALE AND MALE *Xylotrechus colonus*[a]

Peak	Hydrocarbon	Female	Male	Diagnostic ions
1	*n*-C25	+	+	352 (M^+)
2	9-MeC25	+	−	140, 252/253, 366 (M^+)
2	11-MeC25 (trace)	+	−	168/169, 224/225
3	2-MeC25	+	+	323, 351, 366 (M^+)
4	3-MeC25	+	−	309, 337, 366 (M^+)
5	2-MeC26	−	+	337, 365, 380 (M^+)
6	*n*-C27	+	+	380 (M^+)
7	11,13-MeC27	+	+	168/169, 196/197, 224/225, 252/253, 394 (M^+)
8	2-MeC27	+	+	351, 379, 394 (M^+)
9	3-MeC27	+	+	337, 365, 394 (M^+)
10	*n*-C28	+	+	394 (M^+)
11	13-MeC28	+	+	196/197, 238/239
11	12,11-MeC28 (trace)	+	+	168/169, 182/183, 252/253, 266/267
12	C29:1	+	+	406 (M^+)
13	C29:1	+	−	406 (M^+)
14	3-MeC28	−	+	351, 379, 408 (M^+)
15	*n*-C29	+	+	408 (M^+)
16	11,13,15-MeC29	+	+	168/169, 196/197, 224/225, 252/253, 280/281, 422 (M^+)
17	C31:1	−	+	434 (M^+)

[a] Peak numbers correspond with those in Figure 1. "+" indicates a compound is present and "−" indicates it is absent. 11-MeC25 and 12,11-MeC28 coeluted in trace amounts with other compounds. Peaks 12 and 13 represent isomers of the same alkene.

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PLANT CYANOGENESIS OF *Phaseolus lunatus* AND ITS RELEVANCE FOR HERBIVORE–PLANT INTERACTION: THE IMPORTANCE OF QUANTITATIVE DATA

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Abstract—Quantitative experimental results on the antiherbivorous effect of cyanogenesis are rare. In our analyses, we distinguished between the total amount of cyanide-containing compounds stored in a given tissue [cyanogenic potential (HCNp)] and the capacity for release of HCN per unit time (HCNc) from these cyanogenic precursors as a reaction to herbivory. We analyzed the impact of these cyanogenic features on herbivorous insects using different accessions of lima beans (*Phaseolus lunatus* L.) with different cyanogenic characteristics in their leaves and fourth instars of the generalist herbivore *Schistocerca gregaria* Forskål (Orthoptera, Acrididae). Young leaves exhibit a higher HCNp and HCNc than mature leaves. This ontogenetic variability of cyanogenesis was valid for all accessions studied. In no-choice bioassays, feeding of *S. gregaria* was reduced on high cyanogenic lima beans compared with low cyanogenic beans. A HCNp of about 15 μmol cyanide/g leaf (fresh weight) with a corresponding HCNc of about 1 μmol HCN released from leaf material within the first 10 min after complete tissue disintegration appears to be a threshold at which the first repellent effects on *S. gregaria* were observed. The repellent effect of cyanogenesis increased above these thresholds of HCNp and HCNc. No repellent action of cyanogenesis was observed on plants with lower HCNp and HCNc. These low cyanogenic accessions of *P. lunatus* were consumed extensively—with dramatic consequences for the herbivore. After consumption, locusts showed severe symptoms of intoxication. Choice assays confirmed the feeding preference of locusts for low over high cyanogenic leaf material of *P. lunatus*. The bioassays revealed total

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losses of HCN between 90 and 99% related to the estimated amount of ingested cyanide-containing compounds by the locusts. This general finding was independent of the cyanogenic status (high or low) of the leaf material.

Key Words—Cyanogenesis, chemical defense, *Phaseolus lunatus*, *Schistocerca gregaria*, herbivory, cyanogenic potential, cyanogenic capacity, plant–herbivore interactions.

INTRODUCTION

Cyanogenesis is widespread in the plant kingdom (Møller and Seigler, 1999). Over 2500 plant species are known to be cyanogenic and have the capacity to release gaseous HCN from endogenous cyanide-containing compounds, generally cyanogenic glycosides (Jones, 1988; Poulton, 1990; Seigler, 1998; Gleadow and Woodrow, 2002). Cyanogenic glycosides are β-glucosides of α-hydroxynitriles derived from protein and nonprotein amino acids (Zagrobelny et al., 2004). Hydrogen cyanide is toxic to the plant itself. Thus, cyanogenic plants must be able to synthesize and accumulate this toxin as inactive precursors to prevent autotoxicity (Vetter, 2000). Cyanogenic glycosides are separated in the intact plant from one or more specific β-glucosidases that are localized in the apoplast. In addition to separation on the cellular level, the cyanogenic component and the enzyme can be located in different tissues (Thayer and Conn, 1981; Frehner and Conn, 1987; Poulton, 1988; Swain et al., 1992). In case of injury, the β-glucosidase is brought into contact with its substrate. By hydrolysis of the cyanogenic glycosides, α-hydroxynitriles are formed that are relatively unstable and dissociate either spontaneously or are enzymatically accelerated by an α-hydroxynitrile lyase into HCN and an aldehyde or a ketone (Conn, 1980; Hösel and Conn, 1982; Poulton, 1990; Poulton and Li, 1994; Swain et al., 1992).

Hydrogen cyanide is toxic because of its affinity to the terminal cytochrome oxidase in the mitochondrial respiratory pathway and a number of other reactions (Solomonson, 1981; Brattsten et al., 1983). Despite the obvious toxicity of the products of plant cyanogenesis, its role in the scope of plant–herbivore interaction is very complex (Lieberei, 1988, 1989). A range of aspects determines its effectiveness as a mechanism of plant defense. The potential of cyanogenic glycosides as feeding deterrents depends on their concentration in the host plant. The concentration is often highly variable in natural populations. In addition, its phenotypic appearance depends on environmental conditions, the organ and tissue, and its ontogenetic status (Hughes, 1991; Bokanga et al., 1994; Schappert and Shore, 1999a,b; Gleadow and Woodrow, 2000b). If the concentration of cyanogenic precursors is below threshold toxicity, herbivores might not be affected (Feeny, 1976; Gleadow and Woodrow,

2000a). Herbivores can also avoid threshold toxicity by feeding on cyanogenic plants only as part of a mixed diet (Provenza et al., 1992). Furthermore, different herbivores are influenced in different ways by the presence of cyanogenic precursors because of their mode of feeding. Insect herbivores with sucking mouthparts such as phloem feeders (e.g., aphids) cause minimal tissue disruption during the feeding process and, therefore, avoid the release of toxic HCN or even reach tissues with low concentration of cyanogenic precursors depending on the length of their stylus (McMahon et al., 1995). A further important aspect is the degree of adaptation of the particular herbivore to its cyanogenic host. Specialist herbivores have evolved mechanisms to face cyanogenic glycosides (Provenza et al., 1992). Some use the cyanide as source of nitrogen or for their own defense against predators (Schappert and Shore, 1999b; Engler et al., 2000). For such herbivores, cyanogenic glycosides sometimes act as phagostimulants instead of feeding inhibitors (Calatayud and Le Rü, 1996; Mowat and Clawson, 1996). Thus, cyanogenesis may act as plant defense against generalist herbivores, but the effectiveness depends on many factors on both sides of the plant–animal interaction (reviewed by Gleadow and Woodrow, 2002).

Numerous authors have reported observations for defense functions of plant cyanogenesis against herbivores (e.g., Nahrstedt, 1985, 1988; Hruska, 1988; Jones, 1962, 1988, 1998; Crush and Caradus, 1995; Caradus and Forde, 1996; Schappert and Shore, 1999b,c; Tattersal et al., 2001). However, clear experimental results considering quantitative aspects of the action of HCN as causal factor of plant defense are rare (Feeny, 1976; Patton et al., 1997; Gleadow and Woodrow, 2000a,b). Analyses are complicated by the fact that the concentrations of cyanogenic precursors vary within a single plant and in relation to the ontogenetic status of the organs. For example, the concentration of cyanogenic precursors in leaves of different age differed by up to 90%. Furthermore, quantitative and kinetic analysis of the capacity for release of hydrogen cyanide as reaction to tissue disrupture—the basic measure of plant cyanogenesis—is often neglected. Qualitative statements concerning the release of HCN such as “yes” or “no” or semiquantitative test paper methods are insufficient to describe the complexity of cyanogenesis in interaction with a herbivore.

In view of these gaps, the goal of our study was to investigate quantitatively whether or not and how plant cyanogenesis interacts with the feeding behavior and feeding damage invoked by a generalist herbivorous insect. Furthermore, it has to be considered that deterrent properties of cyanogenic plants could depend on the total amount of bitter-tasting, cyanide-containing compounds stored in a given tissue [cyanogenic potential (HCNp)] or the amount of toxic hydrogen cyanide which can be released per unit time [cyanogenic capacity (HCNc)] or both.

For this, we designed a series of no-choice feeding experiments and additional binary-choice bioassays using lima beans (*Phaseolus lunatus*) as cyanogenic plants and the fourth instars of the African locust *Schistocerca gregaria* as generalist herbivores. Using this model system, we measured the HCNp of every leaf used in the bioassays and, in addition, the release of plant hydrogen cyanide per unit time during the course of feeding as response to damage of the leaf material. In this way, we obtained precise quantitative information on the action of plant hydrogen cyanide.

METHODS AND MATERIALS

Plants. P. lunatus seems to be an obligate cyanogenic plant. Extensive screenings of wild and weedy forms and cultivated genotypes revealed no acyanogenic forms (Baudoin et al., 1991). We tested seven defined *P. lunatus* accessions for resistance: L 2357 [origin: Spain; seed color: white/brown/violet, speckled; 100 grain weight (gw): 149.99 g], L 2233, wild type (origin: Cuba; seed color: gray/brown/black; 100 gw: 8.91 g), L 1315 (origin: Peru; seed color: white; 100 gw: 267.73 g), L 1259 (origin: unknown; seed color: brown/red/black, speckled; 100 gw: 82.39 g), L 2441 (origin: Bulgaria; seed color: white; 100 gw: 134.21 g), L 8079 (origin: unknown; seed color: white; 100 gw: 42.65 g), and L 8071 (origin: unknown; seed color: auburn; 100 gw: 45.23 g). In the following, only the numbers are used for specification of the accessions (2357, 2233, 1315, 1259, 2441, 8079, and 8071). Seed material was provided by the Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK) in Gatersleben, Germany.

In addition to these plants, the almost noncyanogenic *Phaseolus vulgaris* L. cultivar "Saxa" was used as control. This cultivar with the stock number 40.176 was purchased from Carl Sperling & Co., Lüneburg, Germany.

Growing and Treatment of Plants. To work with homogenous, biochemically well-defined plant material, single plants of the respective accessions were cultivated and vegetatively propagated as clones. The clones were cut as one node cutting from the mother plant, rooted in water, and, after a 2 wk rooting phase, transferred into standard substrate (TKS®-1-Instant, Floragard®), which was mixed with one third sand of a grain size of 0.3–0.7 mm and one third sand with a grain size of 1–2 mm. Plant containers with a diameter of 18 cm were used.

Between July and August, clonal collections of at least 30 plants were prepared per accession. Plant material was cultivated under greenhouse conditions at a light regime of 16:8-hr light/dark by a photon flux density of 400 μmol s^{-1} m^{-2} at the plant container and 900 μmol s^{-1} m^{-2} on the top of the plants, respectively. Accessional light was provided by 400-W high-pressure

sodium lamps with plant-grow broad-spectrum fluorescent bulbs (Son-Targo 400, Philips®). Temperature in the chamber was 30/20°C in a 16:8-hr light/dark period and ambient relative air humidity (range, 60–70%). Climatic conditions and irrigation of the plants were computer-based and controlled by INTEGRO® software. Plants were fertilized with a nitrogen-phosphate fertilizer (Blaukorn®-Nitrophoska®-Perfekt, Compo GmbH) twice a week.

Leaf material was classified by morphological parameters of development into the three classes "young," "intermediate," and "mature." In addition, the insertion position of the leaves at the stem was documented. Leaves at the apex or a side stem that were unfolded for at least 3 days were classified as "young." These leaves did not exceed one third to one half of the final leaf size. Leaves that were located one to two leaf insertion positions below the category of "young" leaves were defined as "medium" leaves. Leaves assigned to this category were not completely expanded and still showed thin and delicate leaf tissue. "Mature" leaves were located at the stem at least two insertion positions below the leaves that were classified as "medium." These leaves were characterized by a dark green color and a hardened midrib, and were always completely expanded.

The occurrence of pests and diseases was controlled, and the harvest of leaf material of a particular plant was followed by at least 4 wk until the next harvest of leaves to minimize possible effects of various induced defense mechanisms known from the Fabaceae (Fischer et al., 1990; Liu et al., 1992; Underwood, 1999; Underwood et al., 2000; Heil, 2004).

Insects. S. gregaria is a polyphagous insect and an economic pest throughout many regions of Africa (Mainguet et al., 2000). Locusts were provided by the Biocenter Grindel and Zoological Museum (University of Hamburg) and were held under the same conditions as the plants. They were offered a diet consisting of leaf material of different Poaceae (*Poa annua* and *Agropyron repens*) together with fruits (apple and banana). The fourth instars were used as herbivores.

Cyanogenic Features of Plants. Plant cyanogenesis requires at least two basic physiological properties: the ability to synthesize and accumulate cyanide-containing compounds [the cyanogenic potential (HCNp)] and the capacity to release hydrogen cyanide from these endogenous compounds [the cyanogenic capacity (HCNc)]. Therefore, release of HCN is described by two cyanogenic features. The HCNp is defined as the total amount of HCN that can be released from a specific tissue (Loyd and Gray, 1970). HCNc describes the release of HCN per unit time (Lieberei, 1988).

Analysis of Cyanogenic Potential. HCNp was measured by extraction of the cyanogenic precursors from leaf material. To avoid a premature release of HCN by degradation, the complete procedure from harvest of the leaves to extraction of the cyanogenic precursors was conducted with cooled solutions and devices (4°C). In addition, all steps of processing were conducted on ice.

Any injury of leaf material during harvest and transport was avoided, and the harvested leaves were immediately put into water-filled Eppendorf® tubes to minimize effects of wilting. Only the laminae of the leaves were used for analysis. Leaf material was ground with a mortar and pestle for extraction by adding 0.067 mol/l disodium hydrogen phosphate (2 ml/g fresh leaf material). The homogenized sample was filtered and centrifuged for 20 min at $13000 \times g$ and 4°C. The supernatant was used for further analysis.

For enzymatic degradation of the cyanogenic precursors, exogenous β-glucosidase was added to the respective sample to cleave the precursor glycosides and to detect all HCN that can be released. Different cyanogenic substrates require different β-glucosidases. Therefore, the cyanogenic substrate must be matched with appropriate and possibly specific β-glucosidases. The rubber tree *Hevea brasiliensis* (Willd.) Muell. Arg. (1865) contains the cyanogenic glycosides linamarin and lotaustralin, as does *P. lunatus* (Lieberei et al., 1986). Thus, the β-glucosidase of *H. brasiliensis* was assumed to show specific activity for hydrolysis of the cyanogenic precursors found in the lima bean. Fresh leaf material of *H. brasiliensis* was frozen in liquid nitrogen, ground with mortar and pestle by adding cooled phosphate buffer (4°C), and adjusted to pH 6.8 [1 g leaf fw/4 ml buffer (fw = fresh weight)]. The homogenized sample was filtered and centrifuged for 20 min at $13000 \times g$ and 4°C. The supernatant was filtered through membrane caps with a pore size <10,000 kD (Schleicher & Schuell Bioscience GmbH, Dassel, Germany) and frozen at −20°C for further analysis. Generally, β-glucosidase activity was determined by using *p*-nitrophenyl-β-D-glucopyranoside (Merck) as artificial chromogenic substrate. The amount of β-glucosidase added per sample corresponded to 20 nkat. Thunberg vessels were used as reaction flasks for the determination of the HCNp. These were stoppered by a glass stopper with a side bulb of about 1-ml volume. Thus, the Thunberg vessel contained a closed headspace, and the released HCN could not leak from the preparation. The mixture for incubation consisted of 0.05 ml supernatant of the centrifuged sample, 0.45 ml 0.067 mol/l aqueous sodium dihydrogen phosphate solution, 0.10 ml β-glucosidase solution in McIlvaine buffer, pH 5.6 (20 nkat), and 0.60 ml 0.2 mol/l NaOH in the side bulb of the stopper. This mixture was incubated in a water bath for 20 min at a temperature of 30°C. The enzymatic reaction was stopped by the NaOH solution, which was added from the side bulb of the stopper to the incubation mixture. Sodium salt of HCN was formed, and the concentration of cyanide was measured spectrophotometrically by use of the Spectroquant® cyanide test (Merck). This test is based on the formation of cyanogen chloride. This compound reacts with pyridine forming glutacone dialdehyde, which condenses with 1,3-dimethyl barbituric acid to a violet polymethine dye with a maximum absorbance at a wavelength of 585 nm. One mol of polymethine dye corresponds to 1 mol cyanide.

The standard preparation for spectrophotometric measurement of cyanide consisted of one aliquot (0.1 ml sample) that was taken from the stopped incubation mixture. The sample was neutralized by adding an aliquot of 0.1 mol/l HCl (0.1 ml) and made up to 5 ml with 4.8 ml aqua dest. HCl was added for the neutralization of the alkaline sample because pH 7 had to be adjusted to guarantee optimal reaction of Spectroquant® reagents by Merck. The concentration of the chromogenic product was measured spectrophotometrically after 5 min of incubation.

Analysis of Cyanogenic Capacity. We tested the clonal plant material of different accessions for the total quantities of HCN release and the pattern of HCN release, respectively. In these experiments, we also differentiated for the ontogenetic leaf developmental stages "young," "medium," and "mature" of each accession. For this analysis, single trifoliate leaves were treated with 400 μl chloroform. Chloroform disintegrates cell membranes and leads to a breakdown of cellular compartmentation. HCN is released by this chemical tissue rupture from cyanogenic precursors if any active endogenous β-glucosidases with affinity for the cyanogenic precursors are present. Pattern of hydrogen cyanide, which was released to the atmosphere after addition of chloroform, was followed over a time period of 1 hr. The leaf was treated with chloroform a second time later to test for further release of hydrogen cyanide.

HCN Detection System. Both the release of HCN from cyanogenic leaf material as reaction to chloroform treatment and the release from injured leaf tissue in the course of the feeding trials were detected by using the same airflow system (Figure 1). This system was passed by an adjustable airflow provided by a pump with upstream potentiometer for capacity control. The pump was connected with an air humidifier (Figure 1B) consisting of a triple neck flask with a total volume of 2 l filled with 1 l aqua dest. to ensure a sufficient ambient air humidity in the experimental system and to guarantee optimal ambient conditions for locust activity and avoid wilting of the leaf during the bioassays (air humidity was >90%). From the humidifier, air was passed through a 200-ml Erlenmeyer flask (Figure 1C) containing leaf material used for the analysis of the HCNc or for feeding trials with single locust nymphs, respectively. The flask was immersed into a water bath adjusted at 30°C to ensure high activity of the insects in the course of feeding trials, whereas light with an intensity of 200 μmol s^{-1} m^{-2} was provided by a 150-W spot (Philips®). The discharge opening of the flask was connected to a glass capillary inserted into a test tube filled with 0.1 mol/l aqueous NaOH solution (Figure 1D). The capillary released bubbles of a defined volume of 0.83 ml at an immersion depth of 10 cm. The airflow in the equipment was adjusted precisely to 70 bubbles per minute (≈3.5 l/hr). Inflowing air was kept at this relatively low speed because higher speed had a negative impact on locusts. The test tube containing the NaOH solution served as collector of the released HCN and was exchanged at intervals

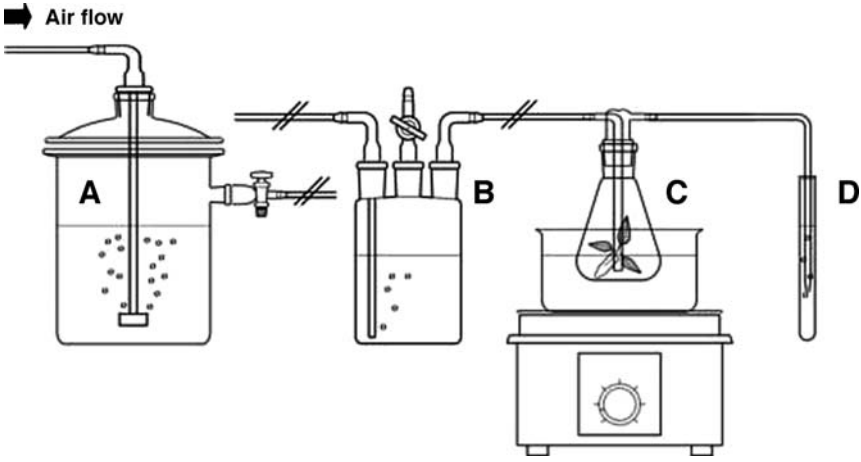

FIG. 1. Equipment for detection of gaseous HCN. The airflow in the vessel system was provided by a diaphragm pump with adjustable capacity. The air passes an air humidifier filled with aqua dest. (**B**) or a HCN generator (**A**) depending on the experiment. The HCN generator was filled with KCN solution (0.025–2.0 mmol/l) to provide a continuous HCN atmosphere within the airflow system for observation of behavior patterns depending on concentration of gaseous hydrogen cyanide. For detection of the cyanogenic capacity (HCNc), single trifoliate leaves were put into an Erlenmeyer flask (**C**) and treated with chloroform. In the course of bioassays, single locust nymphs were put into the flask (**C**) together with a trifoliate leaf. The flask was immersed into a water bath with a temperature of 30°C. Gaseous HCN—provided by the HCN generator, released from leaves as reaction to chemical cell disintegration or released from leaves as a result of tissue damage due to insect feeding—was detected quantitatively at the output of the equipment (**D**).

of 10 min. The pattern of the HCN release from leaf material per unit time was revealed by quantitative measurement of cyanide in the detection solutions by use of the Spectroquant® cyanide test (Merck). A pH of 7 was adjusted by adding 0.1 mol/l HCl to satisfy conditions required for this reagent kit. For analysis of the HCNc by chloroform treatment of particular leaves, the same speed of airflow was chosen, and the air humidifier was kept connected to the vessel system to achieve a similar experimental situation as in the feeding trials.

Bioassays. For no-choice feeding trials, single locust nymphs were placed into the 200-ml Erlenmeyer flask for HCN detection (Figure 1C) containing a trifoliate leaf of a defined accession and developmental stage. *P. lunatus* leaf material was characterized by specific cyanogenic features and was offered to the animals for 1 hr in no-choice feeding trials. In addition to the lima beans, *P. vulgaris* var. Saxa with extremely low cyanogenic concentrations was used

as control. Only locust nymphs of similar size and body weight were chosen for the experiments (mean ± SD, 0.987 ± 0.054 g; $N = 245$). Locust nymphs that molted about 48 hr previously were kept for 24 hr with *ad libitum* access to *Poa annua* leaf material which was part of their normal diet under the conditions mentioned above. They were food deprived for 1 hr prior to the bioassay.

The leaves used in the bioassays were harvested 1 hr after irrigation by the computer-based greenhouse control system. Leaves were put into water-filled Eppendorf® tubes with the petiole immediately after harvest and used in this form in the bioassays to guarantee a constant water supply. The amount of consumed leaf material was determined by weighing the particular leaf before and after the bioassay (MC 1 Analytic AC 210 S, Sartorius®) during constant water supply. This method for estimation of consumed leaf material was favored over leaf area measurements because even slight pressure during scanning in addition to interrupted water supply may lead to premature release of HCN. During the bioassay, release of HCN as a reaction to leaf material consumption by the locusts was consequently detected per unit time, and the duration of feeding on the particular leaf was documented. Detection of released hydrogen cyanide started with the first feeding incidence of the particular locust. Additional binary-choice feeding trials were conducted under the same experimental conditions. In these trials, selected single trifoliate leaves of high and low cyanogenic accessions were presented pairwise to the locusts, and the amount of leaf consumption was determined, respectively.

Behavior of Insects. A general statement as to whether feeding occurred or not did not seem to be sufficient to characterize the herbivore response. In preliminary studies, behavior of locust was observed by offering leaves of *P. vulgaris* and *Poa annua* as standard fodder. The animals were observed for 1 hr under the experimental setup for HCN detection. The HCNp of *P. vulgaris* var. Saxa was close to zero, and *Poa annua* did not contain any detectable cyanogenic components. In these preliminary experiments, very low and acyanogenic leaf material was used to ensure that insects were not affected by cyanogenic precursors or released HCN. These observed characteristics of behavior were interpreted as "normal." The following feeding trials were carried out under the same experimental conditions, but with leaf material of different cyanogenic accessions of *P. lunatus*. Leaf material of *P. vulgaris* var. Saxa was used as a control. Apart from feeding time, the following behavioral patterns were considered to reflect responses of the locusts to leaf consumption: general agility of the insects including the change of location after feeding, cleaning of the mouthparts, antennae, and legs, presence of flight reaction, and posture of the antennae and of the legs.

Behavioral patterns were defined as follows: 0, "normal" behavior (similar to behavior after consumption of leaf material of *Poa annua* and *P. vulgaris*); 1, no change of location, legs are angled and pressed onto the abdomen; 2, like 1,

but loss of any flight reaction and no cleaning of the mouthparts, antennae, and legs; 3, like 2, but, in addition, the antennae are sloped downwards by 45°.

During and after feeding on leaf material, changes of behavior occurred that were classified as defined above. Normal behavior on leaf material of *P. vulgaris* and *Poa annua* was characterized by high agility of the insects and by complete excitability to visual stimuli or vibrations. The feeding interval of the insects was followed by change of the location on the leaf and cleaning of the antennae, the compound eyes, wings, mouth parts, and legs. After intake of cyanogenic leaf material, the legs were pressed on the abdomen, and the locusts were less agile. This behavior was defined as the first level of negative effects (class 1). At this level, the insect still showed complete excitability with respect to visual stimuli or vibrations. The loss of this ability and the loss of comfort behavior together with the posture described above were interpreted as a more severe level of intoxication (class 2). In some cases, the locusts lowered their antennae downwards by about 45° in addition (class 3). In 1.7% (4 of 230 bioassays), the locust nymph died 1–2 hr after the experiment. These most severe signs of intoxication were included in class 3.

Exogenous Application of HCN. To test if application of exogenous hydrogen cyanide leads to similar effects with regard to locust behavior, we added quantified doses of gaseous HCN into the experimental vessel system by replacing the air humidifier (Figure 1B) by a gas generator (Figure 1A). This generator was made of a glass vessel with a total volume of 5 l that was filled with 3 l HCN-containing buffered solutions (phosphate buffer 6.7 mmol/l, adjusted to pH 5.5) and bubbled by a constant stream of air (≈3.5 l/hr). By use of defined cyanide solutions in the gas generator from 0.025 to 2 mmol/l, defined concentrations of gaseous HCN were constantly applied to *S. gregaria* individuals that had *ad libitum* access to almost noncyanogenic leaves of *P. vulgaris*. Gaseous HCN was added over a time period of 60 min. The speed of the airflow was controlled by detection of bubbles with defined volume that were released into a 0.1 mol/l aqueous NaOH solution by passing a capillary at the output of the equipment. The constancy of the HCN atmosphere within the equipment could be proven by spectrophotometric measurement of the amount of cyanide that was fixed in the NaOH solution per unit time using the Spectroquant® cyanide test (Merck). Experiments were conducted at a room temperature of 25°C, whereas the 200-ml Erlenmeyer flask containing the locust and leaf material was immersed into a water bath with a temperature of 30°C to ensure optimal conditions for locust activity. Light intensity was 200 μmol s^{-1} m^{-2} provided by a 150-W spot (Philips®).

HCN Balance. We calculated a HCN balance to investigate in which way the feeding insect gets in contact with HCN. Contact with released HCN could occur mainly during the feeding process, or large amounts of cyanide in bound form could be ingested and HCN might be released within the gastrointestinal

tract of the locust. Five aspects were considered to evaluate the fate of cyanogenic substances during the experiments: (1) amount of cyanide in bound form present in the particular leaf; (2) amount of cyanide consumed by the insect; (3) released HCN from leaf material during the feeding process of the insect; (4) released HCN from the locust bodies after consumption of leaf material; and (5) amount of cyanide in the feces.

The HCNp of every single leaf used in experiments was estimated. Leaf material that was left behind in the particular bioassay was used for analysis of the HCNp, and from these data, the amount of cyanogenic precursors within the consumed part of the leaf was calculated. The total amount of consumed leaf material was determined by weighing the particular leaf before and after the bioassay during constant water supply (MC 1 Analytic AC 210 S, Sartorius®).

The release of HCN from leaf material during the feeding of *S. gregaria* was measured per unit time with the equipment for HCN detection. For detection of hydrogen cyanide released from locust bodies into the atmosphere after leaf consumption, the insect was taken out of the experimental vessel and put in the center of a Conway's microdiffusional apparatus (semimicro type, Shibata Scientific Technology) with the outer ring filled with 10 ml 0.1 mol/l NaOH. The locust was left in there for 30 min. After that, the cyanide concentration of the surrounding NaOH solution was measured spectrophotometrically. The HCN content of the excrements that were obtained for up to 1 hr after the bioassay was analyzed by the method described for the HCNp determination of leaf material.

Statistics. Statistical analyses were run with Statistica 6.0 (Statistica System Reference, 2001).

RESULTS

Cyanogenic Features of P. lunatus. The clonal plants of all accessions studied were cyanogenic. All plants synthesized and accumulated cyanide-containing compounds in their leaves and were capable of releasing hydrogen cyanide from these compounds as reaction to chemical or mechanical damage.

Cyanogenic Potential. The variation of the HCNp was high and ranged from a HCNp of 0.7 μmol HCN/g leaf fw in mature leaves of accession 8071 to 82.0 μmol HCN/g leaf fw in young leaves of accession 2233. In general, the HCNp of young leaves was higher than in leaves of the intermediate or mature developmental stage. This ontogenetic pattern of variation was apparent in all clonal plants of all accessions studied (Table 1; Figure 2). Leaves of the cultivar "Saxa" (*P. vulgaris*) that served as control were characterized by very low concentrations of HCNp. This genotype was almost noncyanogenic.

TABLE 1. CALCULATED BALANCE OF CYANIDE INGESTED BY NYMPHS OF *S. gregaria*

Accession/ leaf development	Cyanogenic status, H = high, L = low	*N*	HCNp of leaf material (μmol HCN/g leaf fw)	Consumed leaf material (mg leaf fw)	Calculated amount of HCN in the consumed leaf material (nmol HCN)	Percentage of detected HCN (%)			
						HCN released to the atmosphere during the feeding process	HCN released from the locust bodies	HCN in the feces	Total loss of HCN
2357, young	H	11	60.5 ± 7.0	10.6 ± 7.5	646.8 ± 478.1	4.4 ± 3.7	0.4 ± 0.5	0.0 ± 0.0	95.1 ± 4.2
2357, medium	H	11	58.4 ± 7.7	15.6 ± 7.5	918.2 ± 522.7	2.1 ± 1.3	0.2 ± 0.1	0.1 ± 0.1	97.6 ± 1.4
2357, mature	H	11	14.9 ± 3.2	53.4 ± 18.3	792.1 ± 313.7	1.4 ± 0.7	0.3 ± 0.1	0.2 ± 0.1	98.2 ± 0.8
2233, young	H	4	60.0 ± 3.1	0.5 ± 0.1	27.0 ± 7.2	6.5 ± 4.4	1.9 ± 2.4	0.0 ± 0.0	91.6 ± 2.4
2233, medium	H	3	24.6 ± 2.7	1.2 ± 1.1	31.6 ± 31.9	15.9 ± 7.1	5.0 ± 7.6	0.0 ± 0.0	79.2 ± 8.4
2233, mature	H	3	6.8 ± 2.1	0.6 ± 0.4	4.3 ± 2.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100 ± 0.0
1315, young	H	11	51.7 ± 3.3	10.0 ± 5.3	514.5 ± 263.3	5.1 ± 2.8	0.3 ± 0.2	0.0 ± 0.1	94.5 ± 2.9
1315, medium	H	11	33.5 ± 4.4	18.3 ± 11.2	593.0 ± 321.0	3.1 ± 1.5	0.3 ± 0.1	0.0 ± 0.0	96.5 ± 1.5
1315, mature	H	10	18.1 ± 6.6	42.6 ± 21.1	677.1 ± 266.7	1.8 ± 0.9	0.4 ± 0.2	0.1 ± 0.1	97.7 ± 1.0

1259, young	H	11	30.5 ± 7.0	18.6 ± 8.5	544.5 ± 213.2	2.7 ± 1.3	0.2 ± 0.1	0.3 ± 0.2	96.9 ± 1.4
1259, medium	H	11	27.9 ± 5.1	23.1 ± 14.2	634.5 ± 401.8	3.6 ± 3.6	0.4 ± 0.4	0.4 ± 0.5	95.8 ± 4.1
1259, mature	H	11	18.0 ± 5.9	32.1 ± 17.5	588.2 ± 330.6	2.1 ± 1.5	0.3 ± 0.2	0.0 ± 0.0	97.1 ± 1.9
2441, young	L	11	18.8 ± 3.3	36.5 ± 25.0	635.6 ± 402.2	3.1 ± 3.5	0.3 ± 0.4	2.1 ± 1.4	94.5 ± 4.0
2441, medium	L	11	13.1 ± 3.9	52.3 ± 35.4	658.3 ± 434.5	2.6 ± 2.7	0.7 ± 1.2	14.3 ± 34.4	82.4 ± 38.1
2441, mature	L	11	6.2 ± 2.1	73.5 ± 26.1	444.1 ± 230.4	1.3 ± 0.7	0.6 ± 0.3	5.5 ± 2.1	92.8 ± 2.4
8079, young	L	8	9.4 ± 1.0	90.4 ± 22.6	849.3 ± 237.1	1.3 ± 0.3	0.1 ± 0.0	0.0 ± 0.0	98.6 ± 0.3
8079, medium	L	8	8.6 ± 0.7	96.4 ± 20.0	835.8 ± 214.7	1.3 ± 0.4	0.1 ± 0.0	0.0 ± 0.0	98.6 ± 0.4
8079, mature	L	7	8.1 ± 1.4	90.7 ± 22.1	726.3 ± 194.5	0.9 ± 0.5	0.2 ± 0.1	0.0 ± 0.0	98.9 ± 0.5
8071, young	L	11	6.5 ± 0.9	78.5 ± 18.3	505.4 ± 127.6	1.1 ± 0.9	0.1 ± 0.1	0.0 ± 0.0	98.8 ± 0.7
8071, medium	L	11	5.1 ± 0.6	52.8 ± 36.0	276.1 ± 200.2	1.0 ± 0.9	0.2 ± 0.3	0.0 ± 0.0	98.7 ± 1.2
8071, mature	L	11	1.2 ± 0.4	64.4 ± 25.1	77.2 ± 37.8	2.4 ± 1.9	0.5 ± 0.6	0.0 ± 0.0	97.1 ± 2.3
Saxa, young	Control	11	0.8 ± 0.2	84.9 ± 30.1	68.6 ± 27.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0
Saxa, medium	Control	11	0.9 ± 0.1	79.7 ± 26.0	71.5 ± 27.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0
Saxa, mature	Control	11	0.7 ± 0.1	87.2 ± 22.5	63.7 ± 20.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0

Notes: Cyanide ingestion by locust is calculated from the weight of consumed fresh leaf material and the cyanogenic potential (HCNp) of the particular leaf. The HCNp of every trifoliate leaf that was used in the bioassays was measured ($N = 230$). Data in percentage are related to the calculated amount of ingested cyanide that was considered as 100%.

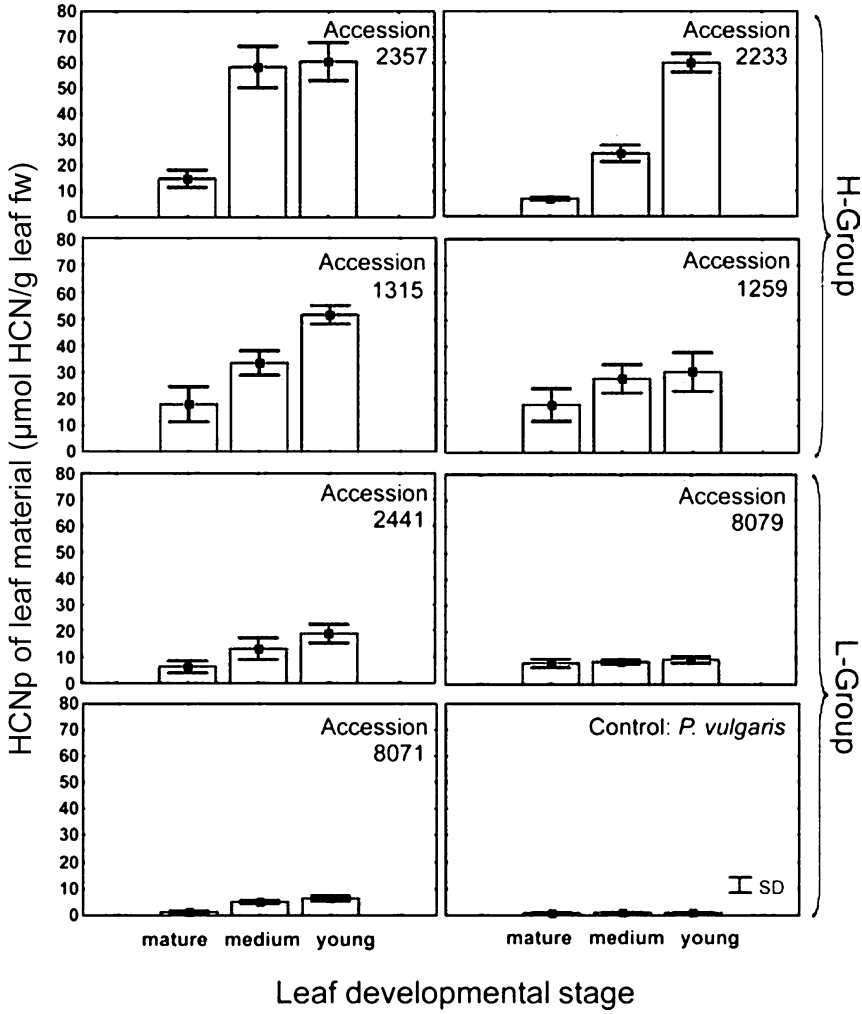

FIG. 2. Cyanogenic potential (HCNp) of *P. lunatus* accessions. Leaves of defined ontogenetic developmental stages of clonal plant material were analyzed. The clearly varying HCNp of the clonal lines allowed to distinguish between a high (H-group) and a low cyanogenic group (L-group) of plants. The control plants (*P. vulgaris* var. Saxa) revealed no substantial HCNp. Values are means ± SD. For number of observations, see Table 1.

Cyanogenic Capacity. The HCNc was estimated by measuring the kinetics of HCN release from leaves as reaction to chemically induced tissue disintegration. The pattern and the total amount of HCN released varied considerably between the accessions and the leaf developmental stages used for analysis (Figure 3).

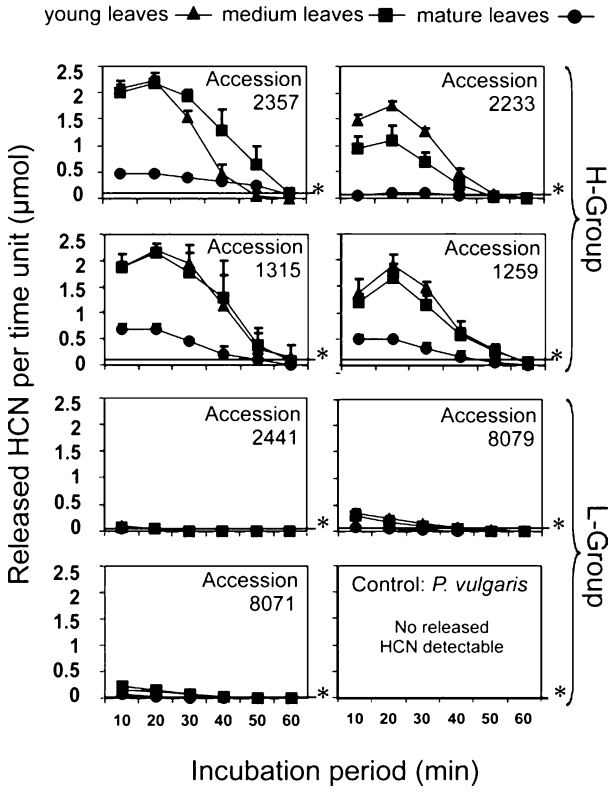

FIG. 3. Cyanogenic capacity (HCNc) of *P. lunatus* accessions. Leaves of defined developmental stages were analyzed for their capacity to release HCN from endogenous cyanide-containing precursors as reaction to chemical tissue disintegration due to chloroform application ($N = 5$ per accession and leaf developmental stage). Values given in the figure are means + SD. The release of HCN was quantitatively estimated per time periods (10 min) between the measurements. The experiments were carried out using the airflow system for detection of gaseous HCN and span a total time period of 1 hr. Application of chloroform to the same leaf a second time later in the experiment revealed no substantial release of hydrogen cyanide. Mean values of released HCN as reaction to this further treatment were below the level marked with an asterisk. The almost noncyanogenic *P. vulgaris* var. Saxa served as control.

Young and intermediate leaf developmental stages of the high cyanogenic accessions 2357, 2233, 1315, and 1259 revealed a rapid release of HCN and also a fast decrease of measurable hydrogen cyanide within 50 min when treated with chloroform. Mature leaves of these accessions were characterized by a lower and more continuous release of HCN that lasted as long as 60 min. No distinct maximum of hydrogen cyanide release was observed for these leaves. HCN was also released from leaves of all developmental stages of the low cyanogenic accessions of *P. lunatus* (2441, 8079, and 8071), but the release of HCN especially from mature leaves was close to zero. Control plants (*P. vulgaris*) had no capacity to release HCN from leaves. Thus, the pattern of HCN release from leaf material of *P. lunatus* depends on the cyanogenic status of the accessions as well as on the developmental stages of the leaves. No additional HCN loss was observed when chloroform was added to the leaves a second time later in the experiments. In addition, grinding the remaining leaf tissue under application of exogenous β-glucosidase isolated from *H. brasiliensis* revealed no substantial release of HCN (Figure 3).

Grouping of Plants for Analysis. Considering the results for the HCNp (Figure 2) and the corresponding HCNc (Figure 3), the accessions were assigned into a high (H-group) and a low cyanogenic category (L-group). Plants of the H-group were characterized by a HCNp of 34.4 ± 18.4 μmol HCN/g leaf fw (mean ± SD; $N = 108$). These plants (accessions 2357, 2233, 1315, and 1259) release more than 1 μmol HCN per trifoliate leaf within the first 10 min after treatment with chloroform. The plants of the L-group (accessions 2441, 8079, and 8071) showed a HCNp of 8.5 ± 5.7 μmol hydrogen cyanide/g leaf fw ($N = 89$). The H- and L-groups differed significantly (ANOVA, $F = 154.97$, $df = 1$, $P < 0.001$). The control *P. vulgaris* var. Saxa was almost noncyanogenic.

No-Choice Bioassays. Different effects of cyanogenesis on the susceptibility of leaf material to *S. gregaria* were observed in the feeding trials in relation to differences of HCNp and HCNc between the accessions of *P. lunatus* and their leaf developmental stages. Below a HCNp of 15 μmol HCN/g leaf fw that corresponds to a release of about 5 nmol HCN/10 min from the beginning of feeding under experimental conditions, there was no deterrent effect of cyanogenic leaf material observed (Figure 4). When feeding on plants of the H-group, locusts consumed less leaf material than when feeding on the L-group ($F = 197.02$, $df = 1$, $P < 0.001$). The intake of plants assigned to the H-group was 22.3 ± 19.6 mg fresh leaf material ($N = 108$). Within the H-group, substantial leaf consumption occurs exclusively on mature leaves with an HCNp near the threshold range of 15 μmol HCN/g leaf fw and a low capacity for HCN release. Leaves of accession 2233 were barely consumed despite the wide range of HCNp from 5.9 μmol HCN/g leaf fw in mature leaves up to more than 81 μmol HCN/g leaf fw in leaves of the young developmental stage. It is assumed that feeding deterrence of these plants is partly because of factors not

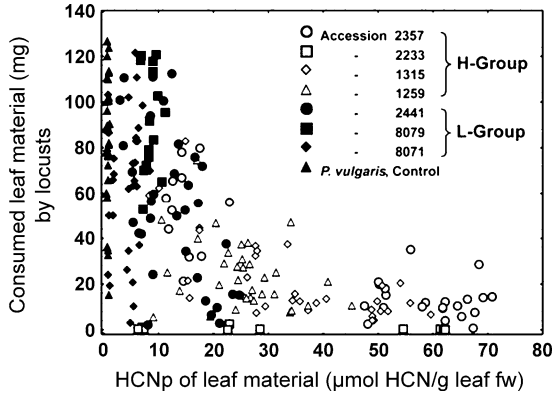

FIG. 4. Cyanogenic potential (HCNp) vs. leaf consumption. Leaves of high (H-group) and low (L-group) cyanogenic accessions of *P. lunatus* were presented to locusts over a time period of 1 hr within the airflow equipment for detection of gaseous HCN. Leaf consumption decreased with increasing HCNp of the presented leaf material. In this figure, there is no discrimination among ontogenetic leaf developmental stages. Results of statistical analyses are given in the text. For number of observations, see Table 1. The almost noncyanogenic *P. vulgaris* var. Saxa served as control.

considered in the present study, but may be a consequence of their outstanding high capacity for HCN release as reaction to feeding (Table 1). The intake of L-group plants of *P. lunatus* was 65.7 ± 32.9 mg fresh leaf material ($N = 89$). A tendency of reduced feeding was observed on the L-group accession 2441 where young leaves revealed the highest HCNp within this group and reached the threshold level of 15 µmol HCN/g leaf fw (Table 1). There was no measurable effect of the HCNp observed within the L-group of *P. lunatus* accessions with regard to leaf consumption. The leaf consumption by locusts on the *P. vulgaris* control plants (83.9 ± 26.6 mg fresh leaf material; $N = 33$) was significantly higher than the leaf intake on plants of the L-group of *P. lunatus* ($F = 7.99$, $df = 1$, $P = 0.006$). The HCNp and the corresponding HCNc affected the feeding behavior of locusts depending on threshold levels.

Binary-Choice Bioassays. Young leaves of the highest and the lowest cyanogenic accession (2357 vs. 8071) and leaves of the second highest and second lowest cyanogenic accession (1315 vs. 8079) were presented pairwise to the locusts (Figure 5). The choice tests between these leaves yielded a significant preference for the lower cyanogenic leaf material ($F = 88.62$, $df = 1$, $P < 0.001$).

Release of HCN as Reaction to Insect Feeding. Similar to the chemically induced release of HCN, the pattern of hydrogen cyanide released from leaf

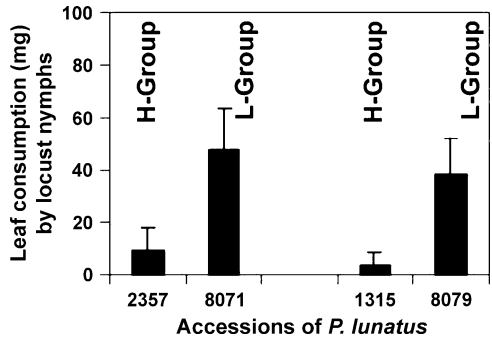

FIG. 5. Binary-choice bioassay. Leaves of H- and L-group plants were offered pairwise to single locust nymphs. Values are means and standard deviations ($N = 9$). Results of statistical analyses are given in the text.

material as reaction to insect feeding revealed differences depending on the general status of the HCNp, the accession, and the ontogenetic leaf developmental stage (Figure 6). Furthermore, the liberation pattern and amount of released HCN depended on the duration of feeding on selected leaf material. The time of feeding on H-group leaf material was 5 ± 4 min ($N = 108$) over the total observed period of 1 hr. On leaves of L-group plants, the locusts fed 18 ± 9 min ($N = 89$). The duration of feeding on H- and L-group plant materials was significantly different (ANOVA, $F = 164.87$, $df = 1$, $P < 0.001$). On high cyanogenic leaf material, further feeding incidences of a few locusts were observed over the time period of 1 hr. These further feeding incidences started 30–60 min after the beginning of the experiment; they were very short, and were not considered as time of feeding, but resulted in visible additional peaks of HCN release (Figure 6).

An interesting fact is that young and intermediate leaves of the L-group accession 2441 showed a substantial capacity for release of HCN as a reaction to feeding (Figure 6). This finding is in contrast to the very low HCNc detected as reaction to chemical tissue disintegration by chloroform treatment (Figure 3) and may indicate the existence of endogenous β-glucosidases in the locusts' saliva.

Effects of Leaf Development. Young leaves of the high cyanogenic accessions that were characterized by the highest HCNp and HCNc were consumed in small amounts. To clarify whether these cyanogenic features were acting as the effective components of plant defense, or if any other chemical or physical factors could be correlated with leaf maturation and might have influenced the feeding behavior of locusts quantitatively, we applied a post hoc test after ANOVA to the data set (Tukey's HSD for unequal N, $P < 0.05$;

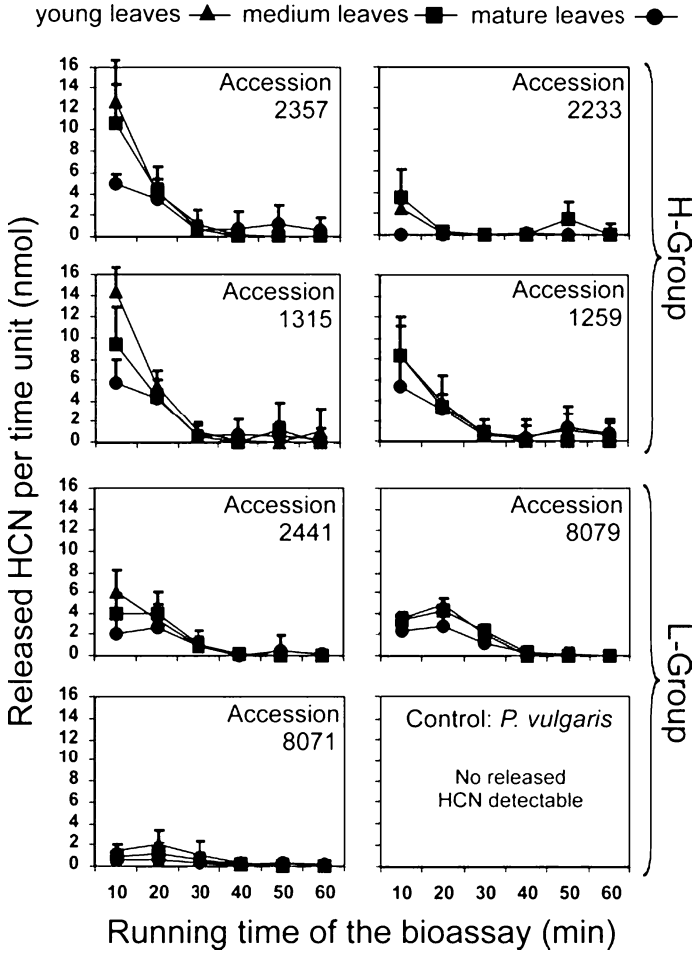

FIG. 6. Release of HCN from leaves as reaction to herbivory. Illustrated is the quantitative release of HCN as reaction to leaf consumption. This experiment was run with the airflow system for detection of gaseous HCN. The total period of detection of HCN release was 1 hr. The experiments started with the first feeding incidence of the locusts where duration is significantly different between the H- and the L-groups of plants. Peaks of HCN release, 30–60 min after start of the feeding trial, were results of further feeding incidents of single locusts. Values given in the figure are means + SD. The duration of the first feeding incidence and results of statistical analyses are given in the text; for number of observations, see Table 1. The almost noncyanogenic *P. vulgaris* var. Saxa served as control.

H-group, $N = 108$; L-group, $N = 89$). Within the H-group, no significant differences in consumption of young and intermediate leaves were found, but there was a significant difference between these leaves and the mature developmental stage characterized by lower HCNp and HCNc. Within the L-group, there was no significant difference in leaf consumption between the developmental stages. Thus, among the H-group plants, leaf consumption decreased with the increase of the cyanogenic features in younger leaves. Young and intermediate leaves of the high cyanogenic plants were not consumed less than mature leaves just because of the leaf developmental stage itself. Deterrent effects on locusts appeared to depend on the high HCNp and HCNc that occurred at this ontogenetic leaf developmental stages.

Effects of Cyanogenesis on Locust Behavior. The state of cyanogenic features affected locust behavior and led to different symptoms of intoxication. The reactions of the locusts feeding on *P. vulgaris* var. Saxa were defined as normal (behavioral pattern 0). This behavior contrasted with reactions of the insects after feeding on cyanogenic plant material of *P. lunatus*. These deviating patterns were interpreted as symptoms of intoxication (behavioral patterns 1–3). Strong symptoms of intoxication occurred after extensive consumption of leaf material of accessions 2441, 8071, and 8079 with a low HCNp and a corresponding low HCNc, whereas feeding on high cyanogenic leaf material (accessions 2357, 2233, 1315, and 1259) resulted in fewer or no symptoms of intoxication (Figure 7). The severity of intoxication strongly interacted with the

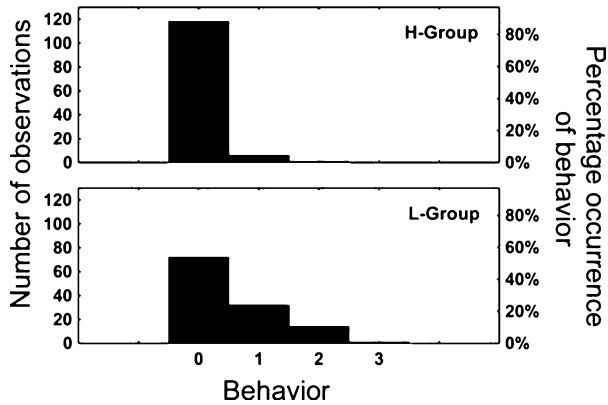

FIG. 7. Behavior of locusts. The behavioral patterns as reaction to consumption of high (H-group) and low (L-group) cyanogenic leaf material were documented and assigned to defined categories. These categories represent different levels of intoxication that range from normal behavior (0) to massive impairment of the herbivore (3). Presented are the total number of observations and the percentage occurrence of defined behavioral patterns among the H-group and L-group of plants.

amount of consumed leaf material ($F = 27.96$, $df = 3$, $P < 0.001$). Furthermore, the release of HCN from leaf material as a reaction to locust feeding was significantly higher for bioassays in which low symptoms of intoxication occurred ($F = 62.94$, $df = 3$, $P < 0.001$). Thus, the results indicate detrimental effects because of extensive ingestion of cyanogenic leaf material and a strong repellent action of high HCNc.

Exogenous Application of HCN. Locusts kept under a HCN atmosphere of 0.260–0.876 μmol/l revealed normal behavior (0). Agility, feeding behavior, and flight reactions were unaffected throughout the 1 hr of incubation under these conditions (Table 2). At a HCN concentration of 1.805 μmol/l in the gas phase, which corresponds to a cyanide concentration in the liquid phase of the generator of 0.5 mmol/l, the locusts showed reduced movements and angled legs that were pressed on the abdomen, whereas other aspects of behavior remained unchanged. This behavior corresponded to pattern 1 that was observed in the bioassays with cyanogenic *P. lunatus* leaf material. At the level of 3.612 μmol/l, the locusts showed reduced movements and, in addition, less feeding on the leaf material and loss of flight reaction. Therefore, this behavior was similar to pattern 2. At the highest HCN concentration of 7.518 μmol/l, the insects stopped feeding on leaf material and the antennae were sloped downwards as was characteristic for behavioral pattern 3 in the feeding experiments. The symptoms of intoxication caused by application of exogenous HCN at higher levels were the same as observed during or after feeding on cyanogenic leaf material. However, an additional characteristic of behavior was the frequent cleaning of the antennae at HCN concentrations above 3.612 μmol/l. All symptoms were completely reversible and disappeared about half an hour after taking the locusts out of the equipment and putting them on fresh leaf material.

In this experiment, we chose relatively low concentrations of cyanide compared to the conditions in cyanogenic leaf material. Even the highest concentrations of hydrogen cyanide in the HCN generator were in the lowest

TABLE 2. LOCUST BEHAVIOR UNDER EXPERIMENTAL HCN APPLICATION

KCN solution (mmol/l)	HCN atmosphere (μmol/l)	Locust Behavior	*N*
0.025	0.260	0	5
0.100	0.312	0	5
0.250	0.876	0	5
0.500	1.805	1	5
1.000	3.612	2	5
2.000	7.518	3	5

Notes: The HCN generator of the airflow system (A, Figure 1) was filled with adjusted KCN solutions to provide defined concentrations of atmospheric HCN. Behavioral patterns of locust on leaf material of control plants (*P. vulgaris* var. Saxa) were documented under these experimental conditions. The meaning of the codes for assignment of locust behavior is given in the text.

range of cyanogenic precursor variation present in *P. lunatus* leaf material. However, the concentration of gaseous HCN in the atmosphere at which first intoxication effects could be observed was 60 times higher than the highest concentrations of released HCN that was measured in the course of the feeding trials as reaction to leaf injury by the locusts.

HCN Balance. The amount of cyanide-containing compounds that were consumed together with the leaf tissue was calculated for every bioassay (N = 230). In addition, the amount of cyanide in the feces of the particular locust and the HCN released to the atmosphere from the locusts' bodies after the bioassay was included into the HCN balance (Table 1). The total calculated intake of cyanide-containing compounds present in the consumed parts of the leaves was not significantly different among all *P. lunatus* accessions ($F = 2.44$, $df = 1$, $P = 0.120$). But, when feeding on leaves on the mature developmental stage of the L-group accession 8071, the intake of hydrogen cyanide was low because of the extreme low HCNp of these leaves. HCN intake was even lower when locusts were feeding on the H-group accession 2233 with a high HCNp because of the small amounts of leaf material that were consumed from this accession. Within the H-group of plants, the intake of leaf material and, therefore, the intake of HCN tended to be lower by locusts feeding on young leaves with high cyanogenic features than by feeding on intermediate and mature leaves with lower cyanogenic properties. Considering the L-group of plants, in contrast, the intake of HCN by feeding on young leaves tended to be higher than when feeding on intermediate and mature leaf developmental stages because of the similar intake of leaf material but the higher HCNp of the young leaves compared with intermediate and mature leaves. The percentage release of HCN to the atmosphere during the feeding process was higher in the H-group than in the L-group of plants ($F = 475.16$, $df = 1$, $P < 0.001$). The young and medium leaf developmental stages especially revealed a high percentage of HCN release compared to the intake of hydrogen cyanide. A special situation was observed in bioassays with the H-group accession 2233. Here, the percentage of HCN released was 6.5 and 15.9% for the young and medium leaf developmental stages, respectively, higher than for any other accession.

Within the L-group, only young and intermediate leaves of accession 2441 and the mature leaves of accession 8071 revealed a considerable percentage release of HCN as reaction to feeding by the locusts. The relatively high percentage release of the latter was because of the fact that the intake of cyanogenic precursors by locust feeding on these leaves was extremely low corresponding to the very low HCNp that was not much higher than in the case of the control plants (*P. vulgaris*). Therefore, even low amounts of released HCN represent a relatively high percentage release. The release of HCN to the atmosphere from the locusts bodies for a time period of 1 hr after the bioassay was low for all accessions with the exception of locust that fed on young and

intermediate leaves of the H-group accession 2233. Here, the mean release of HCN was clearly higher than for the other bioassays.

Considerable amounts of hydrogen cyanide in locust excrement was detected after feeding on leaves of accession 2441 (Table 1). This might depend on the obviously low activity of the endogenous β-glucosidase that resulted in extraordinary low release of HCN as reaction to chemical tissue disintegration (Figure 3).

Among all bioassays, the total loss of HCN in this balance was high. Only for young and intermediate leaves of the H-group accession 2233 and the L-group accession 2441 was the loss lower. In bioassays using this leaf material, a high percentage release of HCN to the atmosphere occurred as a reaction to insect feeding, and high contents of HCN were found in the excrement, respectively.

DISCUSSION

There is substantial evidence that plant cyanogenesis acts as an effective herbivore deterrent against generalist herbivores (Jones, 1962; Hughes, 1991; Schappert and Shore, 1999c, 2000). The potential of cyanogenic glycosides in plants to act as a chemical defense was recently demonstrated by transferring the entire pathway for the synthesis of the aromatic tyrosine-derived cyanogenic glycoside dhurrin from *Sorghum bicolor* to *Arabidopsis thaliana*. The accumulation of dhurrin resulted in resistance of the transformed host plant to the flea beetle *Phyllotreta nemorum*, a Chrysomelid beetle, which is a natural pest on members of the cruciferous plants (Tattersal et al., 2001).

Furthermore, cyanogenesis seems to be beneficial for plants under specific environmental conditions (Kakes, 1989). The distribution of cyanogenesis in populations of *Lotus corniculatus* L. and *Trifolium repens* L. depends on abiotic factors that are also determining the occurrence and activity of snails, the important herbivores of clover (Jones, 1966, 1972, 1988). Hayden and Parker (2002) regarded this plasticity in *T. repens* as an important mechanism to reduce fitness-related defense costs.

According to Bernays et al. (1977), leaves of the cyanogenic tuber crop Cassava (*Manihot esculenta* Crantz) are consumed more frequently by nymphs of *Zonocerus variegatus* (Acrididae) in a senescent or wilted state than young and fresh leaf tissue. These wilted leaves showed a reduced release of HCN from their leaf tissues, but it should be kept in mind that it is not known to what extent other factors also change in a wilted leaf of *M. esculenta*.

In obligate cyanogenic plants such as *M. esculenta* and the lima bean *P. lunatus*, the precursors and the cleaving enzymes are present in the plant without any induction by abiotic stress or attack by pathogens and herbivores.

However, for these obligate cyanogenic plants, the release of HCN during the feeding process of a herbivore has never been quantified. There are several studies which analyze the amount of cyanide-liberating compounds in plants, its spatial distribution, and its dependence on environmental conditions (Jones, 1966; Cooper-Driver and Swain, 1976; Abbott, 1977; Cooper-Driver et al., 1977; Ellis et al., 1977a; Till, 1987; Caradus et al., 1990; Blaise et al., 1991; Hughes, 1991; Shore and Obrist, 1992; Calatayud et al., 1994; Caradus and Forde, 1996; Schappert and Shore, 1999a; Gleadow and Woodrow, 2000a). However, the central aspect of cyanogenesis, the capacity to release hydrogen cyanide from these endogenous compounds to the atmosphere, was just roughly classified by "yes" or "no" or semiquantitative estimation by Feigl–Anger test paper methods (Hayden and Parker, 2002). In addition, the additional measurement of β-glucosidase activity together with analysis of the content of cyanogenic precursors in plant material is not a conclusive measure for cyanogenesis occurring under natural conditions because there may be variation in tissue distribution of both factors (Gleadow and Woodrow, 2000b). Apart from the general ability for release of hydrogen cyanide and its total amount, the kinetics of HCN release may be an important aspect of plant cyanogenesis as protection against herbivores. It has to be assumed that the velocity of HCN release will affect the rate of herbivore deterrence. The fast movement of gaseous HCN in leaf tissue has been pointed out by Lieberei et al. (1996).

P. lunatus is an obligate cyanogenic species with quantitative variation of its cyanogenic properties. The status of HCNp and HCNc of leaves varied considerably depending on genotype, physiological state, and ontogenetic leaf development. Quantitative variation was evident in leaves of defined developmental stages of clonal plant material under controlled greenhouse conditions. Young leaves are generally characterized by a higher HCNp and HCNc than leaves of intermediate and mature developmental stages. Young tissues and organs of reproduction are mostly, but not in general, characterized by high contents of cyanogenic precursors. It may be assumed that these tissues need special protection because of their essential functions (Poulton, 1983, 1988; Gleadow and Woodrow, 2002). Alternatively, there might be investment decisions based on the expected life span of leaves (e.g., Coley, 1980, 1988; Coley et al., 1985). An attractive hypothesis would be that these cyanogenic substances are first used as feeding deterrents by young leaves before nitrogen is being metabolized (Selmar, 1986). Previous studies on the effects of cyanogenic glycosides on *Schistocerca americana* (grasshopper, Neoptera) and *Hypera brunneipennis* (alfalfa weevil, Coleoptera) revealed that these compounds deterred insects from feeding, but were not detrimental if ingested. Grasshoppers and weevils seemed to be more sensitive in detecting cyanogenic glycosides within food plants (Bernays, 1991).

The present study indicates a more complex action of cyanide-containing compounds and plant cyanogenesis. We were able to analyze the effect of

cyanogenesis concerning the herbivore–plant interaction between nymphs of the generalist locust *S. gregaria* and the cyanogenic plant *P. lunatus*. This was accomplished by the precise measurement of the HCNp of the particular leaf presented to the locusts and by quantitative detection of the HCN released during the feeding process of the insects. However, in consideration of these data and by additional observation of the locusts behavior during and after leaf consumption, the results suggest that plant cyanogenesis in this experimental system is involved in at least two levels of plant defense. On the one hand, high HCNp and HCNc result in a clear repellent effect. On the other hand, consumption of leaves with low HCNp and HCNc leads to severe intoxication. So, this complex action is based both on the presence of cyanide-containing compounds and the kinetics of their bioavailability.

The identification of a certain threshold level at which the HCNp and the HCNc become effective is of special interest. Certain thresholds of cyanogenic precursors and released HCN must be reached to achieve repellent action against *S. gregaria*. Severe intoxication of locusts occurred after extensive consumption of low cyanogenic leaf material. Obviously, the insects did not react to the small amounts of HCN released during the feeding process, and, thus, the total intake of cyanogenic leaf material was above a critical threshold for intoxication. It cannot be excluded that factors other than the HCN releasing compounds may be toxic to the locusts and may have caused the detrimental effects. But, the time sequence and symptoms of intoxication were the same regardless of whether we applied exogenous HCN to locusts feeding on almost noncyanogenic leaf material or whether they were feeding on the cyanogenic varieties. During feeding on high cyanogenic leaf material—especially on young leaves—considerably more HCN was released per unit time than during feeding on low cyanogenic leaves. This physiological feature of immediate HCN release in high amounts obviously represents a potent repellent effect. Thus, high and low cyanogenic leaves revealed different modes of action on the herbivore. Calculation of a HCN balance based on the consumed cyanogenic leaf material revealed that the amount of cyanide in the excrements and the amount of HCN released from the locust bodies after feeding were always low. Accordingly, the total loss of hydrogen cyanide within this balance was always high, indicating a metabolization of cyanide by the locust or an interaction of this highly reactive substance with the insect tissues. The HCN balance prepared from feeding trials with leaves of the H-group accession 2233 (wild type) indicated the impact of the HCNc as a feature with high repellent potential. Concerning this accession, bioassays with leaves of all developmental stages revealed extraordinary high percentage rates of HCN release as reaction to locust feeding compared to the other high and low cyanogenic accessions. However, leaves of all developmental stages of this accession were consumed in very low amounts independent of the widely varying HCNp.

This study demonstrates for the first time a quantified protective effect of the HCNp and of the corresponding HCNc against herbivorous insect attack depending on threshold levels. The different modes of action of high and low cyanogenic accessions of *P. lunatus* deserve further discussion, and experiments with respect to the biological function and with special regard to the ecological costs of these systems. *P. lunatus* is an agricultural crop of increasing importance as source for human food and green fodder for livestock (Debouck, 1991). It is important to evaluate possible agroecological advantages or disadvantages of cyanogenesis. HCN release is not only acting on plant–herbivore interaction, but it is also influencing plant–microbe balances (Lieberei et al., 1983) and may also give an allelochemical input to plant–soil relations. The insights gained from this experimental system could easily be transferred to other cyanogenic plants of global economic importance such as cassava (*M. esculenta* Crantz).

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BIOLOGICAL ACTIVITY OF ACYL GLUCOSE ESTERS FROM *Datura wrightii* GLANDULAR TRICHOMES AGAINST THREE NATIVE INSECT HERBIVORES

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Abstract—*Datura wrightii* is dimorphic for leaf trichome type in southern California. "Sticky" plants produce glandular trichomes that secrete acylsugars, whereas velvety plants produce nonglandular trichomes. Glandular trichomes confer resistance to some potential insect herbivores and are associated with reduced feeding in the field by two native coleopteran herbivores: the tobacco flea beetle, *Epitrix hirtipennis*, and a weevil, *Trichobaris compacta*. In contrast, another native beetle, *Lema daturaphila*, damages sticky and velvety plants similarly in the field. A series of choice and no-choice "ester removal" and "ester addition" feeding experiments were performed in the laboratory to evaluate the role of acylsugars in feeding by all three insect species. Consumption of sticky leaves after their esters were removed by washing was compared to consumption of unwashed sticky leaves and velvety leaves in ester removal experiments. Consumption of velvety leaves was measured after acylsugars were applied to those leaves in controlled amounts in the ester addition experiments. Consumption by *E. hirtipennis* was reduced by acylsugars in all experiments. Consumption by *T. compacta* was reduced by acylsugars in the ester removal experiments, but not in the ester addition experiments. The location of the acylsugars at the tip of a long trichome, rather than simply on the leaf surface, may be an important component of the biological activity of acylsugars against *T. compacta* in nature. Consumption by *L. daturaphila* was not significantly reduced by acylsugars in any experiment. The acylsugars caused no significant mortality of any of the three insect species.

Key Words—Acylsugars, bioassays, chemical defense, coleoptera, genetic variation.

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INTRODUCTION

Glandular trichomes may serve many functions in plants, but one of the more important is protecting them from herbivorous insects (Werker, 2000). The glandular trichomes of solanaceous plants contain or secrete a number of phytochemicals that are either toxic or deterrent to a wide variety of herbivorous insects (Dimock and Kennedy, 1983; Duffey, 1986; Goffreda et al., 1989; Neal et al., 1990; Buta et al., 1993; Severson et al., 1994; Yencho et al., 1994; Liedl et al., 1995; Eigenbrode et al., 1996; Wilkens et al., 1996; Liu et al., 1996). One class of compounds produced by glandular trichomes is acylsugars, or esters of simple sugars and one or more fatty acids. These compounds increase the mortality of whiteflies, aphids, mites, and psyllids, and inhibit the growth and survival of phytophagous Lepidoptera (Buta et al., 1993; Juvik et al., 1994; Neal et al., 1994; Puterka and Severson, 1995; van Dam and Hare, 1998a, b). However, the effects of acylsugars vary widely among insect species. They caused no mortality or inhibition of feeding of larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* (Neal et al., 1994), and at least one insect species utilizes these compounds as feeding and oviposition stimulants (van Dam and Hare, 1998a). Despite their potential role in insect pest management (e.g., Puterka et al., 2003), relatively little is known of the role of acylsugar production in plant defense against native insect herbivores.

Datura wrightii Regel, a native perennial plant, is dimorphic for leaf trichome morphology. Within populations, leaves of some plants are covered with relatively long, multicellular trichomes with a secretory gland at their tip and feel sticky when touched. Other plants are covered with a higher density of shorter, nonglandular trichomes, and feel velvety when touched. Trichome morphology is governed by a single gene, and the sticky allele is dominant (van Dam et al., 1999). Glandular trichomes of sticky plants produce several esters of glucose and straight-chain organic acids (van Dam and Hare, 1998b). In laboratory studies, these esters conferred resistance to whiteflies (van Dam and Hare, 1998a) and reduced the growth rate of *Manduca sexta* larvae (van Dam and Hare, 1998b). In the same laboratory studies, however, van Dam and Hare (1998a) showed that addition of acylsugars to velvety *D. wrightii* leaves stimulated feeding and oviposition by *Tupiocoris notatus* (Distant), a mirid bug with specific morphological adaptations to cope with glandular trichomes (Southwood, 1986).

The concentration of acylsugars on sticky *D. wrightii* leaves is strongly influenced by environmental conditions (Forkner and Hare, 2000). Acylsugar concentration per unit leaf area was approximately one-third less for plants that received twice-weekly irrigations than for plants that remained unirrigated for the whole growing season, despite the fact that irrigation did not affect the density of glandular trichomes (Forkner and Hare, 2000). Acylsugar concentra-

tion also differed between years, although this variation could have been the result of sampling plants at different times during the growing season in each of 2 yr (Forkner and Hare, 2000). Environmental variation in acylsugar concentration suggests that the level of herbivore resistance that glandular trichomes confer may vary both among populations growing under different environmental conditions and seasonally within populations.

To better understand the role of natural concentrations of acylsugars as a plant defense mechanism in *D. wrightii*, the following two objectives were addressed. First, the acylsugars produced by sticky plants were quantified monthly from field-grown plants in two irrigation treatments to determine the range of variation in concentration over one growing season. Second, the role of acylsugars as a defense against three common Coleopteran herbivores of *D. wrightii* was evaluated through a series of experiments in which insects were offered sticky and velvety leaves or leaf disks in a series of "choice" and "no choice" experiments. Treatments included unaltered disks from sticky and velvety plants, disks from sticky plants where the acylsugars were removed, and disks or leaves from velvety plants to which esters were added in measured amounts.

The three herbivores were adults of *Lema daturaphila* Kogan and Goeden (formerly *Lema trilineata daturaphila*, see Riley et al., 2003), adults of the tobacco flea beetle, *Epitrix hirtipennis* (Melsheimer), and adults of a weevil, *Trichobaris compacta* (Casey). All are common native insect herbivores of wild and cultivated solanaceous plants. The first species damages sticky and velvety *D. wrightii* similarly in the field, whereas feeding damage by the latter two species is largely confined to velvety *D. wrightii* (Elle and Hare, 2000; Hare and Elle, 2002). Based upon the field observations, I predicted that *E. hirtipennis* and *T. compacta* would be inhibited more than *L. daturaphila* by the acylsugars of sticky *D. wrightii*.

METHODS AND MATERIALS

Variation in Acylsugar Concentration in the Field. Because trichome morphology is a Mendelian trait with the sticky condition being dominant (van Dam et al., 1999), the distribution of phenotypes of backcrossing a heterozygous F1 individual to its homozygous recessive parent is expected to be a 1:1 mixture of velvety (homozygous recessive) and sticky (heterozygous) progeny. This experiment utilized progeny from backcross families # 1, 2, and 4, described in van Dam et al. (1999), and grown in the field in 2000. Although there were initially a total of 216 plants arranged in four blocks for other experiments, data on acylsugar concentration were taken from 48 plants that

were sprayed weekly with acephate to protect them from insect attack. Plants in alternate blocks were assigned to two irrigation treatments. The two "irrigated" blocks were furrow-irrigated for 8 hr once a week from April through November, while the two remaining "unirrigated" blocks were left dry.

The youngest, fully expanded leaf was collected from all surviving plants on July 28, 2000 to determine trichome densities under a microscope using standard procedures (van Dam et al., 1999). Plants were flowering and producing seed capsules at this time.

Acylsugar concentration was determined for protected sticky plants every 4 wk starting on June 21 and ending October 11, 2000 using methods described by Forkner and Hare (2000) and briefly outlined here. The youngest, fully expanded leaf on each plant was collected, placed into a plastic bag, and brought back to the laboratory. The use of plastic bags to transport the leaves may have introduced an error into the experimental design because some exudate might have been transferred from the leaves to the bags. This error is likely conservative, because such potential losses would actually decrease the probability of finding statistical differences in acylsugar concentration between treatments or among sampling dates.

Acylsugars were extracted three times with $CHCl_3$, and leaf areas were measured with a leaf area meter (Li-Cor Model 3000, Li-Cor, Inc., Lincoln, NE, USA). $CHCl_3$ was filtered and removed by evaporation, then the acylsugars were redissolved in MeOH and saponified with 0.04 N NaOH in water. The liberated glucose was quantified spectrophotometrically using a hexokinase-based glucose assay reagent [Sigma Glucose (HK) reagent; Sigma Diagnostics, St. Louis, MO, USA] prepared according to the manufacturer's instructions. The molar concentration of glucose was calculated per leaf from the absorbance, then expressed as μmol of glucose esters per cm^2 of leaf area.

Concentrations were analyzed statistically with a repeated-measures multi-way analysis of variance. The repeated factor was the different sampling dates and the fixed treatment effect was the irrigation treatment. Potential random sources of variation included blocks within irrigation treatments, families, and the interactions of families with irrigation treatment and sampling date.

The data were analyzed using the PROC MIXED procedure of SAS because the design contained both fixed and random factors. PROC MIXED calculates significance tests using a restricted maximum likelihood (REML) method and is more robust for the analysis of mixed models with uneven sample sizes than PROC GLM (SAS Institute, 2000). The logarithmic transformation was applied to ensure normality of residuals.

Ester Removal Experiments. As in other solanaceous species (e.g., Neal et al., 1990), the acylsugars of *D. wrightii* can be removed from sticky foliage simply by washing the leaf for 15 sec in 95% ethanol, followed by a 15-sec rinse in deionized water, without damaging the leaves. In the following bio-

assays, the feeding activity of *L. daturaphila*, *T. compacta*, and *E. hirtipennis* was compared on: (1) sticky vs. velvety leaves, (2) sticky leaves vs. sticky leaves with their esters removed (hereafter "washed sticky leaves"), and (3) washed sticky leaves vs. velvety leaves. Both choice and no-choice experiments were performed. Adult insects of undetermined age and sex were collected from the field on the morning of the trial. For the no-choice experiment, there were 10 trials of 10 replicates, with each replicate comprising a pair of 1.5-cm leaf disks of one of the three leaf treatments above, and a single field-collected adult of *L. daturaphila* or *T. compacta*, or 10 adults of *E. hirtipennis*. Different numbers of adults of each species were used because of the differences in size and feeding mode of each species. Only five trials could be set up for *E. hirtipennis* because of a shortage of insects. After 48 hr, the quantity of foliage consumed by each *L. daturaphila* adult was determined by placing an acetate overlay marked with a grid (1-mm spacing) over the leaf and counting the number of 1×1 mm squares of leaf area removed. For both *T. compacta* and *E. hirtipennis*, the feeding pits per leaf were counted and recorded.

For the choice tests, there were five trials of 15 replicates, where each replicate comprised a pair of leaf disks from each of two treatments and individual adults of *L. daturaphila* or *T. compacta*, or 10 adults of *E. hirtipennis*. Feeding was scored after 48 hr as described above. In some cases, the leaf disks shriveled and the replicate was discarded. Additionally, a few *T. compacta* refused to feed on any leaf disks, and these replicates were also discarded. Both factors led to unequal sample sizes within trials. All bioassays were performed during the months of July and August to try to maximize the difference between sticky and velvety types (see Results), contingent upon the availability of insects in the field.

The "sticky vs. velvety" comparison provides a baseline difference between the leaf types, whereas the "washed sticky vs. sticky" comparison tests the effect of acylsugars on insect feeding on a background of long trichomes, and the "washed sticky vs. velvety" comparison tests the effect of variation in trichome morphology in the absence of acylsugars. In the comparison of sticky vs. washed sticky leaves, the appropriate control was sticky leaves washed for 15 sec in deionized water; in the comparison of washed sticky leaves vs. velvety leaves, the appropriate control was velvety leaves washed identically to sticky leaves, i.e., in both 95% ethanol and deionized water for 15 sec each.

Ester Addition Experiments. Acylsugars were extracted in bulk from various wild-grown sticky *D. wrightii* plants as described elsewhere (van Dam and Hare, 1998b). Numerous extractions were performed in order to obtain the large quantities of acylsugars required for study. Branches were clipped from plants and brought to the laboratory. All leaves were removed from their branches and soaked for 1 hr in 3 l of $CHCl_3$. The extract was filtered through Whatman # 1 filter paper under vacuum to remove solid debris, then the volume of $CHCl_3$ was

reduced by rotary evaporation. The $CHCl_3$ extract was filtered over anhydrous Na_2SO_4 to remove water then further concentrated, transferred to a preweighed vial, and evaporated to dryness under an air stream. The mass of the residue was determined and dissolved in CH_3CN at a rate of 100 ml/g. The material was placed into a sonicating bath (10 min) to ensure that all material was dissolved or suspended in CH_3CN. This material was partitioned three times against equal volumes of hexane to remove leaf waxes and other nonpolar compounds. Then CH_3CN was taken to dryness by rotary evaporation. The residue was dissolved in 50 ml/g of CH_2Cl_2 and partitioned two times against 1 N tartaric acid and three times against water to remove alkaloids and other water-soluble compounds. The CH_2Cl_2 fraction was concentrated by rotary evaporation and transferred into a preweighed vial. Remaining traces of solvent were removed *in vacuo*. The mass of the residue, the "alkaloid-free acylsugars," was determined, and yields ranged from 0.1% to 0.2% of the fresh mass of the leaves.

Acylsugars were applied to velvety leaves using methods derived from Puterka and Severson (1995). On the day that a feeding trial was to be established, a group of 12 young but fully expanded leaves from field-grown velvety *D. wrightii* were collected and brought to the laboratory. Leaves were surface-sterilized by soaking them for 3 min in a solution of 1% commercial bleach (equivalent to 0.0525% $NaHClO_3$). Leaves were rinsed thrice in deionized water and kept turgid by placing their petioles in water reservoirs.

A suspension of acylsugars in water (10 mg/ml) was prepared, and a sonicating bath was used to keep the acylsugars in suspension. This was the highest concentration of acylsugars in water that would remain in suspension during the application process. Acylsugars were applied to velvety leaves by using an ultra-low volume spray device modified from that described in Puterka and Severson (1995). A Nalgene aerosol spray bottle (# 2430-200, Nalgene, Rochester, NY, USA) was modified by affixing a commercial 3/8-in. pipe-to-hose adapter to the side of the spray bottle so that a hose from a cylinder of nitrogen or compressed air could be attached. For each application, 800 μl of the acylsugar suspension were transferred into a 12 × 75 mm test tube. This tube was placed into the spray bottle, and the pick-up tube of the sprayer was placed into the test tube.

To ensure uniform applications, a spraying stand was constructed from ring stands and clamps that consistently held the sprayer at the appropriate distance and angle from the leaf to be sprayed. The concept of the fixed spray platform was taken from Puterka and Severson (1995), but the design was modified in order to accommodate the larger leaves used in this study. The sprayer was clamped into a fixed position, and a 15-cm-diam plastic Petri dish was clamped 50 cm away from the nozzle of the sprayer in which the test leaf was placed. After the leaf and sprayer were set in proper position, the bottle was pressurized to 103 kPa, and all of the acylsugar suspension was sprayed onto the leaf and Petri dish.

In each trial, two of the 12 leaves were treated with 10 mg/ml of acylsugars per leaf surface (top and bottom) to serve as a check on the uniformity of application. Prior to treatment, the areas of these two leaves were determined with a leaf area meter. After treatment, the acylsugars were extracted from the leaves and quantified using methods described above. From this, the quantities of acylsugars actually applied to leaves per unit of leaf area were calculated.

Each trial consisted of five treatments. Control leaves were sprayed top and bottom with 800 μl of water. Leaves were treated with 20, 40, 80, or 120 mg/ml of acylsugars (1, 2, 4, or 6 applications per leaf surface of 800 μl of a 10 mg/ml mixture of acylsugars). These rates were chosen to span the range of acylsugars that occur on field-grown, sticky plants (see Results). Leaves sprayed top and bottom with 800 μl of a 10 mg/ml mixture of acylsugars received an average concentration of 0.097 (±0.003 *SE*, $N = 40$) μmol acylsugars/cm^2 of leaf. Thus, the quantities of acylsugars applied to leaves were approximately equivalent to 0.097, 0.194, 0.388, and 0.582 μmol/cm^2 of leaf. Leaves were returned to their water reservoirs to maintain leaf turgor while the leaf surfaces dried between applications.

For the no-choice bioassays, a piece of filter paper (Whatman #2, 12.5 cm) was placed into the bottom of a 15-cm-diam Petri dish and moistened with deionized water, then a whole treated leaf (above) was placed into the dish. One adult *L. daturaphila* was placed onto each treated leaf, whereas two *T. compacta* adults were used per leaf, and 10 adult *E. hirtipennis* were used per leaf. The dish was sealed with Parafilm® (American National Can, Menuasa, WI, USA) and the dish was placed into a growth room (constant 28°C, 16L:8D photoperiod). Insect feeding was scored after 48 hr as above.

For choice bioassays, only leaves from the control (water only) and 120 mg/ml treatments were utilized. Leaves were treated as described above and allowed to dry, then a cork borer was used to punch out 1.5-cm-diam disks from each leaf, avoiding the midrib. Pairs of disks were placed equidistant from the center of a 9.0-cm Petri dish on a disk of moistened filter paper (Whatman # 2, 7.0 cm) and insects added as above. For these tests, the number of *T. compacta* was reduced from two per dish to one. These experiments were repeated five times for *L. daturaphila* and *T. compacta*, but only three times for *E. hirtipennis* due to a shortage of insects. Additional procedures and data collection were as in the no-choice experiments above.

Data Analysis. Data from both the ester removal and ester addition experiments were analyzed by two-way mixed-model ANOVA using the PROC MIXED procedure of SAS with doses (no-choice) or treatments (choice) being fixed effects, and trials and the interaction between trials and treatments or doses being random sources of variation. For the choice trials, the potential variation among replicates within trials was also included in the model as a random source of variation to control for systematic differences in consumption

rates among individual replicate insects (*L. daturaphila* and *T. compacta*) or groups of insects (*E. hirtipennis*) across treatments. The interaction between treatment and trials was pooled with the error term when not significant at $P \leq 0.25$ prior to testing for significance of the fixed effects, following rules for pooling (Sokal and Rohlf, 1995). The square root ($X + 0.5$) transformation was applied to the feeding data for *L. daturaphila* and *T. compacta*, to ensure that the residuals were normally distributed, but the data for *E. hirtipennis* required no transformation.

RESULTS

Variation in Acylsugar Concentration in the Field. Overall, acylsugar concentration varied from 0.03 to 0.84 μmol/cm^2 of leaf. Acylsugar concentration differed significantly due to irrigation treatment, but not between blocks within treatments (irrigation $F_{1,86} = 4.41$, $P = 0.039$, pooled over blocks). Acylsugar concentration was 27% higher in unirrigated plants (Figure 1). The magnitude of variation over time was statistically significant ($F_{4,86} = 34.26$, $P < 0.001$), and mean concentrations in August were 2.6 times the concentrations in October (Figure 1). The interaction between irrigation treatment and month was not statistically significant ($F_{4,86} = 1.41$, $P = 0.24$). Families did not differ in their response either to irrigation or to season, as there was no significant effect of family or interactions of family with either months or irrigation (all $P \geq 0.15$).

The densities of glandular trichomes on sticky plants averaged 94.6 (±3.0 SE, $N = 82$) per mm^2 and did not differ among families, irrigation treatments, or their interaction (all $P \geq 0.24$, data not shown). Similarly, the density of non-glandular trichomes on velvety plants averaged 242.0 (±4.9 SE, $N = 119$) per mm^2, and also did not differ among families, irrigation treatments or their interaction (all $P \geq 0.23$, data not shown).

Ester Removal Experiments. When given a choice between sticky and velvety disks, both *T. compacta* and *E. hirtipennis* consumed more velvety foliage ($F_{1,63} = 28.12$, $P < 0.001$ for *T. compacta* and $F_{1,59} = 9.26$, $P = 0.0035$ for *E. hirtipennis*, Figure 2). There were more than 2.5 times the number of *T. compacta* feeding pits on velvety as sticky foliage (back transformed means) and 25% more *E. hirtipennis* feeding pits on velvety than sticky foliage (Figure 2). When given a choice between sticky foliage and washed sticky foliage, both species preferred washed sticky foliage ($F_{1,44} = 8.78$, $P = 0.005$ for *T. compacta* and $F_{1,66} = 19.26$, $P < 0.001$ for *E. hirtipennis*). There were more than 2.3 times the number of *T. compacta* feeding pits, and 46% more *E. hirtipennis* feeding pits on washed sticky foliage than on sticky foliage with

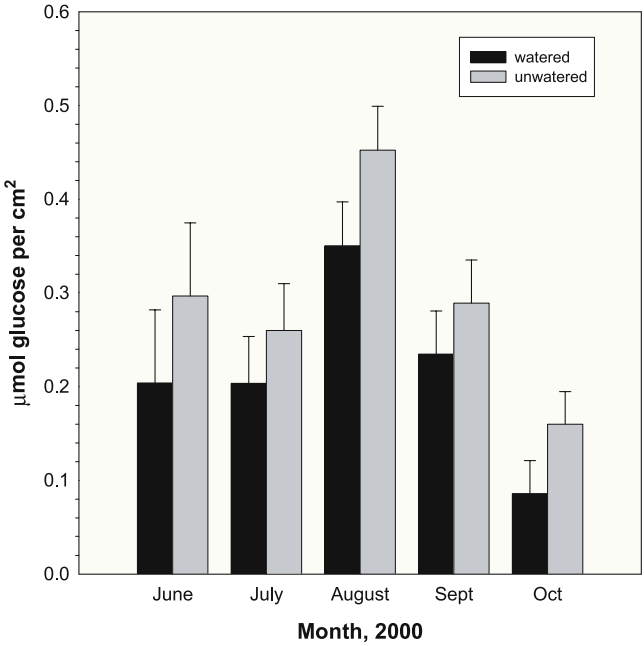

FIG. 1. Least squares means (±SE) quantity of glucose ester produced per cm^2 of leaf surface for watered and unwatered sticky plants sampled monthly. Data are pooled over blocks and families.

acylsugars (Figure 2). When given a choice between washed sticky foliage and velvety foliage, the number of feeding pits from either species did not differ significantly ($F_{1,36} = 2.63$, $P = 0.11$ for *T. compacta* and $F_{1,8} = 0.46$, $P = 0.52$ for *E. hirtipennis*, Figure 2).

L. daturaphila consumed 1.6 times more sticky foliage (back transformed means) when given a choice between sticky and velvety foliage, ($F_{1,8} = 7.65$, $P = 0.025$), but exhibited no significant choice for washed sticky foliage vs. sticky foliage with acylsugars ($F_{1,8} = 1.56$, $P = 0.25$), or washed sticky foliage vs. velvety foliage ($F_{1,8} = 0.03$, $P = 0.87$). In contrast to the bioassays with *T. compacta* and *E. hirtipennis*, the trial × type interactions were sufficiently large in the *L. daturaphila* bioassays that pooling of this potential source of variation with the error was not justified.

In no choice experiments, consumption of the three foliage types by *T. compacta* and *E. hirtipennis* differed significantly ($F_{2,280} = 13.05$, $P < 0.001$ for the former and $F_{2,128} = 3.76$, $P = 0.026$ for the latter.) Both species consumed more sticky foliage with the acylsugars removed than velvety foliage,

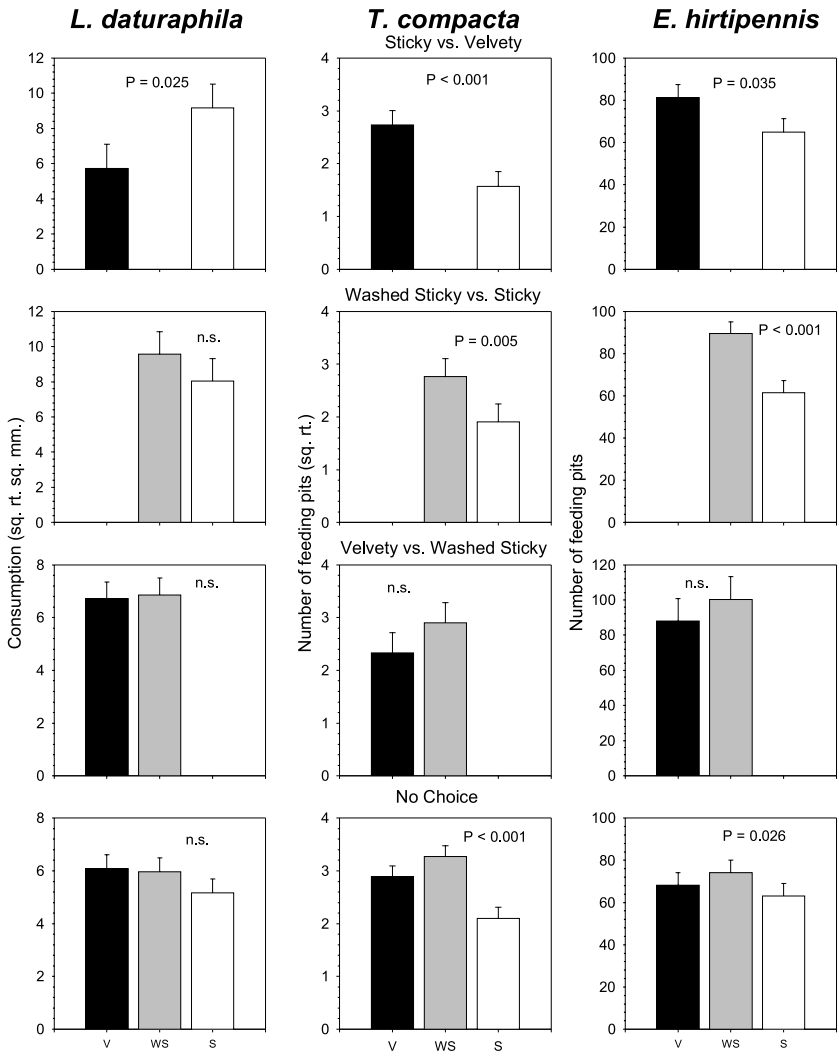

FIG. 2. Least squares means (±SE) feeding by *L. daturaphila, T. compacta*, and *E. hirtipennis* adults on disks of sticky leaves with their acylsugars (S), disks of sticky leaves with the acylsugars removed by washing (WS), and disks of velvety leaves (V) in choice and no-choice bioassays. Top row: sticky vs. velvety choice; second row: washed sticky vs. sticky choice; third row: velvety vs. washed sticky choice; fourth row: no choice. The probability of a statistically significant difference in means is shown. n.s.: Not statistically significant.

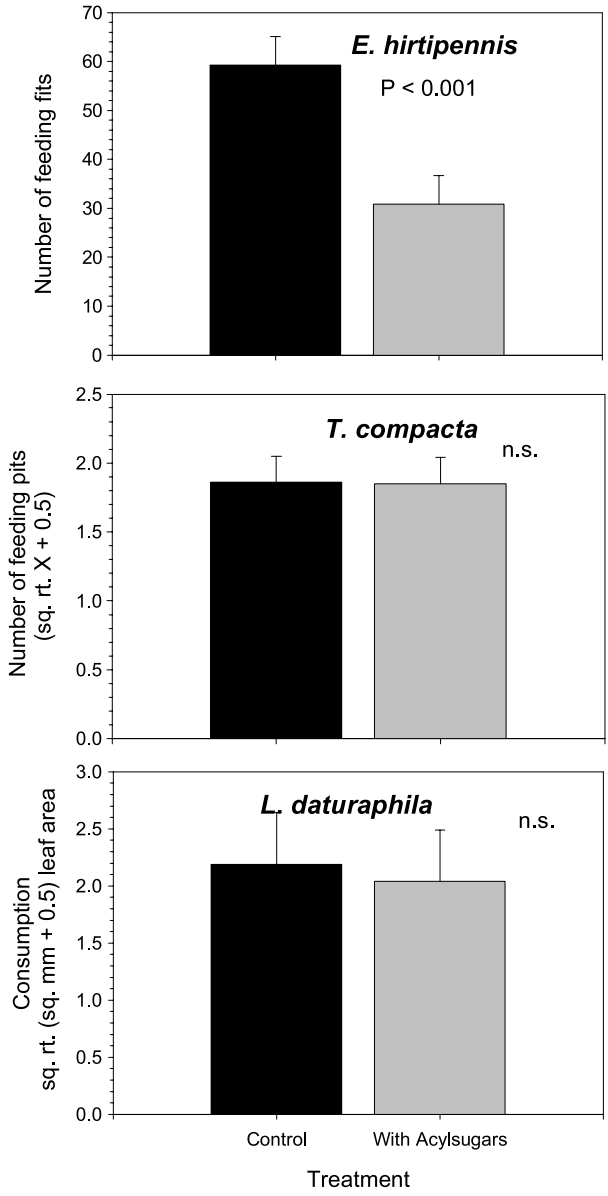

FIG. 3. Least squares means (±SE) feeding by *E. hirtipennis, T. compacta*, and *L. daturaphila* adults on disks of velvety leaves either sprayed with water (Control) or with 0.582 μmol/cm^2 of acylsugars in choice experiments. The probability of a statistically significant difference in means is shown. n.s.: Not statistically significant.

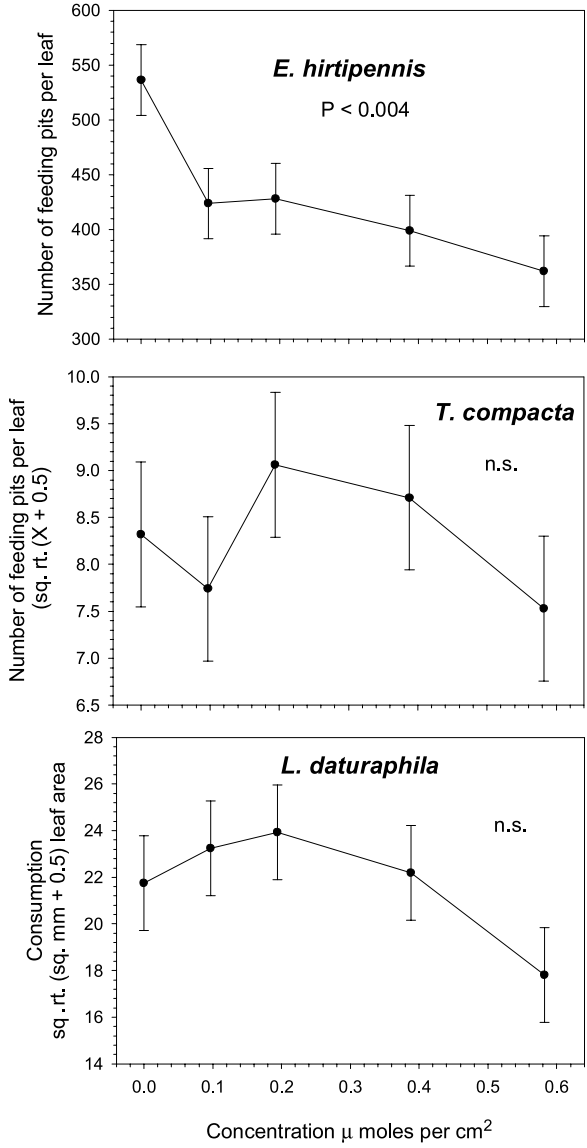

FIG. 4. Least squares means (±SE) feeding by *E. hirtipennis, T. compacta*, and *L. daturaphila* adults on velvety leaves either sprayed with water or with different concentrations of acylsugars in no-choice experiments. The probability of a statistically significant difference among means is shown. n.s.: Not statistically significant.

followed by sticky foliage. Consumption of washed sticky foliage was 30% and 8% greater than velvety foliage, and 161% and 17% greater than sticky foliage for *T. compacta* and *E. hirtipennis*, respectively (Figure 2). *L. daturaphila* consumed similar quantities of all three types of foliage in no-choice experiments ($F_{2,27} = 1.56$, $P = 0.23$, Figure 2). Mortality of all three insect species was negligible in these experiments and was not recorded.

Ester Addition Experiments. In the choice experiments, only feeding by *E. hirtipennis* was significantly reduced by adding 0.582 μmol acylsugars/cm^2 leaf ($F_{1,61} = 43.81$, $P < 0.001$), and the number of feeding pits was nearly cut in half by adding acylsugars to velvety foliage (Figure 3). Similarly, in the no-choice experiments, only feeding by *E. hirtipennis* was reduced by adding acylsugars to velvety leaves ($F_{4,41} = 4.54$, $P = 0.004$), and the highest quantity was no more effective in inhibiting feeding than the lowest (Figure 4). Neither *T. compacta* nor *L. daturaphila* showed any preference for treated or untreated velvety leaves in choice tests ($F_{1,4} = 0.001$, $P = 0.97$ for *T. compacta* and $F_{1,4} = 0.06$, $P = 0.82$ for *L. daturaphila*, Figure 3) nor feeding inhibition by acylsugars in no-choice tests ($F_{4,41} = 0.92$, $P = 0.46$ and $F_{4,40} = 1.65$, $P = 0.18$, respectively, Figure 4).

Survival of *L. daturaphila* adults in the no-choice experiments averaged 98.0 ± 2.0%, whereas survival of *T. compacta* adults averaged 95.0 ± 2.0%, and survival of *E. hirtipennis* adults averaged 85.4 ± 2.4%. In no case was the variation in survival associated with acylsugar dose (all $P \geq 0.32$, data not shown). Insect mortality in the choice experiments was similarly trivial and not analyzed.

DISCUSSION

All three beetle species tolerate the acylsugars of *D. wrightii*, but the acylsugars moderately deter feeding by *E. hirtipennis* and *T. compacta*. The acylsugars were not toxic to any species. This is not surprising because all beetles commonly feed on *D. wrightii* in nature and might be expected to be less affected by the acylsugars of *D. wrightii* than herbivores that encounter *D. wrightii* less frequently, if at all, under natural conditions. The absence of any effect of acylsugars on feeding by *L. daturaphila* was not unexpected because feeding damage by this insect was shown to be independent of trichome type in detailed field studies (Elle and Hare, 2000; Hare and Elle, 2002). Observations of feeding behavior under the microscope show that both adults and larvae of *L. daturaphila* cope with glandular trichomes simply by feeding upon them and ingesting both the gland and the stalk until the trichome layer is short enough for the insects to begin feeding on leaf lamellar tissue. Such

behavior implies that *L. daturaphila* has an effective physiological adaptation to acylsugars, although the mechanism of such an adaptation is not yet known.

T. compacta was more sensitive to acylsugars in the ester removal experiment than in the ester addition experiments. Feeding on sticky foliage was significantly reduced in all comparisons in the ester removal experiments (Figure 2), but not in the ester addition experiments (Figures 3 and 4). The application of acylsugars to velvety leaves does not completely mimic the presence of acylsugars on sticky leaves. Nonglandular trichomes of velvety leaves are less than 30% the length of glandular trichomes of sticky leaves (48 vs. 168 μm, van Dam et al., 1999), but occur at nearly 2.6 times the density. Application of acylsugars to velvety leaves replicates only the overall concentration per unit of leaf area, but not the location of the acylsugars at the tip of a long trichome. For weevils, the present results suggest that the location of the acylsugars on the tips of relatively long trichomes may be more important for deterrence than simply being present on the surface of leaves in a uniform layer. Future experiments to better understand the role of acylsugars on *T. compacta*, and perhaps other insect herbivores, might best be carried out by manipulating and exploiting natural variation in acylsugar production by glandular trichomes rather than by experimentally applying acylsugars to nonglandular foliage.

It is difficult to relate the relatively low biological activity of the acylsugars of *D. wrightii* reported here with the higher levels of other acylsugars against different insect species. High levels of mortality at relatively low doses (e.g., 1 mg/l or 1000 ppm or lower) are commonly reported against aphids, whiteflies, mites, and psyllids (Neal et al., 1994; Puterka and Severson, 1995; Liu et al., 1996), whereas only moderate feeding deterrence and negligible mortality occurred even after multiple applications of acylsugars totaling 120 mg/ml in the current study. It is unclear at this point to what extent the low mortality is the result of specific adaptations of all three coleopterans to *D. wrightii*'s acylsugars, or low overall toxicity of *D. wrightii*'s acylsugars in general. Different insect species may vary widely in their sensitivity to acylsugars (e.g., Neal et al., 1994; Puterka et al., 2003), and changes in either the sugars or the acid components of acylsugars often lead to unpredictable changes in biological activity (Puterka et al., 2003).

The range of acylsugar concentrations found in *D. wrightii* are similar to those found in other solanaceous species. For example, the concentration of total acylsugars in *Lycopersicon pennellii* lines ranged from 0.015 to 0.416 μmol/cm^2 in one study (Goffreda et al., 1990) and from 0.035 to 1.265 μmol/cm^2 in another (Mutschler et al., 1996). In *L. pennellii*, nearly a sevenfold range in concentration also occurred in different populations of the species when grown in a common garden (Shapiro et al., 1994). Although these acylsugars are responsible for resistance of *L. pennellii* to many pests of cultivated

tomato, little is known about the biological significance of variation in the total quantities or composition of acylsugars in natural *L. pennellii* populations (Shapiro et al., 1994).

The prediction that *E. hirtipennis* and *T. compacta* would be more sensitive to the acylsugars of *D. wrightii* than *L. daturaphila* was supported by the ester removal experiments. Additional support for the hypothesis was provided by the ester addition experiments for *E. hirtipennis*, but the ester addition experiments provide more equivocal support of the hypothesis for *T. compacta*. In both the ester removal and ester addition experiments, biological activity appeared to be via inhibition of feeding rather than toxicity. In addition to providing absolute protection against whiteflies (van Dam and Hare, 1998a) and reducing feeding by *Manduca sexta* larvae (van Dam and Hare, 1998b), the acylsugars of *D. wrightii* also reduce feeding by *E. hirtipennis* and *T. compacta*. The more specialized *L. daturaphila* (Kogan and Goeden, 1970) was completely unaffected by *D. wrightii*'s acylsugars.

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DIFFERENCES IN EFFECTS OF PYRROLIZIDINE ALKALOIDS ON FIVE GENERALIST INSECT HERBIVORE SPECIES

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Abstract—The evolution of the diversity in plant secondary compounds is often thought to be driven by insect herbivores, although there is little empirical evidence for this assumption. To investigate whether generalist insect herbivores could play a role in the evolution of the diversity of related compounds, we examined if (1) related compounds differ in their effects on generalists, (2) there is a synergistic effect among compounds, and (3) effects of related compounds differed among insect species. The effects of pyrrolizidine alkaloids (PAs) were tested on five generalist insect herbivore species of several genera using artificial diets or neutral substrates to which PAs were added. We found evidence that structurally related PAs differed in their effects to the thrips *Frankliniella occidentalis*, the aphid *Myzus persicae*, and the locust *Locusta migratoria*. The individual PAs had no effect on *Spodoptera exigua* and *Mamestra brassicae* caterpillars. For *S. exigua*, we found indications for synergistic deterrent effects of PAs in PA mixtures. The relative effects of PAs differed between insect species. The PA senkirkine had the strongest effect on the thrips, but had no effect at all on the aphids. Our

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results show that generalist herbivores could potentially play a role in the evolution and maintenance of the diversity of PAs.

Key Words—Plant–insect interactions, evolution, plant defense, plant secondary metabolites, chemical diversity, *Senecio*, pyrrolizidine alkaloids, senecionine, senkirkine, deterrent.

INTRODUCTION

Plants often synthesize a diversity of structurally related secondary metabolites, such as the 34 glucosinolates in *Arabidopsis thaliana* (Kliebenstein et al., 2001) and the 200 alkaloids in *Catharanthus roseus* (Mersey and Cutler, 1986). The evolution and maintenance of this diversity in related compounds are poorly understood. Many secondary metabolites act as defense against herbivores, and it is often postulated that insect herbivores play an important role in the evolution of these compounds (Ehrlich and Raven, 1964; Rhoades and Cates, 1976). It has been shown that plant chemistry can indeed be under selection by insect herbivores (Mauricio and Rausher, 1997; Shonle and Bergelson, 2000). One explanation for the diversity of structurally related secondary metabolites is that new compounds evolve in a continuous evolutionary arms race between a plant and its insect herbivores, in which a plant that synthesizes new compounds is able to escape herbivory and insect herbivores, in turn, adapt to these compounds. This coevolutionary model implies that structurally related compounds differ in their effects on herbivores, and that more recently developed compounds in a biosynthetic pathway are more effective against present-day insects (Berenbaum and Feeny, 1981; Miller and Feeny, 1983). If the production of less effective compounds is costly, there would be selection against such compounds, although compounds that are earlier in the biosynthetic pathway can remain in the plant in small quantities as necessary precursors. Additionally, related compounds can act synergistically on herbivores (Adams and Bernays, 1978; Lindroth et al., 1988; Berenbaum et al., 1991). Furthermore, the diversity can be maintained through selection by several different herbivores and/or pathogens (Simms, 1990; Mithen et al., 1995; Juenger and Bergelson, 1998).

Although there are a few examples of differential toxic effects of structurally related compounds on specialist insect herbivores (Berenbaum et al., 1986, 1989; Bowers and Puttick, 1988), other studies have shown that related compounds do not differ in their effects on specialist insects (Lindroth et al., 1988; Johnson et al., 1996, Macel et al., 2002). Agrawal (2000) found that induction of a diversity of glucosinolates in *Lepidium virginium* had no effect on a specialist but decreased feeding by a generalist herbivore. Less adapted generalist herbivores could be more susceptible to differences in structure among related compounds and, hence, play an important role in the evolu-

tion and maintenance of the diversity of structurally related secondary plant compounds.

As a model system to study the evolution of the diversity of secondary metabolites, we used the pyrrolizidine alkaloids (PAs) in *Senecio* species. Senecionine-type PAs, comprising over 100 structures, are abundant in the genus *Senecio* (Asteraceae) (Hartmann and Witte, 1995), and senecionine is the primary product in the biosynthesis of these structures (Hartmann and Dierich, 1998). *Senecio jacobaea* L. can contain more than 10 different senecionine-type alkaloids (Witte et al., 1992), and variation in PA patterns has a genetic basis (Vrieling et al., 1993; Macel et al., 2004). PAs are toxic to some mammals (Bull et al., 1968; Mattocks, 1968), have antifungal activity (Hol and van Veen, 2002), and are mutagenic to *Drosophila melanogaster* (Frei et al., 1992). PAs are known to deter generalist insect herbivores (Bentley et al., 1984; Dreyer et al., 1985; van Dam et al., 1995; Hägele and Rowell-Rahier, 2000). Specialist herbivores can detoxify (Hartmann, 1999; Naumann et al., 2002) and sequester PAs (Rothschild et al., 1979; Dobler, 2001; Pasteels et al., 2003). Larval performance of the specialist moth *Tyria jacobaeae* L. was not affected by different PA profiles in its host plant *S. jacobaea* (Macel et al., 2002). Furthermore, larval performance and oviposition preference of this specialist among different *Senecio* species were not correlated with PA patterns (Macel et al. 2002; Macel and Vrieling, 2003). Host plant choice of PA-sequestering specialist flea beetles of the genus *Longitarsus* was also not correlated with PA patterns of *Senecio* species (Kirk et al., unpublished data). These results indicate that it is unlikely that these specialist herbivores play a role in the evolution and maintenance of the diversity of PAs in *Senecio* species. In this study, we focus on the effects of structurally related PAs on feeding and survival of five generalist insect herbivore species.

We addressed the following questions: (1) do related PAs differ in effects on generalists? (2) Do PAs act synergistically? (3) Do the relative effects of individual PAs (most effective PA) differ between insect species? Survival experiments were performed with the western flower thrips (*Frankliniella occidentalis* Pergande) and the green pea aphid (*Myzus persicae* Sulzer) using artificial diets with PAs. The deterrent effects of PAs on insect feeding were tested with the polyphagous Lepidopteran species *Spodoptera exigua* Hübner and *Mamestra brassicae* L. and with the gramnivorous migratory locust (*Locusta migratoria* L.).

METHODS AND MATERIALS

Pyrrolizidine Alkaloids. Most of the pyrrolizidine alkaloids we used in our experiments belong to the structural group of senecionine-type PAs. To specify

structural related activity of PAs, we also selected two PAs from other structural groups: heliotrine (lycopsamine-type PA) and monocrotaline (monocrotaline-type PA) (Hartmann and Witte, 1995). Senecionine-type PAs are mostly 12-membered macrocyclic diesters with a retronecine or otonecine base. Lycopsamine-type PAs are monoesters or diesters containing as a necic acid a hydroxylated 2-isopropylbutyric acid, and monocrotaline-type PAs are 11-membered macrocyclic diesters with retronecine base (Hartmann and Witte, 1995). The structures of the pyrrolizidine alkaloids we used are shown in Figure 1. Senecionine, seneciphylline, retrorsine, jacobine, and senkirkine are all present in *Senecio* species. Senecionine and seneciphylline are commonly found in most *Senecio* species (Hartmann and Zimmer, 1986; Macel et al., 2002). Jacobine is found in *S. jacobaea* together with senecionine and seneciphylline, and senkirkine is found in *S. vernalis* together with senecionine and seneciphylline. Retrorsine occurs in several *Senecio* species but is present in large quantities in *S. inaequidens*. Monocrotaline is found in *Crotalaria* species and heliotrine in *Heliotropum* species. Jacobine was isolated by centrifugal partition chromatography (CPC) from a PA extract of flowering plants of *S. jacobaea* extracted as described by Hartmann and Zimmer (1986). Monocrotaline, retrorsine, and retrorsine N-oxide were obtained from Sigma Chemical Co. Senecionine, seneciphylline, and senkirkine were obtained from Roth Chemical Co., and heliotrine was obtained from LATOXAN, France. We tested the effects of

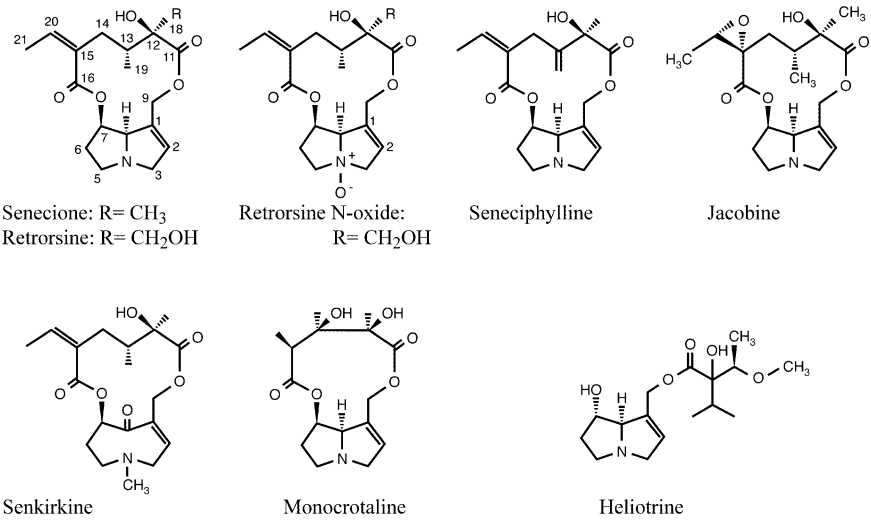

FIG. 1. Structures of pyrrolizidine alkaloids used in this study. Senecionine, R = CH_3, retrorsine: R = CH_2OH; retrorsine N-oxide, R = CH_2OH; seneciphylline; jacobine; senkirkine; monocrotaline; heliotrine.

senecionine, seneciphylline, senkirkine, and monocrotaline on each herbivore. We also tested the other PAs when available. A mixture of senecionine and seneciphylline (commonly found together in *Senecio* species) was tested for all herbivores but *Ma. brassicae*. For *S. exigua* and *L. migratoria*, other PA mixtures were tested as well. The average concentration of PAs found in *S. jacobaea* plants is 5 mg g^{-1} dry weight (Vrieling et al., 1993). This concentration was tested for all herbivores. For some herbivores, a range of concentrations of PAs was tested. PA concentrations differ between plant organs. In *Senecio vulgaris*, PA concentrations range from 0.5 mg g^{-1} dw in the leaves to 60 mg g^{-1} dw in achenes. In *S. vernalis*, PA concentrations in inflorescences can be 30 mg g^{-1} dry weight, more than 10 times the concentration found in the leaves (Hartmann and Zimmer, 1986). The effects of tertiary PAs were tested. In plants, PAs mainly occur as N-oxides (Hartmann and Toppel, 1987). After ingestion, the N-oxides are reduced to tertiary PAs in the gut of insects and then passively absorbed (Hartmann, 1999). For retrorsine, we also tested the effects of retrorsine N-oxide.

Frankliniella occidentalis (Thysanoptera; Thripidae). First instars of *F. occidentalis* were obtained from a lab culture reared on chrysanthemum cut flowers in a growth chamber at 20°C, 12-hr light/dark photoperiod, 60% RH. For the experiments, we used an artificial diet modified after Singh (1983) by de Jager et al. (1996). PAs were added to the diet by dissolving them in dichloromethane, adding the solution to the diet, and heating the diet (40°C) to let the dichloromethane evaporate. As a control treatment, we added dichloromethane without PAs to the diet and heated accordingly. For the experiments, we used "observation plates" as described by de Jager et al. (1996). Each plate contained six separate cells, and each cell contained one thrips. On every plate, six larvae could be observed independently. Experiments were done in the same growth chamber in which the thrips were reared. Senecionine, senkirkine, retrorsine, senkirkine, monocrotaline, and heliotrine were used in three different concentrations per PA: 0.5, 5, and 50 mg g^{-1} dw, equivalent to 0.1×, 1×, and 10× the plant concentration of *S. jacobaea*. Survival of the larvae was measured on 3, 6, 9, and 11 d after the start of the experiment. Per diet, four observation plates ($N = 24$) were used. In a separate experiment, we used control, retrorsine, and retrorsine N-oxide diets at 10× plant concentration and measured survival on each diet on d 3 and d 6 of the experiment. Per diet, five observation plates ($N = 30$) were used. In another experiment, we made diets with a 1:1:1 mixture of senecionine, seneciphylline, and retrorsine, 1:1 mixture of senecionine and seneciphylline, only senecionine, and a control diet. Two concentrations of total alkaloids were used, 2.5× and 5× plant concentration. Survival of the thrips larvae was measured on d 3 and d 6 of the experiment. Per diet, five observation plates ($N = 30$) were used. Thrips survival was analyzed with a Cox regression (Fox, 1993).

Myzus persicae (Homoptera; Aphididae). M. persicae were first reared on chilli pepper plants (*Capsicum frutescens*). Two months before the experiments, they were transferred to an artificial diet as described by Dadd and Mittler (1966). The aphids were reared in petri dishes (200 aphids per dish) (5-cm diam), and 350 μl of diet were applied between two layers of Parafilm®. Once every 3 d, the diet was refreshed. Rearing conditions were 20°C, 12-hr light/dark photoperiod, 50% RH. Senecionine, seneciphylline, senkirkine, monocrotaline, and a 1:1 mixture senecionine and seneciphylline were added to artificial diets. To dissolve the PAs in the diets, the pH was adjusted to pH 5. For each PA, we made four diets of different concentrations: 1.5, 3, 4.5, and 6 mM, equivalent to 0.5×, 1×, 1.5×, and 2× the average plant concentration for *S. jacobaea*. Per experiment, we put 10 adult, apterous aphids on the petri dish. Experiments were repeated 11 times. The experiments lasted 120 hr and were done in a growth chamber at 20°C, 12-hr light/dark photoperiod, 50% RH. Not all PAs could be tested simultaneously. We started experiments with three randomly chosen PA diets and a control per day. The mortality on the control diets did not differ significantly among days. Therefore, we grouped the mortality on the control diets on different days together. The percentage mortality of *M. persicae* on the different PA diets was tested against the percentage mortality on control diet with a Mann–Whitney *U*-test with the sequential Bonferroni correction for the number of PA diets we tested.

Spodoptera exigua (Lepidoptera; Noctuidae). Third instar caterpillars from *S. exigua* were obtained from a lab culture reared on an artificial diet (Singh, 1983) in a growth chamber at 25°C, 16:8-hr light/dark photoperiod, 70% RH. The experiments were performed in the same growth chamber. Senecionine, seneciphylline, retrorsine, retrorsine N-oxide, jacobine, senkirkine, monocrotaline, and heliotrine were dissolved in methanol and applied on lettuce disks (*Latuca sativa*) of 4-cm diam using a pipette to distribute droplets on the upper side of the lettuce disks. For each PA, three solutions of different concentrations were used: 0.15, 0.5, and 1.5 mg g^{-1} fw, equivalent to 0.3×, 1×, and 3× the natural PA concentration found in *S. jacobaea*. As a control, lettuce disks were treated with only methanol. A lettuce disk with a PA was placed on moist filter paper (4.25-cm diam) in a petri dish (10-cm diam) and offered to a caterpillar. After 24 hr, the amount of lettuce eaten was determined by measuring the leaf area (mm^2) eaten. The no-choice experiments were repeated 25 times. Differences in amount eaten among the PAs were analyzed with a Kruskal–Wallis test. In a separate experiment, mixtures of two PAs (1:1 senecionine/seneciphylline, 1:1 senecionine/senkirkine), mixtures of three PAs (1:1:1 senecionine/seneciphylline/jacobine, 1:1:1 senecionine, seneciphylline, senkirkine), and the single senecionine were applied to the lettuce disks (total PA concentration 0.5 mg g^{-1} fw in all cases). The amount eaten of the disk was normally distributed and analyzed with an ANOVA with *post hoc* Bonferroni tests.

Mamestra brassicae (Lepidoptera; Noctuidae). Third and fourth instars from *Ma. brassicae* were obtained from a lab culture where they were first reared on white cabbage (*Brassica oleracea* var. *alba*). One generation prior to the experiments, they were transferred onto an artificial diet (Singh, 1983) in a growth chamber at 20/15°C 16:8-hr light/dark photoperiod, 70% RH. Experiments were performed under the same conditions. Senecionine, seneciphylline, retrorsine, retrorsine N-oxide, jacobine, senkirkine, monocrotaline, and heliotrine were dissolved in methanol and applied on lettuce disks of 4.2-cm diam at a concentration of 0.5 mg g^{-1} fw. The dissolved PAs were applied in the same way as in the *Spodoptera* experiments. As control, lettuce disks were treated with only methanol. Two lettuce disks, one with a PA and one control, were placed on a moist filter paper (4.25-cm diam) in a petri dish (14-cm diam) and were offered to a caterpillar in a two-choice experiment. After 24 hr, the amount eaten from each lettuce disk was determined by measuring the leaf area (mm^2) eaten. The choice experiments were repeated 30 times. For each experiment, the preference was tested with the Wilcoxon signed-ranks matched-pairs test. To be able to compare deterrent effects among PAs, a preference index was generated: preference index = 100 × (amount eaten of PA − amount eaten of control)/(amount eaten PA + amount eaten control).

Differences in preference indices among the PAs were tested with a Kruskal–Wallis test. *Post hoc* Mann–Whitney *U*-tests were done with a sequential Bonferroni correction for significance levels.

Locusta migratoria (Orthoptera; Acrididae). The fifth instar hoppers of *L. migratoria* (gregarious form) that were used for experiments had been reared on dried grass. The experiments were performed in a growth chamber at 20/15°C, 16:8-hr light/dark photoperiod, 70% RH. Senecionine, seneciphylline, retrorsine, retrorsine N-oxide, jacobine, senkirkine, monocrotaline, and heliotrine were dissolved in methanol and applied on glass microfiber filters (Whatman® GF/A, 4.7-cm diam). To each filter, 0.5 ml of distilled water was added. The PA concentration was 0.5 mg g^{-1} fw (fw of filter with water), and to the control, only methanol was added. Two disks, one with PA and one control, were offered in a choice situation to the nymphs. The filters were placed in a white plastic box (17 × 12.5 × 6 cm) covered with a transparent lid. After 16 hr, the amount eaten from each filter was determined by measuring the number of mm^2 eaten. In the same way, the preference between a single PA and a 1:1(:1) PA mixtures was tested by offering the nymphs a disk with one PA and a disk with a mixture of two or three PAs in a two-choice test. Experiments were repeated 19–26 times. For each experiment, the preference was tested with the Wilcoxon signed-ranks tests. For comparison between PAs, preference indices were calculated and compared in an identical way as for *Ma. brassicae*.

RESULTS

F. occidentalis. The survival of the thrips larvae differed among the PA diets. Heliotrine did not affect survival of the larvae, whereas the other PAs significantly reduced larval survival at 10× plant concentration (Figure 2). At 1× plant concentration, only senkirkine decreased survival, although this was only marginally significant. Because senecionine is the precursor for senecionine-type PAs, we tested if survival on the senecionine diets differed from the other PAs. Over all concentrations tested, survival on senecionine diets was lower than on the heliotrine diets but higher than on the senkirkine diets (Table 1). We found no indications for a synergistic effect of PAs in PA mixtures. At both 2.5× and 5× plant concentration, survival on the PA diets did not differ among diets with only senecionine and diets with PA mixtures (Cox regression, senecionine–mixtures: $P > 0.05$). Both the retrorsine N-oxide and

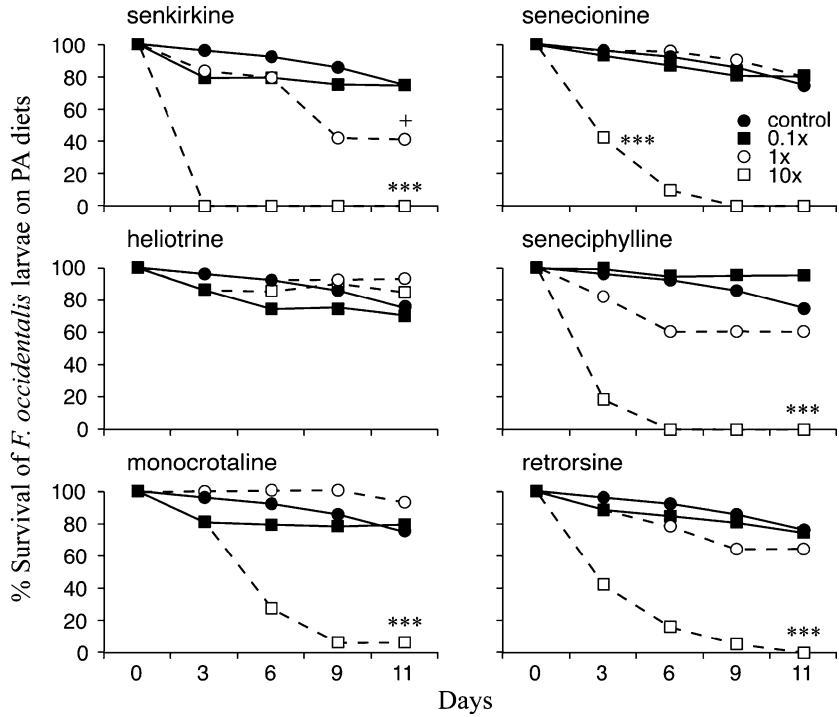

FIG. 2. Percentage survival of larvae of the thrips *F. occidentalis* on diets with pyrrolizidine alkaloids. Three different pyrrolizidine alkaloid concentrations were used, 0.1×, 1×, and 10× the plant concentration ($N = 24$ thrips per diet). ***($P < 0.001$) $^{+}$($P < 0.07$) (Cox regression, alkaloid diet compared to control).

TABLE 1. SURVIVAL ANALYSIS (COX REGRESSION) OF LARVAE OF THE THRIPS *F. occidentalis* ON PYRROLIZIDINE ALKALOID (PA) DIETS

	B	SE	Wald	df	P	R
Senecionine–PAs			32.70	5	<0.001	0.129
Retrorsine	0.192	0.265	0.525	1	0.469	0
Seneciphylline	0.199	0.280	0.504	1	0.478	0
Monocrotaline	−0.355	0.289	1.508	1	0.220	0
Heliotrine	−1.479	0.378	15.28	1	<0.001	−0.099
Senkirkine	0.568	0.265	4.576	1	0.032	0.043

All alkaloids are tested against senecionine with concentrations as strata in the model. See also Figure 2.

the free-base retrorsine significantly decreased survival compared with the control, but the N-oxide was less toxic than the free base (Figure 3).

M. persicae. The survival of *M. persicae* differed among the PA diets (Figure 4). Senecionine and seneciphylline reduced survival at plant concentration levels. Monocrotaline reduced survival only at the highest PA concentration (2× plant concentration). Senkirkine did not affect the survival of the aphids at all. There was no synergistic effect of PAs in the PA mixture (Figure 4).

S. exigua. We found no deterrent effects of any individual PA on the feeding of *S. exigua* caterpillars. At all three PA concentrations (0.3×, 1×, and 3× plant concentration), the amount consumed by the caterpillars from the lettuce disks with PAs did not differ from the control disks or among the PAs (Kruskal–Wallis, all $P > 0.30$). We did find indications for synergistic effects of PAs in the PA mixtures (Figure 5). The amount eaten tended to differ among the

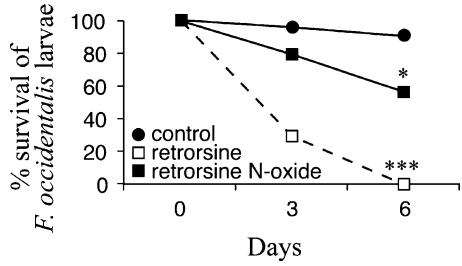

FIG. 3. Percentage survival of larvae of the thrips *F. occidentalis* on diets with retrorsine and retrorsine *N*-oxide and a control diet. PA concentration was 10× plant concentration ($N = 30$ thrips per diet). ***($P < 0.001$), *($P < 0.05$) (Cox regression, alkaloid diet compared to control).

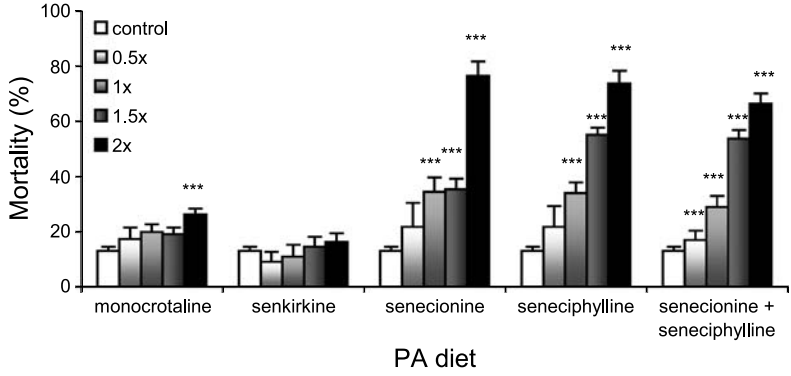

FIG. 4. Survival (±SE) of the aphid *M. persicae* on diets with pyrrolizidine alkaloids (N = 11 replicates per diet, 10 aphids per replicate). Concentrations of 0.5×, 1×, 1.5×, and 2× the plant concentration of *S. jacobaea* were used. Significance levels of Mann–Whitney *U*-test for independent samples, alkaloid compared to control, corrected with sequential Bonferroni test: *($P < 0.05$), ***($P < 0.001$).

PA mixtures (ANOVA, $F = 2.13$, $df = 4$, $P = 0.081$). *Post hoc* tests showed that the caterpillars consumed less from the PA mixture of senecionine/seneciphylline/senkirkine than from senecionine. The feeding from the other mixes did not differ significantly from senecionine.

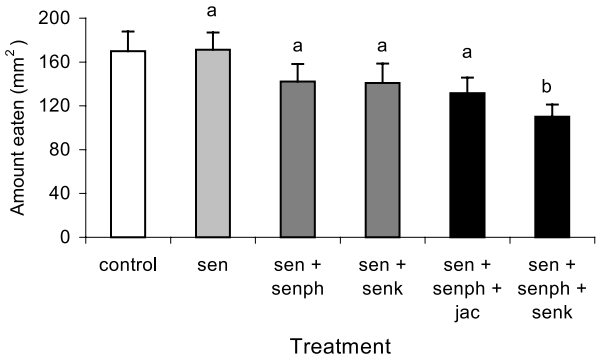

FIG. 5. Amount eaten (±SE) by caterpillars of *S. exigua* of lettuce disks with single pyrrolizidine alkaloids, alkaloid mixtures, or control (methanol). Total pyrrolizidine alkaloid concentration was 1× plant concentration. Different letters indicate significant differences [one-way ANOVA among alkaloids (P = 0.081) followed by *post hoc* Bonferroni tests ($P < 0.05$)] N = 25 replicates per test. Pyrrolizidine alkaloid codes: sen = senecionine; senph = seneciphylline; senk = senkirkine; jac = jacobine.

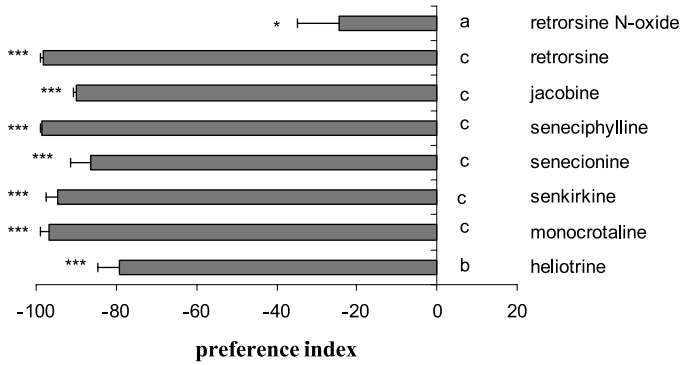

FIG. 6. Preference indices (±SE) for *L. migratoria* feeding on glass fiber filters with pyrrolizidine alkaloids. $N = 21–25$ replicates per test. Comparison per alkaloid for testing for preference for control: *($P < 0.05$), ***($P < 0.001$) (Wilcoxon signed-ranks matched-pairs test). Comparison of preference indices: different letters indicate significant differences between preference indices [Mann–Whitney *U*-test with sequential Bonferroni correction ($P < 0.05$)].

Ma. brassicae. No deterrent effects of PAs on the feeding of *Ma. brassicae* caterpillars were found in any of the two-choice experiments (Wilcoxon signed-ranks match-pairs tests, all $P > 0.05$). There were no differences in preference indices between the PAs (Kruskal–Wallis, $\chi^2 = 8.04$, $df = 6$, $P = 0.24$).

L. migratoria. All PAs deterred feeding of *L. migratoria*, but not all PAs were equally deterrent (Figure 6). Heliotrine was the least deterrent of the free-base PAs. All other PAs were equally deterrent when tested against the control (Figure 6). However, when senecionine and seneciphylline were tested against

TABLE 2. FEEDING OF *L. migratoria* FROM GLASS FIBER DISKS WITH PYRROLIZIDINE ALKALOIDS IN TWO-CHOICE EXPERIMENTS

Choice	mm^2 eaten (±SE)	*N*	P^a
Senecionine	157.9 (15.4)	19	0.082
Seneciphylline	10.6 (1.2)		
Senecionine	186.0 (14.9)	19	0.006
Senecionine + Seneciphylline	4.3 (0.3)		
Seneciphylline	14.9 (11.1)	20	0.149
Senecionine + Seneciphylline	39.9 (6.5)		
Senecionine + Seneciphylline	17.3 (2.0)	24	0.016
Senecionine + Seneciphylline + Jacobine	2.8 (0.3)		

[a] *P* values of Wilcoxon signed-ranks matched-pairs test.

each other, the locusts tended to prefer senecionine to seneciphylline (Table 2). Senecionine alone was less deterrent than a mixture of senecionine + seneciphylline, but seneciphylline alone was equally deterrent as the senecionine + seneciphylline mixture (Table 2). These results show that seneciphylline is more deterrent than senecionine. The senecionine + seneciphylline mixture was less deterrent than the mixture of senecionine + seneciphylline + jacobine (Table 2), indicating possible synergistic effects. Although the retrorsine N-oxide deterred the locusts, it was less deterrent than the free-base PAs (Figure 6).

DISCUSSION

Our results showed that the effects of PAs differed among the insect species. All PAs significantly deterred feeding of the locust. Individual PAs did not deter feeding by the caterpillars of the two polyphagous moth species. The two moth species are truly polyphagous and are possibly less sensitive to various secondary metabolites in their diet than the migratory locust that is a specialist on grasses. We performed no-choice tests for *S. exigua*, and choice tests for *Ma. brassicae* and *L. migratoria*. Perhaps if we had used choice tests for *S. exigua*, our results would have been different. van Dam et al. (1995) found that the single PAs from *Cynoglossum officinale* deterred feeding of *S. exigua* in choice tests. These PAs are of a different structural type than the PAs we tested, which could also explain the contrasting results. The PA N-oxide had less effect on the locust and the thrips than the free-base form of the same PA. Other studies have also shown that PA N-oxides are less deterrent to insects than the free-base PAs (Dreyer et al., 1985; van Dam et al., 1995). Nevertheless, the PA N-oxide still significantly deterred feeding or decreased survival in our experiments. In *Senecio* species, young leaves and flowers have a relatively high concentration of PAs compared with old leaves (Hartmann and Zimmer, 1986; De Boer, 1999). The concentration-dependent effect of PAs on the aphids and thrips showed that in *Senecio* species, the more valuable plant parts, flowers, and young leaves are better defended against these generalists than less important organs, as is predicted by the optimal defense theory (Zangerl and Bazzaz, 1992; van Dam et al., 1996).

The coevolutionary arms race hypothesis between plants and insect herbivores (Ehrlich and Raven, 1964) implies that related compounds have different effects on herbivores. Our results showed that structurally related PAs differed in their effects on the locust *L. migratoria*, the aphid *M. persicae*, and the thrips *F. occidentalis*, indicating a potential for selection on individual PAs by insects. Differences in (deterrent) effects among structurally related PAs were also found on spruce budworm larvae (Bentley et al., 1984) and the pea aphid *Acyrthosiphon pisum* (Dreyer et al., 1985). The arms race hypothesis

further implies that evolutionary older compounds are less effective than more recently developed ones (Feeny, 1976; Berenbaum and Feeny, 1981). It is generally assumed that successive steps in biosynthesis of compounds represent successive evolutionary stages (Rodman, 1981). We found that some, but not all, senecionine-derived PAs were more effective than the precursor senecionine, and this depended on the herbivore species that was tested.

Another explanation for the diversity of plant secondary metabolites could be that related compounds act synergistically (Adams and Bernays, 1978; Lindroth et al., 1988; Berenbaum et al., 1991). However, we did not find any evidence for synergistic effects of PAs on survival of the thrips or aphids. We did find indications that a mixture of PAs is more deterrent than single PAs for the locust and *S. exigua* caterpillars. Therefore, we cannot exclude that plants with a more diverse PA composition will be more successful in deterring generalist insect herbivores than plants with a simpler PA pattern.

The diversity in related secondary metabolites may also be maintained by selection pressure from different herbivores (Simms, 1990; Mithen et al., 1995; Juenger and Bergelson, 1998). The experiments with the thrips and the aphid show that generalist insect species can respond differently to the same PA. While senkirkine was the most effective PA against the thrips at plant concentration level, it had no effect on the aphid. Senecionine and seneciphylline were the most effective against the aphid. Herbivore populations will change over time and, thus, selection pressure on plant defense chemistry will change accordingly. Many authors have linked variation in plant secondary metabolites with contrasting selection pressures from generalist and specialist herbivores (e.g., Linhart, 1991; van der Meijden, 1996). Our results show that it is likely that contrasting selection pressures from different generalist herbivores will also maintain variation in plant secondary compounds. In conclusion, we found that there is a potential for selection of generalist insect herbivores on plant PA profiles and PA diversity.

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ARE CHEMICAL COMPOUNDS IMPORTANT FOR SOYBEAN RESISTANCE TO *Anticarsia gemmatalis*?

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Abstract—The identification and quantification of flavonoids (rutin and genistin) present in extracts of soybean genotypes, and their effects on the biology and physiology of *Anticarsia gemmatalis* Hübner (Lep.: Noctuidae) were studied. Analysis of covariance and bicoordinate utilization plots were used to remove the effect of feeding time from pupal weight and consumption as well as to separate pre- and postingestive effects of treatment on *A. gemmatalis* growth. Genotypes PI 274454, PI 227687, and "IAC-100" extracts in general, caused higher mortality, negatively influenced initial larval and pupal weight, and elongated larval cycle. Larvae fed on the "IAC-100" extract diet ingested larger amounts of food per unit of time, but were less efficient in its conversion to biomass. Leaf extracts of PI 227687 had the largest concentration of rutin (quercitin 3-*O*-rhamnosylglucoside), followed by PI 274454, and "IAC-100"; PI 74454 also had the highest genistin (genistein 7-*O*-glucoside) content. The susceptible cultivar "BR-16" showed only a kaempferol-based flavonoid in its chemical profile, indicating that after successive crosses, secondary compounds responsible for plant defenses were eliminated. Genotypes PI 274454, PI 227687, and "IAC-100" showed accentuated resistance characteristics and were considered inadequate sources for the development of *A. gemmatalis*. Considering rutin and genistin concentration in these genotypes, it is suggested that flavonoids are important factors conferring resistance to *A. gemmatalis*.

Key Words—Resistance, velvet bean caterpillar, rutin, genistin.

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INTRODUCTION

The velvet bean caterpillar (*Anticarsia gemmatalis* Hübner) (Lep.: Noctuidae) is considered the main defoliator pest of soybean [*Glycine max* (L.) Merrill] in Brazil. Utilization of insect-resistant cultivars have advantage of being less harmful to the environment and, at the same time, do not require adoption of complex technologies by growers. Resistance to insect attack is due largely to chemicals present in the host plant (Kubo and Hanke, 1986). These compounds, which generally are secondary metabolites, can have behavioral (preingestive) and physiological (postingestive) effects on insects (Berenbaum, 1986).

Efforts have been made with many crops to obtain lines and cultivars with moderate levels of resistance to insects. Wild soybean introductions PI 171451, PI 227658, and PI 229358 have been used since the early 1970s as sources of resistance to defoliator insects such as *Epilachna varivestis* (Van Duyn et al., 1971, 1972), *Trichoplusia ni* (Luedders and Dickerson, 1977), *Diabrotica speciosa*, and *Colaspis* sp. (Rezende and de Miranda, 1980), *Pseudoplusia includens* (Killen et al., 1977; Beach and Todd, 1988), *Spodoptera* spp. (Beach and Todd, 1987), and *A. gemmatalis* (Beach and Todd, 1988; Lambert and Killen, 1984; Oliveira et al., 1993). In addition, these PIs were considered moderately resistant to some seed-sucking insects (Jones and Sullivan, 1979; Piubelli et al., 2003a,b). In field trials, breeding lines with PI 274454 in their genealogy were less defoliated than cultivars used as susceptible controls (Rezende et al., 1980). Cultivar IAC-100, which in addition to other sources of resistance, has PI 274454 and PI 229358 in its genealogy, was released by the breeding program of the Instituto Agronômico (Campinas, São Paulo State, Brazil) because of its resistance to leaf feeders and stink bugs (Rossetto et al., 1995; Veiga et al., 1999).

Few attempts have been made to investigate the chemical basis for insect resistance in soybean. Consumption by *P. includens* larvae of an artificial diet, in which crude CH_2Cl_2 (methylene dichloride) leaf extracts of PI 227687 had been incorporated, caused strong allelochemical effects, such as weight reduction and increased mortality (Smith and Fischer, 1983). Sharma and Norris (1991) reported the extraction, separation, and identification of isoflavonoids of PI 227687 foliage as well as their antifeedant and/or antibiotic properties against *T. ni*.

In general, soybean and other leguminous species lack some potent secondary metabolites that are present in other plant families, such as Cruciferae and Solanaceae (Kogan, 1986). Nevertheless, some constitutive (Hoffmann-Campo, 1995) or induced flavonoids (Kogan and Fisher, 1991) were identified in diverse soybean organs. Those phenolic compounds also play important functions in defense against microorganisms and insects (Dixon and

Steele, 1999). Some of them may be antifeedant and/or antibiotic to soybean pests (Neupane and Norris, 1991; Sharma and Norris, 1991, 1994; Hoffmann-Campo et al., 2001).

Extracts from leaves of the insect-resistant genotype PI 227687 negatively affected the physiology and behavior of *Heliothis virescens* larvae (Hoffmann-Campo, 1995) and *T. ni* (Hoffmann-Campo, 1995; Hoffmann-Campo et al., 2001). The effects persisted when the most polar fraction, named fraction A, was added to the artificial diet. This fraction was composed mainly of rutin (quercitin 3-*O*-rhamnosylglucoside), quercitin 3-*O*-glucosylgalactoside, and genistin (genistein 7-*O*-glucoside). In further studies, rutin negatively affected the development of *H. virescens* (Hoffmann-Campo, 1995) and *T. ni* (Hoffmann-Campo et al., 2001). These insects are occasional and none of them are main pests of soybean (Kogan and Turnipseed, 1987) and, thus, likely not exposed to compounds of this plant. The aglycone quercitin and its glycoside rutin increased mortality and elongated larval period of *A. gemmatalis* (Gazzoni et al., 1997), although the physiological effect of flavonol was not investigated.

This study was conducted to evaluate the effects of different soybean genotype extracts with respect to the biology and physiology of *A. gemmatalis*, and also to quantify the flavonoids rutin and genistin present in these genotypes that are used as sources of resistance to insect pests in the breeding programs of Embrapa Soybean. Furthermore, we tested the hypothesis that, as a leguminous specialist and major defoliator, *A. gemmatalis* could cope with the mixture of allelochemicals present in soybean leaves by postingestive mechanisms.

METHODS AND MATERIALS

Plant Material. Soybean leaves of the genotypes "BR-16," PI 229358, PI 227687, PI 274454, and "IAC-100," produced under greenhouse conditions ($T = 23 \pm 2°C$, RH = 78%), were harvested at growth stage V6 (Fehr and Caviness, 1977) to carry out bioassays and chromatographic analyses. The bulk leaves were collected to perform bioassays, and samples of the fourth trifoliolate were taken to the Phytochemistry Laboratory to be analyzed by high-performance liquid chromatography.

Plant Extract for Diet Incorporation. To carry out the feeding experiments, 50g of dried leaves of each genotype were ground, mixed with 40% aqueous ethanol (EtOH), and left overnight (approximately 18 hr) in a shaker at 100 rpm. The extract was filtered through Framex paper and reduced by rotary evaporator. The aqueous extract was passed into a glass-wool plugged column (3×38 cm), with a nonionic Amberlite XAD-4 resin as adsorbent. Ultrapure water (pH 2.0 and 7.0) was passed through the column to eliminate the bulk of

non-flavonoid compounds. Methanol (MeOH; 80%) was used for flavonoid elution. The resulting extract was concentrated on a rotary evaporator, maintained frozen (−17°C) until addition to the artificial diet of *A. gemmatalis* (Greene et al., 1976, modified by Hoffmann-Campo et al., 1985), and offered to the larvae. A diet without addition of extract was used as control.

Feeding Experiments. A. gemmatalis larvae were obtained from the Embrapa Soybean Laboratory of Insect Mass Rearing, located in Londrina, PR, Brazil. Since eclosion, the larvae were reared on the diet containing each soybean extract. From late second and early third instars, larvae were weighed and housed individually in 30-ml acrylic cups (Fill-rite Corp., Newark, NJ, USA). Insects were maintained in controlled environmental chambers (25 ± 2°C; RH: 70 ± 10%; 14 L:10 D photoperiod) and observed daily to evaluate the average duration of each larval stage, until reaching the prepupal stage; numbers of dead insects were recorded to calculate percent mortality. Pupae were frozen (−50°C), oven-dried (60°C, 72 hr), and weighed to obtain dry pupal mass. Remaining food and frass were placed into tubes, oven-dried (60°C, 72 hr), and weighed. To estimate the initial dry weight of the larvae, five (second/third instar) were taken from each treatment, weighed, frozen (−50°C), oven-dried (60°C, 72 hr), and reweighed. The correction factor for initial fresh to dry weight was calculated. Values obtained were multiplied by the fresh weight of each set of experimental larvae. The same procedure was used to calculate the dry weight of the food.

Plant Extract Chemical Analysis. The fourth trifoliolate leaves of each genotype were excised and cut into small pieces. Aliquots of each sample (500 mg) were placed into glass tubes, macerated, and homogenized with 5.0 ml of 80% MeOH. After heating (50°C), extracts were maintained with constant shaking (150 rpm) for approximately 18 hr, and the supernatants were transferred into 50-ml beakers, and kept in fume cupboards until dryness (±96 hr). Samples were redissolved in 1.0 ml of 80% MeOH, and 20-µl aliquots were injected into an HPLC (Shimadzu, model SPD-M10A VP) and developed on a reverse-phase column (CLS-ODS-C18-M, 4.6-mm internal diam × 250 mm in length). Flavonoids were eluted with a linear gradient system (Hoffmann-Campo, 1995) composed of two solvents: (A) 2% acetic acid (HOAc, Vetec, UV/spectrometry grade) and (B) a solution composed of MeOH (J.T. Baker, HPLC grade), HOAc, and water (18:1:1). The initial gradient consisted of 75% solvent A and 25% B, which changed to 35% A and 65% B in 23 min, and returned to the initial condition at 25 min. This condition was maintained for 5 min for column cleaning. Solvent flow rate was 1.0 ml/min, and absorption was measured at 260 nm.

Flavonoid concentrations in the genotypes were estimated by comparing the concentration of rutin ($C_{27}H_{30}O_{16}$, quercitin 3-*O*-rhamnosylglucoside) and genistin ($C_{21}H_{20}O_{10}$, genistein 7-*O*-glucoside) in the genotype samples with

those obtained from HPLC-injected standards (Sigma). Standard concentrations of rutin (0.01, 0.02, 0.04, 0.08, 0.16, 0.32, and 0.64 mg/ml) and of genistin (0.01, 0.02, 0.04, and 0.08 mg/ml) were injected onto the HPLC to construct the compound equations. After the injection of samples, scanning by photodiode array was performed to compare HPLC trace spectra and retention times with those of the standards. The area corresponding to each compound trace was calculated and applied to the equation (standards) to estimate the concentrations of rutin and genistin in each genotype.

Statistical Procedures, Feeding Experiments. Bioassays were developed in a completely randomized design, with six treatments and 30 replicates. Mortality in each treatment was compared by using a χ^2 test of homogeneity, according to Banzatto and Kronka (1992), at a 5% probability. All other data were analyzed using the SAS statistical package for PC (SAS Institute, 1996). The effects of treatments on larval initial weight, pupal weight, food consumption, frass production, and feeding time (days) were analyzed through ANOVA, performed by the general linear model (GLM) procedure. If a significant effect of treatments at minimal 5% of probability was observed, the means were compared by least significant difference (LSD). Analysis of covariance (ANCOVA), proposed by Raubenheimer and Simpson (1992), followed by bicoordinate utilization plots (Raubenheimer and Simpson, 1994), was used to remove the effect of covariate feeding time from consumption and pupal weight. This statistical procedure was also used to separate pre- and postingestive effects of treatment on *A. gemmatalis* growth. Thus, differences in pupal weight were adjusted for covariates consumption and digested food (consumption − frass produced). When the interaction among covariate and treatments was not significant, the parallel line model was used, and the main effect of treatment and/or covariate was considered. If the effect of treatment was significant at a minimal 5% probability, means were compared by ANCOVA means (least square means).

Statistical Procedures, HPLC Analyses. A completely randomized design was used in the HPLC data analyses, with four genotypes and five replicates, injected in duplicate. Data were analyzed by ANOVA, and the means of each genotype were compared by Tukey's test at a 5% probability, by using the SAS statistical package (SAS Institute, 1996).

RESULTS

Differences in larval mortality rates were dependent on the treatment as indicated by a significant χ^2 test. Mortality rates were higher when larvae were fed on PI 274454, "IAC-100," or PI 227687 extract diets compared with the control, PI 229358, and "BR-16" extract diets (Table 1).

TABLE 1. LARVAL MORTALITY, LARVAL DRY WEIGHT (2ND/3RD), PUPAL DRY WEIGHT, FOOD CONSUMPTION, FRASS PRODUCTION, AND FEEDING TIME (FROM 2ND/3RD TO PREPUPATION) OF *Anticarsia gemmatalis* FED ON ARTIFICIAL DIETS CONTAINING EXTRACTS OF DIFFERENT SOYBEAN GENOTYPES OR CONTROL DIET

Diet	Mortality (%)	Weight (mg) ± SEM				Feeding time (days) ± SEM
		Larvae DW	Pupae DW	Food consumption	Frass production	
Control	2.2	0.56 ± 0.01 a	87.9 ± 3.52 ab	279.1 ± 4.80 a	144.4 ± 3.29 ab	7.3 ± 0.06 c
"BR-16"	1.7	0.31 ± 0.01 bc	86.0 ± 5.58 abc	281.5 ± 8.06 a	161.1 ± 4.81 a	9.9 ± 0.28 b
PI 229358	5.1	0.41 ± 0.01 b	96.6 ± 5.73 a	258.4 ± 8.08 ab	145.1 ± 5.24 ab	10.7 ± 0.30 b
PI 227687	26.7	0.29 ± 0.01 d	64.4 ± 4.41 c	259.5 ± 8.41 ab	144.9 ± 5.15 ab	13.7 ± 0.42 a
PI 274454	33.4	0.36 ± 0.01 bc	68.3 ± 5.98 bc	235.3 ± 7.49 b	132.7 ± 5.37 b	14.0 ± 0.60 a
"IAC-100"	33.4	0.26 ± 0.01 d	74.6 ± 6.44 abc	289.7 ± 10.00 a	147.4 ± 6.33 ab	13.0 ± 0.57 a
F values	58.61[a]	59.14***	5.22***	5.55***	3.13***	75.46***

Means followed by the same letter are not significantly different by Tukey test at 5% probability level.
[a] $\chi^2_{(5;\ 0.05)}$.
***$P < 0.001$

There was a significant effect of diet, by ANOVA, on larval and pupal weight, consumption, frass produced, and feeding time (Table 1). Larval weight was negatively affected by extracts of any soybean genotype, including "BR-16" (our control cultivar) as compared with plain diet. The lowest pupal weight was observed for larvae fed on diet containing extracts of PI 227687 (64.4 ± 4.41 mg) and PI 274454 (68.3 ± 5.98 mg). When larvae fed on PI 229687 extract diets, pupal weight was 96.6 ± 5.73 mg. Larvae fed on "IAC-100," "BR-16" extract diets, and control diet consumed larger amounts of food than those fed on PI 274454 extract; larvae feeding on the latter diet also produced the lowest amount of frass, whereas larvae fed on "BR-16" produced the largest amount of frass. The feeding time of *A. gemmatalis* larvae was negatively affected by all diets containing soybean extracts. The larval cycle was more elongated

TABLE 2. ANCOVA TESTING FOR THE EFFECT OF DIETS CONTAINING EXTRACTS OF DIFFERENT SOYBEAN GENOTYPES OR CONTROL DIET ON THE FOOD EATEN ADJUSTED FOR FEEDING TIME (TIME) AS COVARIATE (A, B), ON THE WEIGHT OF PUPAE ADJUSTED FOR CONSUMPTION AS COVARIATE (C, D), AND ON THE WEIGHT OF PUPAE ADJUSTED FOR DIGESTED FOOD (FOOD EATEN – FRASS) AS COVARIATE (E)

Source of variation	*df*	*F* values	
		Pupae	Food
(a) Time (covariate)	1	34.0***	19.1***
Diet	5	3.15**	0.99, ns
Time × diet	5	3.00*	0.58, ns
Residual	290	–	
(b) Time (covariate)	1	–	19.2***
Diet	5	–	4.28**
Residual	295	–	–
(c) Consumption (covariate)	1	82.4***	–
Diet	5	0.73, ns	–
Consumption × Diet	5	1.67, ns	–
Residual	284	–	–
(d) Consumption (covariate)	1	81.5***	–
Diet	5	6.24***	–
Residual	289	–	–
(e) Digested food (covariate)	1	27.2***	–
Diet	5	1.76, ns	–
Digested food × diet	5	2.98*	–
Residual	278	–	–

ns, Not significant.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

when extracts of PI 274454, PI 227687, and "IAC-100" were added to the diet in comparison with the plain diet, "BR-16," and PI 229358 extract diets.

Feeding time (covariate) and diets containing soybean extracts (treatments) affected pupal weight (Table 2a), indicating that *A. gemmatalis* growth depended on an interactive effect of covariate and treatments. Positive slopes were observed in the adjusted lines for the control and PI 227687 extract diets (Figure 1). All other treatments yielded negative slopes, indicating that increases in feeding time did not contribute to increased insect growth.

The interaction of covariates feeding time (Table 2a) and consumption (Table 2c) with diets was not significant for their relationships with the amount of ingested food or weight of pupae. Thus, a parallel line model was fitted. The

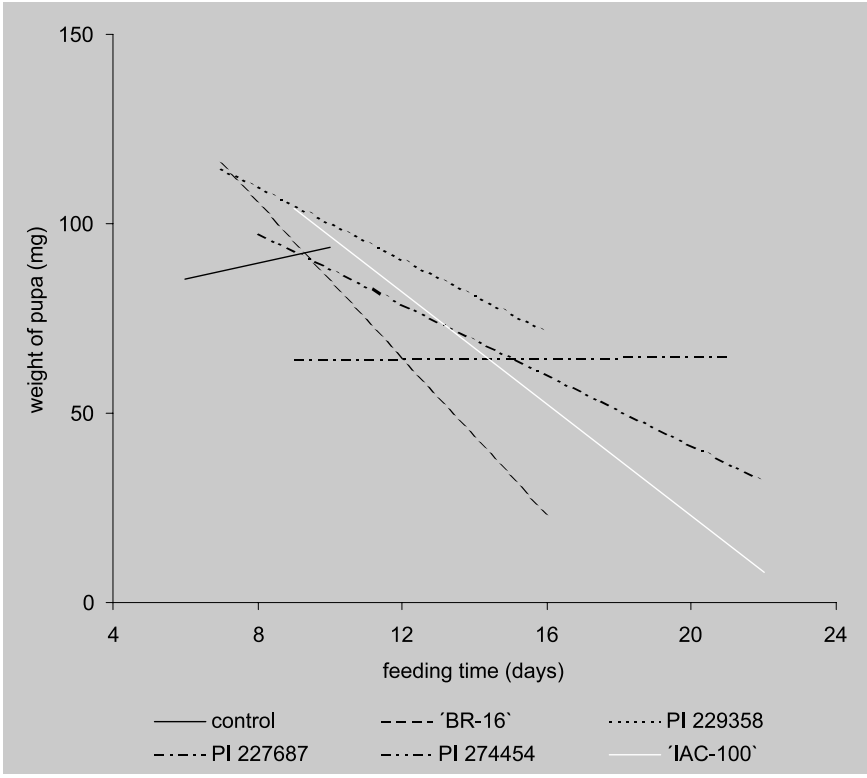

FIG. 1. Relationship between pupal weight and feeding time of *Anticarsia gemmatalis* fed on artificial diets containing extracts of different soybean genotypes or control diet. For statistical analysis, see Table 2a.

main effect of the diet and feeding time (Table 2b) or consumption (Table 2d) as covariate was significant, showing that the amount of ingested food depended on both factors, but not of the interaction between them. ANCOVA means of consumption adjusted by time (Figure 2) shows that larvae fed on "IAC-100" extract diet ingested a larger amount of food per unit of time, in comparison with the other treatments. Insects that fed on the PI 229358 extract diet converted more ingested food to biomass, as indicated by ANCOVA means of weight of pupae adjusted by consumption (Figure 3). Differently, lower conversion was observed when larvae were fed on extracts of "IAC-100" and PI 227687 compared with control diet, "BR-16," and PI 229358 extract diets.

The covariate digested food and diets significantly influenced pupal weight (Table 2e, Figure 4). Therefore, insect growth was dependent of the interactive effect between digested food and treatment. A positive relationship among the

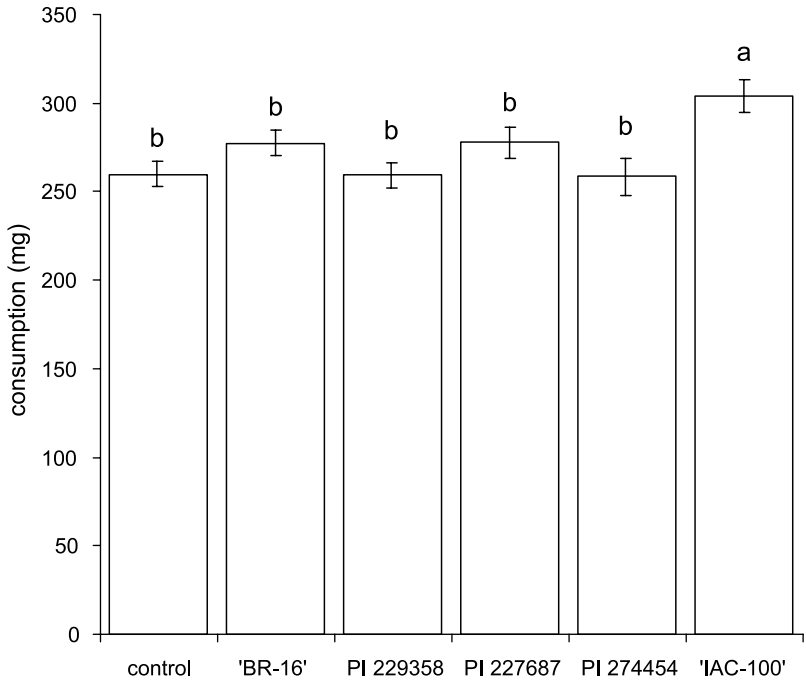

FIG. 2. ANCOVA means of consumption adjusted by feeding time of *A. gemmatalis* fed on artificial diets containing extracts of different soybean genotypes or control diet. For statistical analysis, see Table 2a and b. Columns followed by different letters are statistically significant.

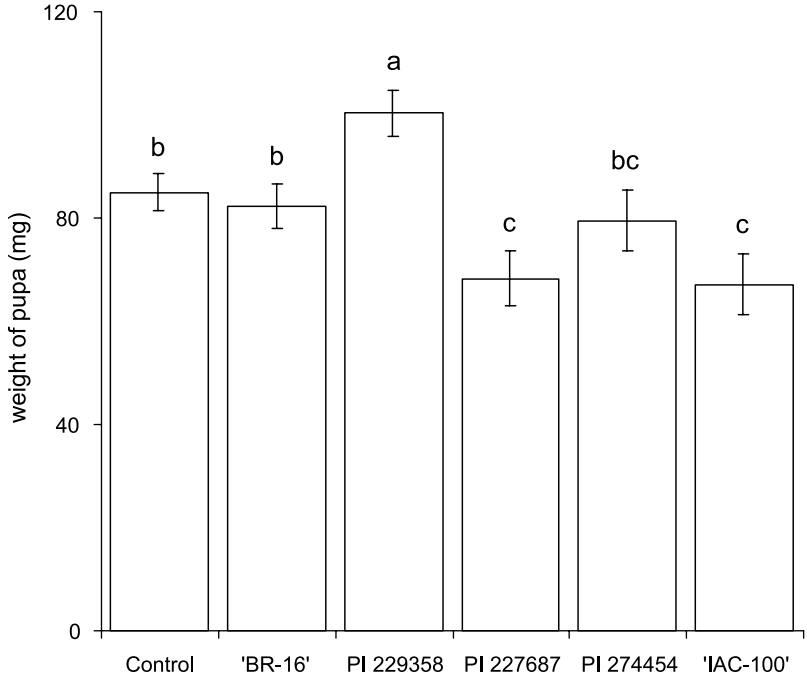

FIG. 3. ANCOVA means of weight of pupae adjusted by consumption of *A. gemmatalis* fed on artificial diets containing extracts of different soybean genotypes or control diet. For statistical analysis, see Table 2c and d. Columns followed by different letters are statistically significant.

factors is observed in Figure 4 for most of treatments, suggesting that those larvae that had higher amount of digested food (ingestion–excretion) became heavier. An exception was observed in larvae fed on the "IAC-100" extract diet, which presented a shallow negative slope, indicating that even with increased digested food, pupal weight remained at the same level.

The highest concentration of rutin (3.682 mg/g dried leaves) was observed in the extracts of PI 227687 (Table 3). PI 274454 and "IAC-100" presented intermediate, whereas PI 229358 had the lowest rutin content. The concentration of genistin in PI 227454 was higher than in the other tested genotypes. The susceptible cultivar BR-16 showed a smaller number of HPLC traces in comparison with the genotypes considered resistant. Rutin or genistin were not detected in its leaf extract, and, in fact, only a kaempferol-based compound was observed.

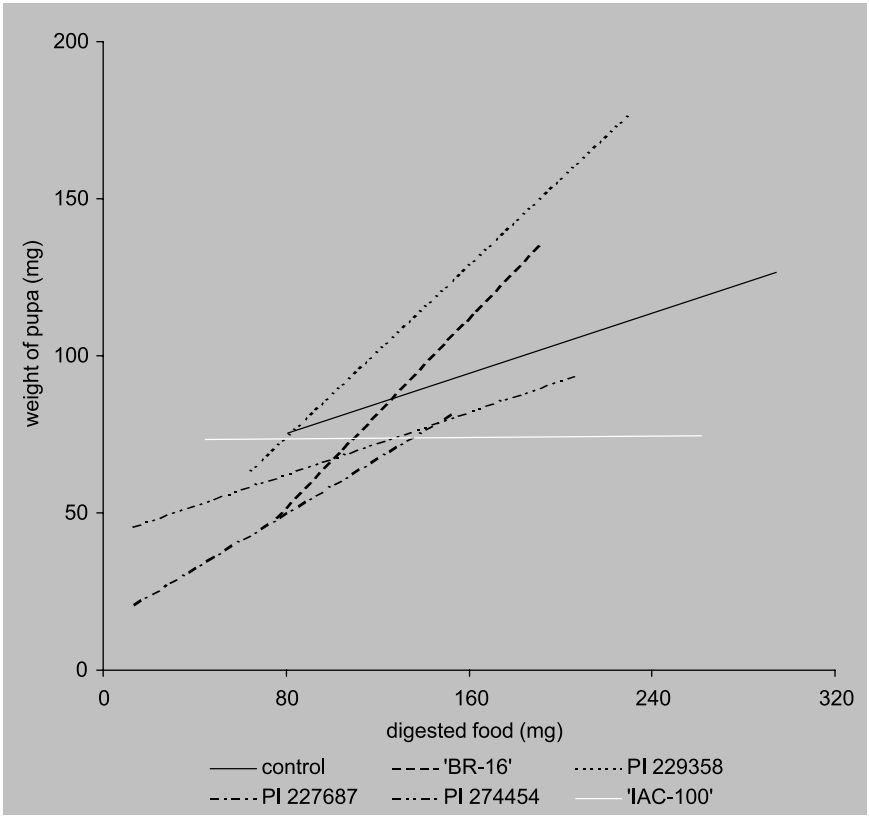

FIG. 4. Relationship between pupal weight and digested food of *A. gemmatalis* fed on artificial diets containing extracts of different soybean genotypes or control diet. For statistical analysis, see Table 2e.

TABLE 3. CONCENTRATION OF RUTIN AND GENISTIN IN SOYBEAN GENOTYPES WITH RESISTANCE CHARACTERISTIC TO INSECTS

	Concentration (mg/g)	
Genotypes	Rutin	Genistin
PI 227687	3.682 ± 0.416 a	0.122 ± 0.005 b
PI 274454	1.472 ± 0.282 b	0.258 ± 0.030 a
"IAC-100"	0.972 ± 0.082 bc	0.142 ± 0.011 b
PI 229358	0.212 ± 0.021 c	0.136 ± 0.005 b
F values	37.65***	13.28***

Means followed by the same letter are not significantly different by Tukey test at 5% probability level.
***$P < 0.001$.

DISCUSSION

For this study, we selected PI 227687, PI 229358, and PI 274454. The first two are those most used globally as sources of resistance to defoliating insects, and the latter is used similarly in Brazil. We also tested the cultivar IAC-100, which is regarded as possessing multiple insect-resistance characteristics (Rossetto et al., 1995), and "BR-16" as a nonresistant cultivar. Host-plant resistance can negatively affect insect physiology (antibiosis) or behavior (antixenosis). As morphological factors (trichomes, etc.) were ruled out, resistance was probably due to chemical factors (Soo Hoo and Fraenkel, 1966). These authors also stated that insects achieve appropriate growth when they have shorter development time (here we evaluated feeding time), heavier larva and/or pupa, and low mortality rate. Larvae fed on diet containing genotypes PI 227687, PI 274454, and "IAC-100" had protracted growth rates, higher mortality, and longer feeding times. The weights of pupae after adjustment by consumption were lower for insects fed on PI 227687 and "IAC-100" compared with other treatments, including the control and "BR-16," and PI 229358 extract diets, indicating that those insects were not able to efficiently transform food eaten in body mass.

In contrast, larvae fed on PI 229358 extract diet had lower mortality (5.1%), were heavier, and took the same time in the feeding period as those on "BR-16." When studying the biology of *H. virescens* in diets containing extracts of soybean genotypes, Hoffmann-Campo (1995) observed 45 and 10% mortalities when larvae were fed on PI 227687 and PI 229358 extracts, respectively. In field experiments, Hatchett et al. (1979) observed 87% (PI 227687) and 100% (PI 229358) larval mortalities. It should be pointed out that Hoffmann-Campo (1995) studied the effects of soybean crude extracts added to artificial diet, whereas Hatchett et al. (1979) evaluated the direct effects of insect feeding on resistant-genotype leaves. Thus, resistance of PI 229358 probably resulted from some morphologic factor than in this experiment, as in Hoffmann-Campo (1995), was removed by extraction, or else the whole plant has more resistant factors than could be detected with the extract provided in the present study.

An apparent contradiction was observed regarding the behavior of *A. gemmatalis* fed on the "IAC-100" extract diet. Larvae showed high mortality but consumed larger amounts of food, and pupal weight was similar to larvae fed on plain diet and on "BR-16" extract diet. In fact, this is not a completely atypical behavior for insects; they sometimes eat more to compensate for low nutrient levels (Slansky and Wheeler, 1989) and for intermediate level of allelochemicals in the diet (Paradise and Stamp, 1990). Consequently, mortality of larvae fed on the "IAC-100" extract did not result from preingestive, but likely postingestive effects. By ANOVA, larvae fed on such diet produced heavier pupae compared with those fed on PI 227687 extract diet, but after

adjustment by consumption, they showed lower ANCOVA means of pupal weight (similar to PI 227687). Additionally, despite retaining (digesting) large amounts of food, "IAC-100" extract diet was not able to transform this into heavier pupae, as indicated by the shallower slope, showed in Figure 4.

Higher consumption rates result in ingestion of greater doses of active compounds (Wheeler and Slansky, 1991), and *A. gemmatalis* probably failed to detect detrimental compounds present on "IAC-100" extract and, in the sequence, had to deal with their toxic effect in the diet. The same effect was also observed with *Helicoverpa* (*Heliothis*) *zea* (Duffey and Isman, 1981; Isman and Duffey, 1982a,b), *Manduca sexta* (Stamp and Scrobola, 1993), and *T. ni* (Hoffmann-Campo, 1995) when fed on rutin-enriched diet.

The highest concentration of rutin was found in PI 227687 compared with the other genotypes. Rutin concentration in "IAC-100" foliage was similar to PI 274454 and PI 229358, which are part of its genealogy (Veiga et al., 1999). Genistin was observed in all resistant genotypes, and its concentration in PI 274454 was nearly two times higher than in the other resistant genotypes. Before analysis, our crude leaf extracts were passed into a nonionic XAD-4 column to eliminate the bulk of nonflavonoid compounds, without any further fractionation. The identification of genistin and daidzin were performed by comparison of their ultraviolet spectra (from photodiode array), and retention time with authentic genistin and daidzin, and by coelution from HPLC. Neither daidzin nor daidzein (aglycone) were observed in our crude, unfractionated extracts of soybean leaf. Differently, Sharma and Norris (1991) reported the aglycone daidzein in methanolic extracts of PI 227687 after TLC fractionation in ethyl acetate. These authors did not refer to genistein (and its glycoside genistin) and rutin in PI 227687 leaf extracts. Also, there was no remark regarding how they identified daidzein.

The flavonoid aglycones, according to Markham (1982), are more reactive than the glycosides because the glycosylation makes possible their storage in the cell vacuoles. Flavonoid aglycones are rarely found as internal constituents, but regularly encountered on the external surface of leaves and fronds (Markham, 1989). In many years of analysis, we have not found isoflavone aglycones in healthy plants in our lab, even in pods/seeds. However, it is known that daidzein and genistein conjugates (7-*O*-glucosyl and 6″-*O*-malonyl-7-glucosyl) are constitutively present in large quantity in soybean seedlings after fungal infection (Graham et al., 1990) and in immature seeds after stinkbug damage (unpublished data). Graham et al. (1990) observed that daidzin conjugates can be rapidly hydrolyzed into free daidzein, which is the precursor of glyceollins, phytoalexins produced *de novo* in all soybean seedling organs.

Leaf extracts of genotype PI 227687 contain the isoflavone genistin and seven flavonol glycosides, i.e., three kaempferol-, two isorhamnetin-, and two quercitin-based compounds, one of them rutin (Hoffmann-Campo, 1995). This

compound was not observed in "BR-16," used as the susceptible control in the present study; kaempferol was the only flavonoid observed in other susceptible cultivars, such as Embrapa-1, Embrapa-4, IAS-5, and Davis (Hoffmann-Campo, 1995). Chan et al. (1978) observed that kaempferol was less toxic to *H. virescens*, *H.* (*Heliothis*) *zea*, and *Pectinophora gossypiella*, compared to quercitin-based compounds that possess a catechol group. According to Chan et al. (1978) and Elliger et al. (1980), there is a relationship between the presence of a catechol B-ring in a flavonoid and the inhibition of insect growth. This fact suggests that soybean-breeding programs usually do not allot priority to resistance to insects and, in practice, have eliminated secondary compounds otherwise responsible for plant defense.

Hoffmann-Campo (1995) qualitatively examined the flavonoid profile of 16 soybean genotypes. Rutin was found in one resistant cultivar (IAC-100), one breeding line (BR82-12547), and two wild soybean genotypes (PI 227687 and PI 229358). The present study reports that rutin concentration in PI 229358 was very low, which may explain the good performance of *A. gemmatalis* fed on diet containing this extract. Flavonoids are frequently used by monophagous and oligophagous insects to recognize their host plants (Harborne and Grayer, 1993), and, as *A. gemmatalis* is a leguminous specialist insect, it would be expected to be less affected by flavonoids from soybean leaves. However, this important growth inhibitor of lepidopterans is being removed after successive breeding crosses, and likely, *A. gemmatalis* has lost the ability to cope with this toxic compound.

The information obtained in the present work provides elements for future studies on the genetics of chemical compounds conferring resistance to defoliators. Consequently, the knowledge that PI 227687 and PI 274454 possess high rutin and genistin concentrations, respectively, and caused deleterious effects on *A. gemmatalis* physiology has various potential applications in IPM and in breeding programs, contributing to the sustainability of soybean-based agricultural systems. The identification of chemicals responsible for plant defense and their role in the interactions with insects can help breeders in the development of cultivars resistant to pests. Recent progress in biotechnology, especially in molecular biology, has opened new opportunities in developing host-plant resistance (Panda and Khush, 1995), and, according to Dixon and Steele (1999), flavonoids possess strong potential for metabolic engineering. In addition, with continual exposure to *Bacillus thuringiensis* (Bt) toxins, mainly in transgenic crops, resistance may develop in several insect pests (Tabashnik, 1994). Combining traditional chemically based breeding programs and genetically engineered *B*t toxin-based resistance has a potential to be much more sustainable and easily adopted than the usual higher dose/refuge strategies (Cooper et al., 2004). Thus, considering the concentrations of rutin and genistin in these genotypes, it is reasonable to suggest that flavonoids are important

factors in terms of resistance to *A. gemmatalis*. Effort is needed to maintain or increase flavonoid concentration in soybean cultivars. Finally, further studies regarding the effect of rutin, genistin alone, and interactively are necessary for a complete understanding of the role of flavonoids in the defense of soybean to *A. gemmatalis*.

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LABORATORY EVALUATION OF *Artemisia annua* L. EXTRACT AND ARTEMISININ ACTIVITY AGAINST *Epilachna paenulata* AND *Spodoptera eridania*

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Abstract—Ethanolic extract of aerial parts of *Artemisia annua* L. and artemisinin were evaluated as anti-insect products. In a feeding deterrence assay on *Epilachna paenulata* Germ (Coleoptera: Coccinellidae) larvae, complete feeding rejection was observed at an extract concentration of 1.5 mg/cm^2 on pumpkin leaf tissue. The same concentration produced a feeding inhibition of 87% in *Spodoptera eridania* (Cramer) (Lepidoptera: Noctuidae). In a no-choice assay, both species ate less and gained less weight when fed on leaves treated with the extract. Complete mortality in *E. paenulata* and 50% mortality in *S. eridania* were observed with extract at 1.5 mg/cm^2. Artemisinin exhibited a moderate antifeedant effect on *E. paenulata* and *S. eridania* at 0.03–0.375 mg/cm^2. However, a strong effect on survival and body weight was observed when *E. paenulata* larvae were forced to feed on leaves treated at 0.03 and 0.075 mg/cm^2. *Artemisia annua* ethanolic extract of aerial parts at 1.5 mg/cm^2 showed no phytotoxic effect on pumpkin seedlings.

Key Words—*Artemisia annua*, *Epilachna paenulata*, *Spodoptera eridania*, insecticide, artemisinin.

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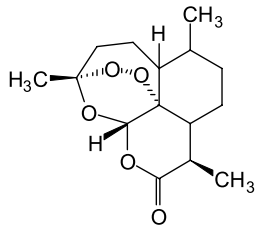
INTRODUCTION

Many wild plants are capable of synthesizing secondary metabolites with biological properties that are important in the fight against insect pests (Matthews, 1993; Enriz et al., 2000; Calderón et al., 2001; Céspedes et al., 2001; Gonzalez-Coloma et al., 2002). For these to be practical and economical, the selected species must meet some requirements, such as being cultivatable, and having a potent active principle, high stability, and a good yield. In our search for naturally occurring insecticide products, we previously studied the anti-insect activity of extracts of *Melia azadarach* L. (Meliaceae) (Valladares et al., 1997, 1999; Carpinella et al., 2002, 2003), and now we have focused on *Artemisia annua*.

The genus *Artemisia* is a rich source of biologically active natural products. Approximately 200 *Artemisia* sp. grow in China, where more than 50 of them have been used in traditional Chinese medicine (Tan et al., 1998). *Artemisia annua* L., known as "sweet Annie" or "annual wormwood," is an annual herb native to Asia (China) where it is known as "quinghao." The plant has become naturalized in many countries including Argentina, Bulgaria, France, Hungary, Italy, Spain, and the United States (Klayman, 1989, 1993), and it is well known for its antimalarial activity, attributed to the presence of artemisinin (**1**) (Klayman et al., 1984). This compound is also phytotoxic (Duke et al., 1987) in laboratory assays (Chen et al., 1991), but has performed poorly in the field (Duke et al., 2000). The essential oil from aerial parts of the plant has been used in cosmetics and pharmaceuticals, and it has been recently reported as protecting stored products and plants against insect attack (Rao et al., 1999; Tripathi et al., 2000, 2001).



Artemisinin **(1)**

As far as we know, neither organic extracts from aerial parts of this plant nor artemisinin has been assayed on insects of agronomical interest,

although some authors have apparently noted that artemisinin has anti-insect activity (Jia, 1997; Wang and Wang, 2002), but have not published in a peer-reviewed journal. In the present paper, we study the effects of an ethanolic extract of *A. annua* aerial parts on the feeding behavior, development, and survival of the leaf-feeding coccinelid, *Epilachna paenulata* Germ. (Coleoptera: Coccinellidae), and also on the polyphagous pest, southern armyworm, *Spodoptera eridania* (Cramer) (Lepidoptera: Noctuidae). The anti-insect activity exhibited by **1** is also reported here.

METHODS AND MATERIALS

Plant Material. Aerial parts of the *A. annua* plant were collected in the hill area of Córdoba, Argentina, after the plants bloomed in March 2000, and were identified by Prof. Luis Ariza Espinar from the Botanic Museum of Córdoba National University, Argentina. The vegetable material was air-dried at 20°C, crushed and Soxhlet extracted with ethanol (133.68 g). After exhaustive solvent removal, a viscous residue was obtained (22.21 g, 16.6% yield), and then a weighed amount of extract was dissolved in enough 95% ethanol to make concentrations of 1% and 10%. The presence of artemisinin in the extract was confirmed and quantified by high-performance liquid chromatography (HPLC) (Na-Bangchang et al., 1998), yielding 0.53 g (2.41%). Pure artemisinin was dissolved in 95% ethanol at concentrations of 2, 5, and 25 mg/ml for testing for anti-insect activity.

Insects. Larvae for the experiments were obtained from continuous colonies, reared on a natural diet of pumpkin, *Cucurbita maxima*, maintained in a growth chamber at 24 ± 1°C, 70–75% relative humidity, with a photoperiod of 16:8 light–dark cycle, and periodically renewed with field specimens (Aranda et al., 1996).

Chemicals. Artemisinin was purchased from Sigma (St. Louis, MO, USA). All organic solvents were of HPLC-grade and purchased from Merck (Darmstadt, Germany). Acetonitrile and acetic acid were obtained from Fisher Scientific (New Jersey, NJ, USA).

Equipment. HPLC was performed on a Shimadzu liquid chromatography system equipped with an LC-10AS pump and an L-ECD-6A electrochemical detector. A Phenomenex Luna C18 5 μm particle size (250 × 4.6 mm i.d.) reversed-phase column was used. The compound was eluted with 45% (v/v) MeCN in water containing 0.1 M acetic acid (pH 4.8), as previously reported (Na-Bangchang et al., 1998). The electrochemical detector was operated in the reductive mode at an applied potential of 1.0 V.

Phytotoxicity Assay. Pumpkin, *Cucurbita maxima*, seedlings were used for a phytotoxicity assay. Pumpkin seeds were germinated, and seedlings grown in a growth chamber for 5 d, in pots (250 ml) containing a sterile soil mix (2:2:1 sand/loam/peat, by volume). For every 2 d, the 5 d-old seedlings (N = 15) were sprayed with a 10% solution of *A. annua* ethanolic extract (approximately 2.4 mg/ml of **1**) dissolved in water (~500 µl per plant), during 20 d. This dosage was equivalent to 1.5 mg/cm^2 of extract and 0.03 mg/cm^2 of **1**. A control, sprayed with water, was run in parallel. The number of new leaves and symptoms of chlorosis were determined. Data were analyzed with analysis of variance (ANOVA) and Tukey's honestly significant difference test.

Feeding Deterrence Assay. To survey the antifeedant properties of the extract and of artemisinin, a modified leaf-disk choice test (Carpinella et al., 2003) was used. Two cotyledon leaves from a *C. maxima* seedling (both the same size, age, and from the same plant) were placed in a 9-cm-diam. Petri dish, and a glass disk with two 1.3-cm-diam. holes was placed on top. A third-instar *E. paenulata* or *S. eridania* larva was placed equidistant from a leaf disk treated with 20 µl of the test solution, and a control leaf disk treated with 20 µl ethanol. The larvae were allowed to feed for 24 hr. Ten replicates were used for each treatment. The relative amounts of the treated and untreated leaf areas eaten (recorded in percentages from 0 to 100) were visually estimated by dividing the leaf area into imaginary quarters. Measurements were always made by the same operator. Data were then compared by using the Wilcoxon paired-sample test (α = 0.05). The Antifeedant Index (AI%) was calculated as $(1 - T/C) \times 100$, where T and C represent the consumption on treated and untreated disks, respectively (Gonzalez-Coloma et al., 1998). After calculation of the AI% for compound **1**, the relative potency (ED_{50} values, the effective dosage for 50% feeding reduction) for **1** was determined by linear regression of AI% on log dose. These, and all the following experiments, were carried out at 24 ± 1°C and with a 16:8 (L/D) photoperiod.

No-Choice Assay. This test was carried out to analyze the effects of the extract and of artemisinin on the development and survival of larvae of both insect species. A first-instar *E. paenulata* and a third-instar *S. eridania* were each placed into a Petri dish and fed with fresh pumpkin leaves (renewed every 48 hr), treated either with 20 µl of the ethanolic extract solution, artemisinin solution, or with ethanol (control). Ten replicates were used for each treatment. A similar set of larvae of both insects were not fed at all, and acted as starved controls. Leaf consumption, development, and survival were recorded regularly. Data were analyzed with analysis of variance (ANOVA), Tukey's honestly significant difference test (Zar, 1996) and the Kaplan–Meier method (Hollander and Wolfe, 1999).

RESULTS AND DISCUSSION

In order to assess the insect control effects of *A. annua* extract, and the possibility of developing it as a natural insecticide for use in pest management programs, two phytophagous species belonging to two different orders, *E. paenulata* and *S. eridania*, were used.

Before starting with the insecticide assays, an evaluation of phytotoxicity was made on 5-d-old pumpkin seedlings, and no differences were observed in the development of new leaves or in phytotoxicity symptoms, such as chlorosis and death, between treatment and control (data not shown) during 20 d. These results suggest that phytotoxicity did not occur at the highest concentration (1.5 mg/cm^2 of extract, *vide infra*) evaluated in our feeding deterrence and no-choice assays.

Both species, *E. paenulata* and *S. eridania*, were deterred from feeding on pumpkin leaves treated with 1.5 mg/cm^2 *A. annua* extract (approximately equivalent to 1% w/w), consuming significantly less food (Wilcoxon, $P < 0.05$) than the control (Table 1). Feeding inhibition of *E. paenulata* larvae was almost complete at this concentration, and a significant difference was still observed at 0.15 mg/cm^2 (approximately equivalent to 0.1% w/w), where an AI of 78.8% was observed. These values indicate the potent antifeedant activity of *A. annua* extract, at the same level of activity as that previously shown by *M. azedarach* extract against *E. paenulata* (Carpinella et al., 2003).

After 3 d, larvae of *E. paenulata* confronted with leaves treated with 0.15 mg/cm^2 of ethanolic extract had consumed approximately half the leaf area of those receiving untreated leaves, and this trend was maintained throughout the experiment (Table 2). When larvae were fed on leaves treated with 1.5

TABLE 1. FEEDING DETERRENCE EFFECTS OF *Artemisia annua* AERIAL PARTS ETHANOLIC EXTRACT ON *Epilachna paenulata* AND *Spodoptera eridania*

	Leaf area eaten (%) ± SE[a]		
Extract dosage (mg/cm^2)	Treated	Control	AI%[b]
Epilachna paenulata			
0.15	7 ± 6.8**	33 ± 10.3	78.8
1.5	0.5 ± 1.6**	31.3 ± 15.7	98.5
Spodoptera eridania			
0.15	15.3 ± 8.3	16.3 ± 12	6.1
1.5	2.5 ± 4.3*	19.5 ± 22.7	87.1

[a] Consumption significantly lower on extract-treated food, *$P < 0.05$; **$P < 0.01$, Wilcoxon signed paired rank test.

[b] Results observed at 24 hr. Antifeedant Index (AI) = $(1 - T/C) \times 100$. Means of ten replications are presented.

TABLE 2. MEAN LEAF AREA CONSUMED BY *Epilachna paenulata* AND *Spodoptera eridania* LARVAE ON LEAVES TREATED WITH *Artemisia annua* AERIAL PARTS ETHANOLIC EXTRACT IN NO-CHOICE ASSAY (% PER INDIVIDUAL/DAY)

Days	Area consumed (%)[a]		
	Control	0.15 mg/cm^2	1.5 mg/cm^2
Epilachna paenulata			
3	21.2a	11.3b	2.4c
5	22.5a	24.6a	5.3b
7	40.1a	23.3b	5.0c
13	33.3a	17.6b	0.0
Spodoptera eridania			
2	16.1a	14.5a	18.6a
6	20.2a	13.7b	13.2b
9	48.6a	38.4b	22.1c
13	72.5a	53.3b	9.6c

[a] Means within rows followed by the same letter are not significantly different in ANOVA test with repeated measures (Tukey's test, $P < 0.05$).

mg/cm^2, an abrupt decrease in the area consumed was observed during the experiment, amounting to at least four times less than that consumed by the control larvae ($F = 52.9$; $df = 27$; $P < 0.001$).

From d 3 onwards, there were significant differences in the mean weight of *E. paenulata* larvae between treatments and control (data not shown). Whereas control larvae steadily increased their body weight, larvae treated with 1.5 mg/cm^2 lost weight. At d 5, significant differences were observed between both dosages ($F = 8.14$; $df = 27$; $P < 0.001$).

The distribution of survival for *E. paenulata* was significantly different between the treatments at 5, 7, and 13 d (data not shown). On the fifth day, 30% survival was observed at the 1.5 mg/cm^2 dosage, while 90% of the starved larvae had survived. These data suggest the presence of one or more toxic compounds, whose toxicity was evident at the highest concentration. The small amount of leaf ingested was enough to incorporate these compounds in toxic doses. At d 7, complete mortality was observed for these two treatments showing significant differences with both control and 0.15 mg/cm^2 dosages. At this time, the mortality could be attributed either to toxic metabolites present in the extract, or to the highest concentration provoking a strong antifeedant effect on larvae, causing 100% mortality through starvation.

In the no-choice assay, *S. eridania* larvae consumed less of leaves treated with 0.15 mg/cm^2 than controls (Table 2). When larvae were fed on 1.5 mg/cm^2 treated leaves, there were significant differences in the consumed area compared with controls from d 6 to the end of the study ($F = 3.86$, $df = 54$, $P = 0.04$).

These findings show that the inhibitory effect of the *A. annua* extract was more effective on *E. paenulata* larvae than on *S. eridania*, and probably acted in the latter as a secondary antifeedant, where reduction of food intake follows some initial consumption (Ascher, 1993).

There was a great difference in mean body weight of *S. eridania*, which began to be significant on d 2 and continued until the end of the assay for both treatments (data not shown). Moreover, from d 9 the body weight of larvae eating 1.5 mg/cm^2 was half or less than that of those fed with the lower dose ($F = 5.18$, $df = 54$, $P = 0.021$). The distribution of survival for *S. eridania* was significantly different between treatments at d 9 and 13 (data not shown). The survival rate by d 2 of larvae fed on 1.5 mg/cm^2 was 70%. However, survival declined to 50% at the end of the study, while, with starved larvae, 0% survived at d 9.

The dose of 1.5 mg/cm^2 artemisinin contained approximately 0.03 mg/cm^2 of **1**. We, therefore, checked the antifeedant effect of **1** at concentrations around this value. Artemesinin exhibited a moderate antifeedant effect on *E. paenulata* and *S. eridania* at 0.375 mg/cm^2 with an AI% of 87.9 and 81.0, respectively (Table 3). Lower doses (0.075 and 0.03 mg/cm^2) showed an AI% of approximately 60% to 75%. This did not account for the strong feeding inhibition of the extract, suggesting that the activity is only partially attributable to the presence of **1**, and that an additive or synergistic effect between **1** and other active principles could be present. From the data from both species (Table 3), the effective dosage for 50% feeding reduction (ED_{50}) calculated for compound **1** was 0.0136 mg/cm^2. Comparing the antifeedant activity of

TABLE 3. ANTIFEEDANT INDEX OF ARTEMISININ ON *Epilachna paenulata* AND *Spodoptera eridania* LARVAE UNDER FEEDING DETERRENT ASSAY

Dosage (mg/cm^2) artemisinin	Leaf area eaten (%) ±SE[a]		AI%[b]
	Treated	Control	
Epilachna paenulata			
0.01	29.5 ± 37.9	54.5 ± 42.3	45.9
0.03	17.5 ± 28.5	46 ± 34.4	61.9
0.075	33.3 ± 14.4*	96.5 ± 4.1	65.4
0.375	11.5 ± 4.6*	95.3 ± 3.5	87.9
Spodoptera eridania			
0.01	16 ± 17.1	19 ± 10.4	15.8
0.03	7.5 ± 14.8	18.5 ± 26.8	59.4
0.075	12.3 ± 15.2*	51 ± 33.5	75.8
0.375	7.5 ± 9.5*	49 ± 23.31	81.0

[a] Consumption significantly lower on extract-treated food, *$P < 0.01$, Wilcoxon's signed paired rank test.

[b] Results observed at 24 h. Antifeedant Index (AI) = $(1 - T/C) \times 100$, 10 replications.

TABLE 4. MEAN LEAF AREA CONSUMED BY *Epilachna paenulata* LARVAE ON LEAVES TREATED WITH ARTEMISININ IN NO-CHOICE ASSAY (% PER INDIVIDUAL/DAY)

Days	Area consumed (%)[a]		
	Control	0.03 mg/cm^2	0.075 mg/cm^2
3	15.5a	3.6b	6.8b
5	46.7a	24.5b	26.2b
7	39.5a	1.4b	–
9	47.8	–	–
11	32.5	–	–

[a] Means within rows followed by the same letter are not significantly different in ANOVA test with repeated measures (Tukey's test $P < 0.05$).

1 with that reported (Carpinella et al., 2002) for meliartenin (ED_{50} = 0.0008 mg/cm^2), azadirachtin (ED_{50} = 0.00072 mg/cm^2), and toosendanin (ED_{50} = 0.0037 mg/cm^2), **1** is approximately 17, 19, and 4 times less active than meliartenin, azadirachtin, and toosendanin, respectively.

Larvae of *E. paenulata*, forced to feed on pumpkin leaves treated with 0.03 and 0.075 mg/cm^2 of **1**, consumed less than the control ($F = 16.15$; $df = 2$; $P < 0.001$) (Table 4). At d 7, the 0.03 mg/cm^2-leaf area consumed by larvae was 28 times lower than that ingested of the control. At the same time, larvae exposed to 1.5 mg/cm^2 of extract ate eight times less than the larvae fed with untreated leaves (Table 2). Although **1** showed a moderate antifeedant effect, it provoked a strong inhibition of feeding once a significant quantity of treated food was ingested in no-choice assays.

Body weight of control larvae increased throughout the experiment (data not shown), while the two treatments showed almost no change in body weight ($F = 53.47$, $df = 2$, $P < 0.001$), and at d 5 weighed at least four times less than the controls.

Larvae were observed to tremor, move without coordination, and collapse when they were forced to feed on **1**-treated leaves. This behavior was similar to symptoms of insects under the effects of neurotoxins (Wang et al., 2000).

The distribution of survival for *E. paenulata* (data not shown) was significantly different $P < 0.001$ between the treatments at d 7, 9, and 11. Complete mortality was reached at d 7 and 9 for 0.075 and 0.03 mg/cm^2, respectively, while all starved larvae died at d 7. These data would suggest that death was attributable to the inhibition of feeding by **1**, although the possible neurotoxic effect observed suggests that other mechanisms may be involved.

In summary, *A. annua* ethanolic extract inhibited feeding and produced negative effects on the survival and development of the leaf-feeding coccinelid, *E. paenulata*, and the polyphagous pest, southern armyworm, *S. eridania*.

High mortality was observed in both *E. paenulata* and *S. eridania* with the extract at a dose of 0.15 mg/cm^2. Its activity at concentrations of 0.15 and 1.5 mg/cm^2 opens up the possibility of using *A. annua* ethanolic extract for the control of these pests. Artemisinin affected the development and survival of *E. paenulata* at a dose of 0.03 mg/cm^2, equivalent to 1.5 mg/cm^2 of *A. annua* extract, indicating that **1** is its main active principle. The strange behavior of larvae of this species fed on **1**, as if under a neurotoxic effect, warrants further investigation.

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OLFACTORY RESPONSES OF BANANA WEEVIL PREDATORS TO VOLATILES FROM BANANA PSEUDOSTEM TISSUE AND SYNTHETIC PHEROMONE

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Abstract—As a response to attack by herbivores, plants can emit a variety of volatile substances that attract natural enemies of these insect pests. Predators of the banana weevil, *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae) such as *Dactylosternum abdominale* (Coleoptera: Hydrophilidae) and *Pheidole megacephala* (Hymenoptera: Formicidae), are normally found in association with weevil-infested rotten pseudostems and harvested stumps. We investigated whether these predators are attracted to such environments in response to volatiles produced by the host plant, by the weevil, or by the weevil–plant complex. We evaluated predator responses towards volatiles from banana pseudostem tissue (synomones) and the synthetic banana weevil aggregation pheromone Cosmolure+ in a two-choice olfactometer. The beetle *D. abdominale* was attracted to fermenting banana pseudostem tissue and Cosmolure+, whereas the ant *P. megacephala* was attracted only to fermented pseudostem tissue. Both predators were attracted to banana pseudostem tissue that had been damaged by weevil larvae irrespective of weevil presence. Adding pheromone did not enhance predator response to volatiles from pseudostem tissue fed on by weevils. The numbers of both predators recovered with pseudostem traps in the field from banana mats with a pheromone trap were similar to those in pseudostem traps at different distance ranges from the pheromone. Our study shows that the generalist predators *D.*

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abdominale and *P. megacephala* use volatiles from fermented banana pseudostem tissue as the major chemical cue when searching for prey.

Key Words—Aggregation pheromone, *Cosmopolites sordidus*, Curculionidae, infochemicals, prey searching, synomone, olfactometer, natural enemies, plant volatiles.

INTRODUCTION

During host searching, natural enemies of herbivorous insects (predators and parasitoids) are known to utilize volatile chemicals emitted by plants or herbivorous insects (Vinson, 1976; Vet and Dicke, 1992; Turlings et al., 1995; Dicke and Vet, 1999). Predators and parasitoids that forage for herbivorous prey by using infochemicals may have a problem concerning reliability and detectability of these stimuli (Vet and Dicke, 1992; Wiskerke et al., 1993). Stimuli from the prey's food are detectable but are not reliable in indicating prey presence. In contrast, prey-derived stimuli are generally the most reliable sources of information but usually not easily detectable at long distances (Vet and Dicke, 1992). Natural enemies have evolved different mechanisms to cope with this reliability–detectability problem (Vet and Dicke, 1992). One of these mechanisms is that natural enemies can exploit pheromones from their victim as kairomones in long distance herbivore location (Lewis et al., 1982; Noldus et al., 1991; Wiskerke et al., 1993; Hedlund et al., 1996; Hendrichs and Hendrichs, 1998; Hoffmeister and Gienapp, 1999; Wertheim et al., 2003; Francis et al., 2004; Fatouros et al., 2005). The use of chemical information that is both reliable and easy to detect enhances natural enemy searching efficiency (Vet and Dicke, 1992).

Infochemicals, both those used within and between species, can be utilized in pest management by either exploiting the way the natural enemy responds, or by manipulating the source of the infochemical (Dicke et al., 1990; Vite and Baader, 1990; Foster and Harris, 1997; Degenhardt et al., 2003; Powell and Pickett, 2003). For example, infochemicals can be used to enhance the searching efficiency, host utilization, and reproductive capacity of natural enemies (Renwick, 1992; Turlings et al., 1995; Scutareanu et al., 1997; Steidle and van Loon, 2003; McGregor and Gillespie, 2004). There are a few studies on the application of infochemicals to manipulate the behavior of predators or parasitoids in the field (e.g., Drukker et al., 1995; Shimoda et al., 1997; Bernasconi et al., 2001; James, 2003; James and Price, 2004). However, data on the role of infochemicals in predator foraging have become available for several groups such as predatory mites (Sabelis and Dicke, 1985), pentatomids (van Loon et al., 2000), anthocorids (Dwumfour, 1992; Drukker et al., 1995; James and Price, 2004), chrysopids (Reddy et al., 2002; James and Price, 2004), and

coccinellids (Le Ru and Makosso, 2001; Ninkovic et al., 2001; Steidle and van Loon, 2002; James and Price, 2004). The predator *Rhizophagus grandis* (Gyll.) (Coleoptera: Rhizophagidae) is attracted to traps baited with a kairomone produced by the bark beetle *Dendroctonus micans* Kug (Coleoptera: Scolytidae) (Aukema et al., 2000), and this can be exploited to monitor the predator's distribution in the field.

The banana weevil *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae) is a major pest of bananas in East Africa. Yield losses of up to 100% have been reported (Sengooba, 1986, unpublished). The weevil oviposits in the leaf sheaths and corm at the base of the banana mat (a banana mat consists of plants arising from a common corm/rhizome) (Abera et al., 2000). The larvae tunnel in the corm and pseudostem (the plant part between the corm and leaves), damaging the vascular system and weakening the stability of the plant. In Uganda, distribution studies showed that over 60% of the adult weevils in the banana field were associated with crop residues such as rotting pseudostem (Gold et al., 2004). The weevil has predators that have been mostly found in environments harboring weevils such as banana pseudostem traps and fermenting banana pseudostem tissue, often in larval weevil tunnels (Koppenhofer et al., 1992; Koppenhofer, 1993; Tinzaara et al., 1999; Abera, 2004). Some ant species that have the potential to control *C. sordidus* include *Pheidole megacephala* and *Tetramorium guineese* (Mayr) (Hymenoptera: Formicidae) (Gold et al., 2001; Abera, 2004). Nonant predators known to prey on weevil eggs and larvae include *Dactylosternum abdominale* (Fabricius) (Coleoptera: Hydrophilidae), *Euborellia annulipes* (Lucas) (Dermaptera: Carcinophoridae), and *Thyreocephalus interocularis* (Eppelscheim) (Coleoptera: Staphylinidae) (Koppenhofer et al., 1992). Of these five predator species, *D. abdominale* and *P. megacephala* are the most abundant predators in environments preferred by weevils in Uganda (Tinzaara et al., 1999; Gold et al., 2001; Abera, 2004).

D. abdominale and *P. megacephala* are generalist predators that feed on microfauna and -flora of decomposing plant tissues, eggs, and small larvae of insects. Decomposing tissue is more attractive to these predators than fresh tissue (Koppenhoefer, 1993). Generalist predators are known to use infochemicals during prey location (Dwumfour, 1992; Scutareanu et al., 1997; Haberkern and Raffa, 2003; Steidle and van Loon, 2003; McGregor and Gillespie, 2004). Information on how volatiles from decomposing banana pseudostem tissue influence prey location by generalist predators is not available. Therefore, we have investigated the behavior of *D. abdominale* and *P. megacephala* predators under laboratory and field conditions to assess whether they use volatile infochemicals associated with banana weevils and/or their food.

An aggregation pheromone has been identified for *C. sordidus*, which is specific to the weevil (Jayaraman et al., 1997). A synthetic pheromone source containing a mixture of the four sordidin isomers is sold under the trade name

Cosmolure+. The pheromone has been studied in the laboratory and in the field for the management of *C. sordidus* (Tinzaara et al., 2000, 2003) and attracts both male and female weevils (Alpizar et al., 1999; Tinzaara et al., 2000). The pheromone-baited trap captures up to 18 times more weevils than a conventional split pseudostem trap (Tinzaara et al., 2000). Information on the effect of this aggregation pheromone on the behavior of the weevil's predators has not been investigated. Several species of predators have been reported to use the aggregation pheromones of their hosts during host searching and location (Dwumfour, 1992; Vet and Papaj, 1992; Hedlund et al., 1996; Scutareanu et al., 1997; Haberkern and Raffa, 2003; Steidle and van Loon, 2003).

The objectives of this study were to determine whether: (1) volatiles from banana pseudostem tissue and *C. sordidus* pheromone attract the predators *D. abdominale* and *P. megacephala*; (2) the predators respond to host plant volatiles and whether this response is dependent on dose or weevil feeding; (3) the pheromone enhances the predators' response to weevil-damaged pseudostem tissue; and (4) the pheromone affects the predators' distribution around pheromone-baited traps in the field.

METHODS AND MATERIALS

Site Description

Laboratory and field studies were conducted at Kawanda Agricultural Research Institute (KARI) (0°25′N, 32°51′E, 1190 m), 13 km north of Kampala, Uganda. The site has two rainy seasons (March–May and September–November) with an average precipitation of 1180 mm per year. Average daily temperatures range between 16 and 29°C. Relative humidity in the laboratory ranged from 60 to 80%.

A field experiment was conducted in banana plots at KARI planted with cultivar Nabusa (*Musa* spp., AAA-EA group). The plot size was 12 × 10 mats at a spacing of 2.5 × 3 m (Figure 1). The plots were weeded after every 2 mo and were not mulched.

Odor Sources

Pieces of fresh pseudostem (less than a week after harvest) from the banana cv Nabusa collected from banana fields at KARI were placed in plastic containers for 7 d at room temperature to get fermented pseudostem tissue. Fresh pseudostem tissue was collected at the time of the bioassays. Fifty grams of either fresh or fermented pseudostem tissue was used for bioassays. This same dose was previously successfully used for studies of the weevil's response to infochemicals (Tinzaara et al., 2003).

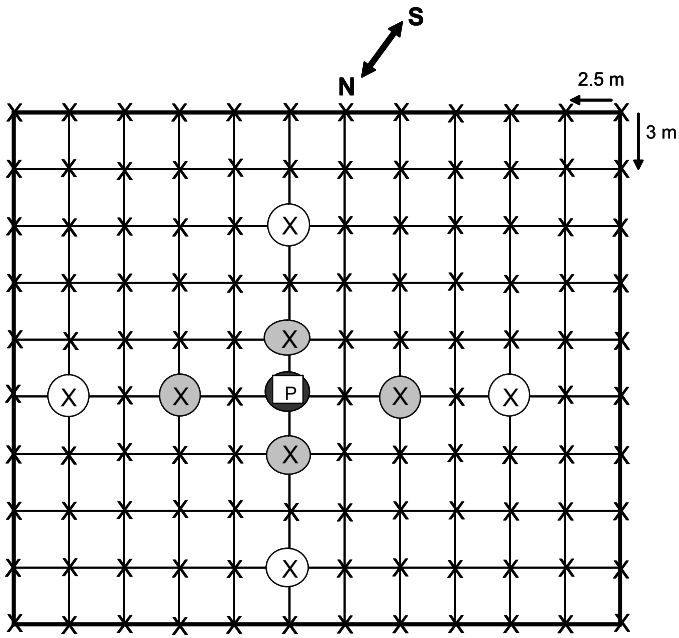

FIG. 1. A schematic diagram of the plot used in the field experiment to determine the effect of the aggregation pheromone on the distribution of predators. Pseudostem trap pieces were placed on banana mats (X) at different distance ranges indicated by circles (black = 0 m, grey = 0.1–5 m, white = 5.1–10 m) from the pheromone trap (P).

Pheromone lures for use in laboratory bioassays and field experiments were obtained from ChemTica International in San Jose, Costa Rica. They were sealed in plastic sent by a courier (transit time <1 wk) and subsequently stored in a freezer at −5°C upon arrival until use. Each pheromone pack contained 90 mg of Cosmolure+ with a release rate of 3 mg/day (Oehlschlager, personal communication). The pheromone packs were individually used as odor sources in their original plastic package material.

Predators

Adults of the beetle *D. abdominale* and the ant *P. megacephala* were selected for use in laboratory bioassays to assay their response to infochemicals. Predators were collected by hand searching in rotten banana pseudostems and corms from the field and kept on a nonsubstrate tissue (wetted tissue paper) in the laboratory for 24–48 hr before use in bioassays. Neither age nor sex of the

collected *D. abdominale* beetles was known. Worker ants of *P. megacephala* of unknown age were used.

Olfactometer

An olfactometer similar to that employed by Lofgren et al. (1983) and Cordova-Yamauchi et al. (1998) to study laboratory response of ants to banana weevil aggregation pheromone was used in all experiments. The apparatus consists of a petri dish with 19-cm diam and 4 cm in height, without a lid. Two holes were made through the sides of the dish close to the base, and two delivery tubes were inserted into them. A filter paper was placed at the floor of the petri dish and wetted with about 50 ml of water before each test. One of the (arms) tubes of the olfactometer was connected to a jar (125 ml) containing a test odor source and the other to a jar containing clean air as control. Volatiles entered the arena by diffusion.

A single predator was placed at the center of the olfactometer arena. Each predator was observed for a maximum of 10 min and was considered to have responded when it entered one arm of the olfactometer or when at the end of the 10 min, the predator was within less than 1 cm from the entry port of the arms. After testing five individuals for each odor set, the odor sources were replaced with fresh ones. For all experiments, five individuals of each predator species were tested for all odor sets per day. The first experiment was repeated during 6 d (N = 30, total number of individuals per predator species per odor set), whereas the rest of the laboratory experiments were repeated on 10 d (50 individuals per predator species per odor set, unless mentioned otherwise). Each predator individual was tested only once and then discarded. Treatment and control arms were exchanged after testing each predator by connecting the tubes at the opposite side to avoid trail formation. This had been observed to occur especially in the case of *P. megacephala* in preliminary tests. This procedure also precluded the effects of unforeseen asymmetry in the setup. The apparatus was washed with ethanol and air-dried before using a new predator species (i.e., after testing five individuals).

Experiments

We conducted five experiments with *D. abdominale* and *P. megacephala*. The first four were done in the laboratory using the olfactometer, and the fifth was done in the field.

Testing of the Olfactometer. This experiment was conducted to test whether any directional bias interfered with the responses of the two predator species in the olfactometer. The following odor sets were compared in this

experiment: (1) clean air vs. clean air and (2) fermented pseudostem tissue vs. fermented pseudostem tissue.

Predator Response to Pseudostem Tissue and Weevil Pheromone. The response of the predators to banana pseudostem tissue and the weevil's aggregation pheromone was evaluated in this experiment. Odor sets that were tested in the olfactometer were (1) fresh pseudostem tissue vs. clean air, (2) fermented pseudostem tissue vs. clean air, (3) fermented vs. fresh pseudostem tissue, and (4) pheromone vs. clean air.

Predator Response to Different Doses of Fermented Pseudostem Tissue. This experiment was conducted to determine whether predator response to infochemicals was dose-dependent. Odor sources were 1, 5, 25, and 125 g of fermented pseudostem tissue. Predator response to volatiles emanating from these amounts of tissue was compared to clean air in the olfactometer.

Predator Response to Weevil-Damaged Pseudostem Tissue in the Absence and Presence of Pheromone. This experiment was conducted to determine whether feeding by weevil larvae influences predator response to the banana pseudostem tissue and to evaluate whether the presence of pheromone enhances the predators' responses to volatiles from banana pseudostem tissue without weevil larvae feeding. Treatments were as follows: (1) fresh pseudostem, fed on by weevil larvae for 48 hr and larvae removed (F-LR); (2) fresh pseudostem, fed on by weevil larvae for 48 hr and larvae present (F-LP); and (3) weevil larvae alone. The following odor sets were compared: (1) F-LR vs. clean air, (2) F-LP vs. clean air, (3) larvae vs. clean air, (4) F-LP vs. larvae, (5) F-LR vs. F-LP, and (6) F-LR plus pheromone vs. F-LR.

Pseudostems of the cultivar Nabusa were collected from the fields at KARI. Weevil larvae (third to fifth instar) collected from the field were allowed to feed on fresh pseudostem tissue for 48 hr. Five larvae were placed on a pseudostem piece measuring 30 × 10 cm. After 48 hr at 22–28°C, the tissue had been tunneled, and tissue had turned dark brown and was used in bioassays with or without the larvae present. The larvae that were tested without food were collected from the field 24 hr before bioassays. They were placed in petri dishes (9-cm diam) with a nonsubstrate food material (moist tissue paper).

Field Distribution of Predators Around the Pheromone-Baited Traps. A field experiment was conducted at KARI to determine the distribution of banana weevil predators around pheromone-baited traps. We evaluated the hypothesis that predators aggregate around the trap mat as a result of a response to the pheromone and/or weevil-related volatiles. A pitfall pheromone-baited trap (Tinzaara et al., 2000) was placed at the center of each plot (Figure 1). Soapy water was placed in the trap to retain the predators that had entered. The soapy water was renewed at every sampling occasion. Pheromone traps were checked every 37 d, and predators captured in the traps were recorded and taken to the laboratory in vials for sorting and identification.

Ten fresh split pseudostem pieces (each 30 cm long) were placed in the plots at the time of installing the pheromone traps. In each plot, pseudostem pieces were placed on the trap mat and on four mats in each of the distance range of 0.1–5 and 5.1–10 m from the trap. Selection of the distance ranges was based on previous data on response by the weevil to the aggregation pheromone (Tinzaara et al., 2000). Six replicate plots were used. To determine distribution of predators around the pheromone-baited traps, predators were searched for in banana pseudostem pieces after 30 d at different distances from the trap.

Statistical Analysis

The χ^2-test for goodness of fit was used to determine the preference for one of the stimuli tested during the olfactometer bioassays (distribution of expected values 50:50). Field data on the number of predators and the weevils distributed around the pheromone-baited traps relative to distance were subjected to analysis of variance (ANOVA) using the GLM procedures of SAS software (SAS, 1990). The means were compared using the Student–Newman–Keuls (SNK) test. A regression analysis was used to determine the relationship between weevil and predator catches in pheromone-baited traps.

RESULTS

Both predator species, *D. abdominale* and *P. megacephala*, showed equal distributions when offered clean air vs. clean air and when offered fermented pseudostem tissue vs. fermented pseudostem tissue ($P > 0.05$) (Table 1). There were fewer nonresponders for *P. megacephala* (22%) than *D. abdominale* (40%) in the olfactometer apparatus used. The data indicate that the apparatus has no symmetrical bias and can be used for evaluating responses of these predators to banana pseudostem tissue and the pheromone.

TABLE 1. NUMBER OF PREDATORS RESPONDING TO CLEAN AIR AND FERMENTED BANANA PSEUDOSTEM ODORS IN A TWO-CHOICE OLFACTOMETER ASSAY IN THE LABORATORY

	Dactylosternum abdominale			*Pheidole megacephala*		
Comparison odor sources (A/B)	A	B	No response	A	B	No response
Clean air/clean air	10	9	11	12	11	7
Fermented/fermented tissue	9	8	13	11	13	6

A total number of 30 individual predators were tested per comparison set. The responses of the predators to the two odor sources did not differ significantly in either experiment ($P > 0.05$, χ^2 test).

Both *D. abdominale* and *P. megacephala* preferred fermented pseudostem tissue over clean air ($P < 0.001$ and $P < 0.05$, respectively). Neither predator species discriminated between fresh pseudostem tissue and clean air (Figure 2). In a direct comparison, more beetles and ants chose the fermented rather than the fresh pseudostem tissue, but this was only statistically significant for the ant *P. megacephala* ($P < 0.05$). Significantly more beetles moved to the side of the olfactometer with the pheromone ($P < 0.01$) than to the one with clean air, whereas the ants were not attracted to the weevil's pheromone.

The response of both *D. abdominale* and *P. megacephala* to fermented pseudostem tissue was dose-dependent. At all doses, the number of *D. abdominale* and *P. megacephala* choosing the side of the olfactometer with

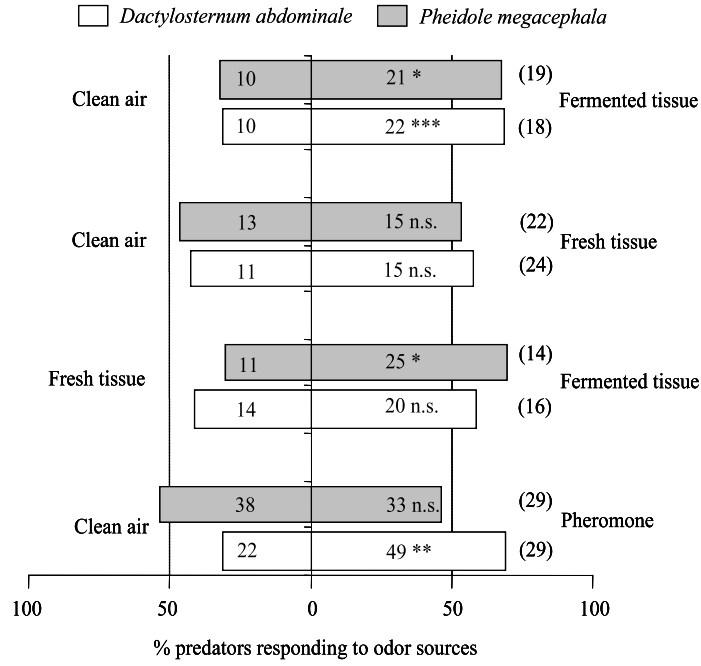

FIG. 2. Response of the banana weevil predators *Dactylosternum abdominale* and *Pheidole megacephala* to volatiles from banana pseudostem tissue and the pheromone in an olfactometer: percentage of responding individuals of each predator per comparison set, choosing one odor source or the other. Significantly different values are indicated with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$; n.s. = nonsignificant at $P > 0.05$, χ^2 test. The total number of individuals tested per odor set was 50, except for pheromone vs. clean air when 100 individuals were tested for response. The nonresponding predators are indicated in brackets at the right of bars.

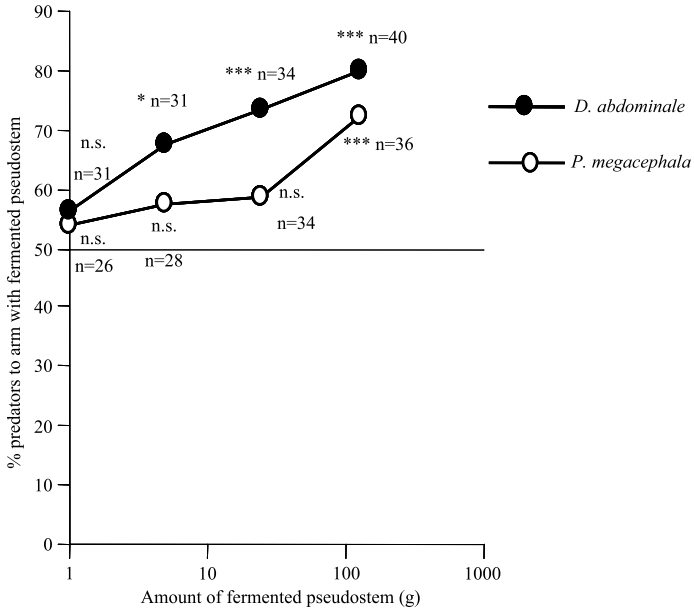

FIG. 3. Dose response of the predators *D. abdominale* and *P. megacephala* to volatiles from fermented banana pseudostem tissue in an olfactometer when the alternative was clean air. *$P < 0.05$, ***$P < 0.001$, n.s. = nonsignificant ($P > 0.05$) (χ^2 test, N indicates total number of responding predators out of 50 individuals tested per dose).

the fermented banana tissue was higher than the number choosing the side with clean air (Figure 3), even when small amounts were used (down to 1 g). However, only the following responses were significant: more *D. abdominale* chose for fermented pseudostem tissue when 5, 25, and 125 g were used than for clean air, and more *P. megacephala* chose for the pseudostem tissue when 125 g was used compared to clean air. Both *D. abdominale* and *P. megacephala* also significantly preferred 50 g of fermented tissue over clean air (Figure 2).

The presence of weevil larvae did not influence the predators' responses to pseudostem tissue. More *D. abdominale* chose fermented pseudostem tissue with or without feeding larvae present than clean air ($P < 0.05$) (Figure 4). There was no significant ($P > 0.05$) difference between the numbers of beetles choosing larvae vs. clean air, fermented pseudostem with larvae vs. larvae only, and fermented pseudostem tissue with larvae either present or absent. The effect of the pheromone when tested in the presence of fermented pseudostem tissue (without larvae) vs. the fermented tissue alone was not significant ($P > 0.05$).

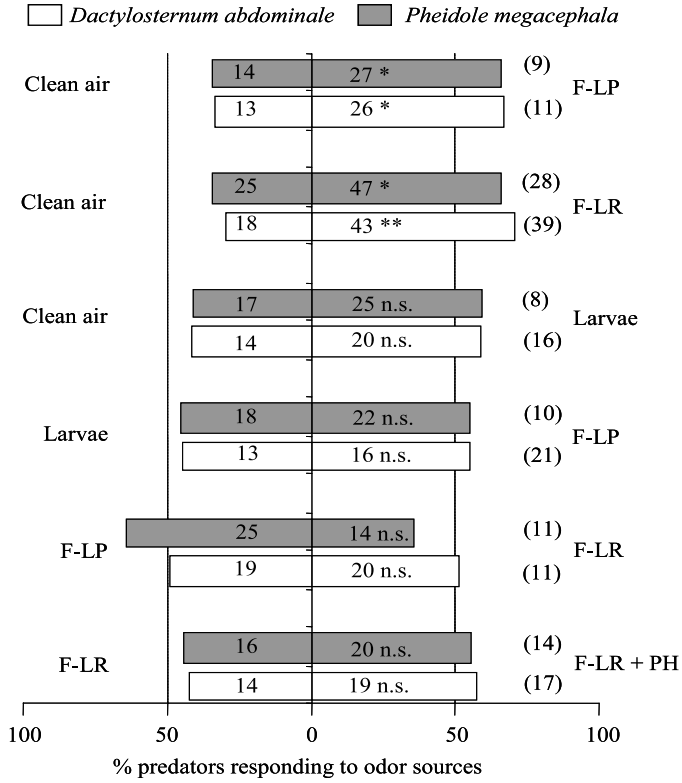

FIG. 4. Response of predators in an olfactometer to fermented tissue with (F-LP) or without (F-LR) feeding weevil larvae and in combination with the pheromone (PH): percentage of responding individuals of each predator per comparison set, choosing one odor source or the other. Significantly different values are indicated with $*P < 0.05$ and $**P < 0.01$; n.s. = nonsignificant at $P > 0.05$, χ^2 test. The total number of individuals tested per odor set was 50, except for F-LR vs. clean air when 100 individuals were tested for response. Nonresponding predators are indicated in brackets at the right of bars.

More *P. megacephala* ants chose the side of the olfactometer with fermented pseudostem with larvae present or absent than the side with clean air ($P < 0.05$). There was no significant difference in the responses between the other odor sources tested.

The presence of pheromone traps had no effect on predator distributions in the field. The numbers of *D. abdominale* and *P. megacephala* that were recovered on the mats where pheromone traps were present compared to those that

TABLE 2. MEAN NUMBER (±S.E.) OF PREDATORS AND *C. sordidus* RECOVERED FROM PSEUDOSTEM PIECES PLACED AT DIFFERENT DISTANCES FROM THE PHEROMONE-BAITED TRAP IN BANANA PLOTS AT KARI, UGANDA

Predators and *C. sordidus*	Number of insects recovered from different distances (m)		
	0	0.1–5	5.1–10
Labia spp. (Dermaptera: Labiidae)	2.0 ± 0.9a	0.8 ± 0.3a	1.3 ± 0.6a
D. abdominale (Coleoptera: Hydrophilidae)	4.8 ± 1.0a	3.2 ± 0.9a	3.3 ± 1.0a
P. megacephala (Hymenoptera: Formicidae)	2.0 ± 1.2a	0.3 ± 0.3a	0.6 ± 0.3a
Banana weevil larvae, *C. sordidus*	3.8 ± 2.0a	1.1 ± 0.4a	1.1 ± 0.7a
Adult banana weevils, *C. sordidus*	21.5 ± 6.3a	7.1 ± 2.3b	6.5 ± 1.2b

In each of the six plots, there were 10 pseudostem traps at each of the points per distance range. Means for each predator and *C. sordidus* followed by similar letter in a row are not significantly different ($P < 0.05$, SNK).

were recovered from mats less or more than 5 m from the trap were similar (Table 2). Numbers of adult weevils were significantly higher at the pheromone trap mat than on mats less than 5 m and in the range of 5.1–10 m from the trap. The numbers of weevil larvae recovered at all distances from the pheromone trap were similar.

The ants *P. megacephala* were the only predators that were captured in the pheromone-baited traps in the field. The mean number of weevils and *P. megacephala* captured in pheromone traps was 2.4 (±0.4 S.E.) and 4.2 (±1.2 S.E.) per plot per 3 d, respectively. There was no significant relationship between the numbers of *P. megacephala* and the number of weevils caught in pheromone-baited traps ($r^2 = 0.04$, $P = 0.61$).

DISCUSSION

Predators of the banana weevil are often observed in decomposing banana tissue such as harvested stumps, and often in tunnels where banana weevil eggs, larvae, and pupae are normally found (Koppenhofer, 1993). The results of our olfactometer experiments demonstrate that the predators *D. abdominale* and *P. megacephala* respond to volatiles from fermented banana pseudostem tissue. Attraction to the food of its host was similarly reported for several natural enemy species, such as *Leptopilina* parasitoids (Hymenoptera: Eucoilidae) (Vet, 1985; Vet and Van Opzeeland, 1985), *Anthocoris nemorum* (Heteroptera: Anthocoridae) (Dwumfour, 1992), *Orius tristicolor* (Hemiptera: Anthocoridae)

(Van Laerhoven et al., 2000), and *Chrysoperla carnea* (Neuroptera: Chrysopidae) (Reddy et al., 2002).

Many predator species are known to discriminate between volatiles from herbivore-damaged and undamaged plants (Geervliet et al., 1994; Dicke, 1999). Damaged plants become more attractive soon after the herbivores start feeding on them (Dicke et al., 1990, Turlings et al., 1990; Vet and Dicke, 1992; Geervliet et al., 1994). In our study, the predators did not discriminate between volatiles from larva-damaged pseudostem tissue with or without the larvae present. In addition, neither predator species discriminated between volatiles from weevil larvae and clean air. Similar data have been reported for other tritrophic systems as well (e.g., Turlings et al., 1990; Vet and Dicke, 1992; Geervliet et al., 1994), including a system consisting of fermenting substrates, a fungivore, and its parasitoid (Dicke et al., 1984). Fermented pseudostems that had not been damaged by weevil larvae were also attractive to the predators, which has also been recorded for parasitoids of fungivores (Dicke et al., 1984; Vet, 1985). Stimuli originating from the host habitat may influence host habitat location, although volatile stimuli originating from the host are more reliable (Vet and Dicke, 1992). Our results indicate that the predators *D. abdominale* and *P. megacephala* exploit volatiles from fermented pseudostem tissue, and that prey-related odors do not play a role in prey location.

Several species of natural enemies have been reported to use the aggregation pheromones of their hosts during host searching and location (Aldrich et al., 1984; Wiskerke et al., 1993; Hedlund et al., 1996; Bruni et al., 2000; Reddy et al., 2002; Wertheim et al., 2003, 2005). In our study, the predatory beetle *D. abdominale* was observed to respond significantly to the pheromone compared to clean air in the laboratory. Contrary to what was expected, the pheromone did not enhance the response of *D. abdominale* to volatiles from weevil-damaged pseudostem tissue in the laboratory. The ant *P. megacephala* was not attracted to the banana weevil's aggregation pheromone in the laboratory.

Our field data indicate that banana weevil aggregation pheromone has no effect on the predator distribution around the trap. Although volatiles from the herbivore itself would provide reliable information to the predator (Vet and Dicke, 1992; Wiskerke et al., 1993), the distribution of both predator species in the field was not related to the number of adult weevils captured in pheromone traps indicating that the aggregation pheromone released by male *C. sordidus* is not used by the predators in the field. In addition to the lack of response to the aggregation pheromone, generalist predators such as formicine ants have not been reported to use prey-derived chemicals as kairomones during foraging (Cosens and Toussaint, 1985). In contrast, several other predator species have been reported to use pheromones of their prey during prey searching and location (Dwumfour, 1992; Hedlund et al., 1996; Haberkern and Raffa, 2003; Steidle and van Loon, 2003).

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A NOVEL LONG-CHAINED ACETATE IN THE DEFENSIVE SECRETION OF THRIPS

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Abstract—Defensive secretions of adult and larval *Suocerathrips linguis* (Phlaeothripidae, Thysanoptera) were found to contain a long-chained acetate, (11*Z*)-11,19-eicosadienyl acetate, that was not previously known to occur naturally. This substance occurred together with octadecyl acetate and other long-chained acetates. The eicosadienyl acetate repels ants and spreads on the surface of such potential predators. The mixture can provide a long-lasting surface coating.

Key Words—*Suocerathrips linguis*, Thysanoptera, Phlaeothripidae, defensive secretion, repellent, octadecyl acetate, (11*Z*)-11,19-eicosadienyl acetate, GC-MS.

INTRODUCTION

Defensive secretions with low volatility produced by *Suocerathrips linguis* Mound and Marullo (Thysanoptera, Phlaeothripidae) were investigated in this study. The production of volatile substances by a species of Thysanoptera was noticed long ago by Hodson (1935) as "a pungent and distinctive but not unpleasant odour." During the last three decades, there have been more than 30 papers dealing with secretions of thrips. Several thrips species, when they are attacked by an enemy, raise and lower their abdomen with a drop of a fluid on the abdominal tip (Lewis, 1973). The secretory reservoir seems to be the hindgut

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(Howard et al., 1983). Some of the complete secretions, as well as single components, e.g., lactones such as mellein, have been found to be ant repellents (Howard et al., 1983; Blum, 1991; Blum et al., 1992). They may also function as contact irritants, alarm pheromones, or in some cases fumigants.

S. linguis was described from specimens occurring in the Royal Botanical Garden Kew, London, UK, where it was living on cultivated *Sansevieria* plants (Mound and Marullo, 1994). Despite being restricted to members of this genus, *S. linguis* feeds on fungi growing on the surface of these plants (Moritz, 2002). *Sansevieria* is an endemic Afrotropical genus, and presumably *S. linguis* is also African in origin. Like many other Phlaeothripidae, *S. linguis* shows aggregation and subsocial behavior (Moritz, 2002; Moritz et al., 2003).

METHODS AND MATERIALS

Rearing and Sample Collection. S. linguis was reared on *Sansevieria trifasciata* under a constant light regime of 16:8-hr light/dark (light on at 6:00 am), a temperature of around 23°C, and a relative humidity of 80%. Thrips were stimulated mechanically to produce droplets, and these were collected on the inner wall of a fine capillary glass tube (Minicaps 5 µl, Hirschmann Laborgeräte). The tube was then rinsed with the following solvents: distilled methanol (Aldrich, 49,429-1), hexane (Fluka, 52767), and, for preparation of derivatives, carbon disulfide (Aldrich, 18,017-3). For GC-MS, droplets were collected from 10 to 20 thrips and investigated within 2 hr. For derivative reactions, droplets from 50 thrips were used. For comparison and to examine differences between males and females, whole-body extracts were obtained by soaking 10–20 adults in methanol or hexane for 2 hr. From second instars, 20 droplets were collected and dissolved in methanol.

Chemical Analysis. For GC-MS analyses, samples were dissolved in a small amount (50–100 µl) of methanol or hexane, and then 5 µl were injected into an HP 5890 GC equipped with 5972 MSD and an HP5-MS column (30 m $\times$ 0.25 mm ID; stationary phase: 0.25 µm cross-linked 5% phenyl methyl silicone) that used electron impact ionization. The carrier gas was helium at a flow rate of 1 ml/min, the injector temperature was set to 300°C, and the detector temperature to 280°C. Column temperature was maintained at 100°C following the injection and then linearly increased to 300°C at a rate of 7K/min. The NIST database, literature data, and the software AMDIS (NIST, version 2.1) and NIST MS Search (version 1.7) were used to analyze mass spectra and to compare retention times.

To determine position of double-bonds, samples were treated with dimethyl disulfide (DMDS, Aldrich, 52,801-3) as follows. They were dissolved in 50 µl carbon disulfide for 10 min, then 10 µl of a 5% iodine solution (in CS_2)

and 60 µl DMDS were added and mixed together with the help of a microliter syringe. After 10 hr at 40°C, the reaction was quenched by the addition of 50-µl 5% solution of sodium thiosulfate (Aldrich, 21,726-3) in H_2O. The organic phase was evaporated on a slide, and the solid residue was dissolved in methanol and investigated as described above. Pure solvent without thrips secretion was treated and checked for impurities. The resulting mass spectra of the derivatives were evaluated by self-written software ACE_FIND, based on the findings of Buser et al. (1983) and Vincenti et al. (1987). The software calculates the diagnostic mass peaks of DMDS derivatives of aliphatic acetates in a selected chain length range with all possible positions of two double-bonds. It considers aliphatic derivatives, cyclic derivatives, and special properties in conjugated double-bonds as well and compares the resulting mass peaks with up to 16 input values. The output is a list of substances sorted by the number of mass peaks that fit the input data.

Bioassay. For the first preliminary bioassay tests, two colonies of *Myrmica rubra* (Linnaeus, 1758) (about 25 individuals each, obtained from Antstore, Berlin) were reared separately in glass tanks $0.2 \times 0.3 \times 0.2$ m^3, filled to a depth of 50 mm with garden soil. In the test, ants had to choose between two pieces of food (ca. 75 mg of turkey meat). The pieces were placed on a sheet of filter paper at a distance of 50 mm, and a circle (radius 20 mm) was drawn in pencil around each. The edge of one circle was soaked with 0.5 µl of test substance dissolved in 50 µl methanol, and the second was treated only with 50 µl methanol as a control. The sheet of filter paper was placed on a glass slide in the tank to prevent contamination from the soil. The number of ants inside the circles was recorded every 10 min until the food was consumed. Test and control were not replaced during each test.

RESULTS

The main components of the droplets were long-chained acetic acid esters (acetates) of one saturated (C18) (Figure 1, peak at 17.53 min retention time) and several unsaturated alcohols (C16, . . . , C20) (other peaks from 17.11 to 19.82 min retention time). These constituents have a low volatility. In all cases, it was possible to find the peak for the molecular (M^+) ion, as well as a fragment at m/z 61 (CH_3CHOOH) and a fragment M^+-60 because of McLafferty rearrangement in acetates.

No differences were detected between males, females, and second instar larvae. The secretion does not contain any compound from the fungi on which the thrips feed, nor from the host plant (*Sansevieria*). Sex-specific concentrations of substances with high volatility were found only if the whole thrips were extracted (Tschuch et al., 2002). Thus, the thrips must have additional

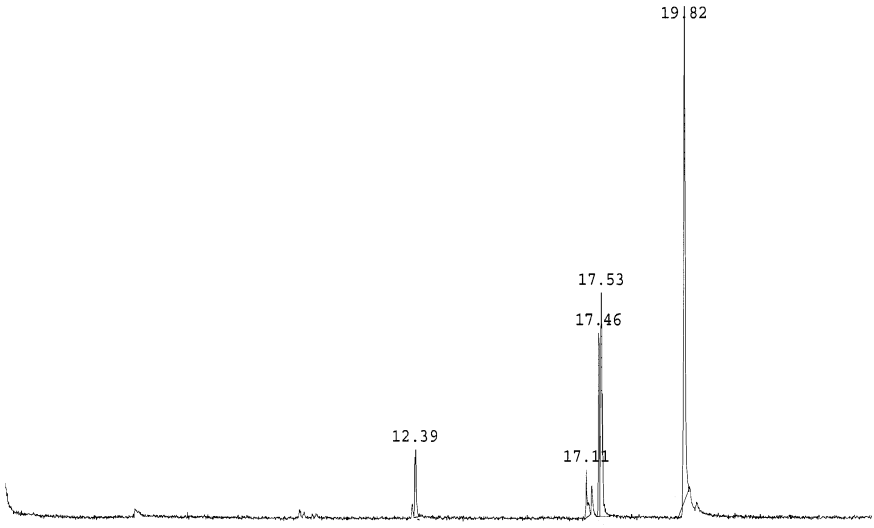

FIG. 1. Total ion gas chromatogram of 20 secretion droplets (numbers are retention times in minutes).

secretory glands to produce volatile semiochemicals, although these are not the topic of this paper.

The molecular (M^+) ion of the main component is 336. A fragment at *m*/*z* 61 ($CH_3COOH+H$) and a small fragment at *m*/*z* 276 suggested that this compound was an eicosadienyl acetate (C20:2-OAc). The other mass peaks were similar to some octadecadienyl acetates (C18:2-OAc, library data). In the DMDS derivatives of the secretion, one substance had a characteristic mass spectrum with *m*/*z* 61 as the highest peak (spectrum measured from *m*/*z* 50). There are only four peaks >5% above *m*/*z* 120: 121, 169, 217, and 259. With software, all derivatives of acetates between C18:2 and C22:2 were investigated. The only DMDS derivatives fitting all four peaks according to Vincenti et al. (1987) were C20:2-OAc with one double-bond at position 11 and the other double-bond at position 17, 18, or 19. The 11,17-isomer must have a prominent peak at *m*/*z* 89, the 11,18-isomer at 75, and the 11,19-isomer at 61. *m*/*z* 89 and 75 could not be found as high peaks, so the result must be 11, 19-C20:2-OAc. The mass peaks are as follows: 61 $[c]^+$ together with the 61 from CH_3CHOOH (therefore, this *m*/*z* has the highest abundance), 121 $[bc\text{-}3*CH_3SH]^+$, 169 $[bc\text{-}2*CH_3SH]^+$, 217 $[bc\text{-}CH_3SH]^+$, and 259 $[a]^+$ (where a = 259 is the part from the acetic acid to former double-bond at position 11, b = 204 is the part between the former two double-bonds, and c = 61 is the remaining part). Small peaks are also visible at 403 and 429 corresponding to

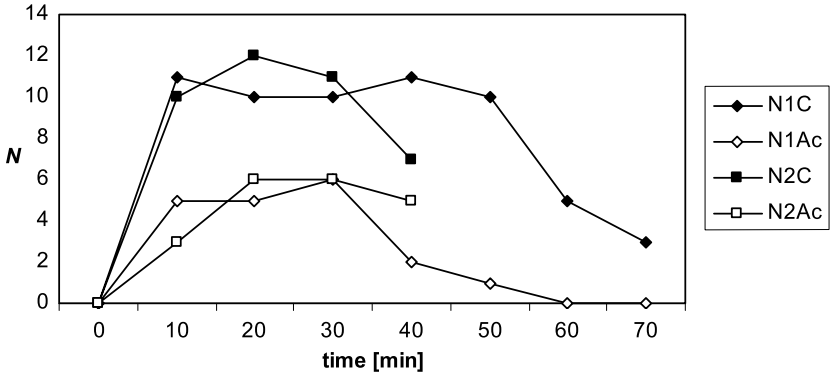

FIG. 2. Number of *Myrmica rubra* ants choosing between two pieces of food, each surrounded by a ring. One ring was treated with (11*Z*)-11,19-eicosadienyl acetate solved in methanol (Ac), and the second only with methanol (C). N1 and N2 are the nests 1 and 2; *N* is the number of ants inside the ring.

$[ab\text{-}CH_3CHOOH]^+$ and $[M\text{-}2*CH_3SH]^+$. Thus, the main component is an 11,19-C20-OAc.

The identified constituents were compared with authentic substances: octadecyl acetate was bought (Sigma, S-5003 stearyl acetate), and (11*Z*)-11,19-eicosadienyl acetate was synthesized as described in Csuk et al. (2004) because (*E*) isomers are seldom found in insect allomones or pheromones (with the exception of Lepidoptera sex pheromones). Mixtures of authentic substances with *S. linguis* secretion were analyzed by GC-MS and yielded only a single peak. Furthermore, the mass spectra of the constituents and authentic compounds matched closely.

Preliminary bioassay tests showed that the (11*Z*)-C11,19-OAc alone is an effective repellent against *M. rubra* (Figure 2). For 1 hr, the number of ants inside the acetate ring was at all times lower than inside the control ring. The food in the control ring was removed by the ants in small portions, whereas the food in the acetate ring was left more or less untouched. The test with nest 2 (N2) ended after 40 min because the food in the control (C) was consumed. Only two tests could take place because of the limited amount of substance synthesized.

DISCUSSION

The main constituent of the anal secretion of *S. linguis* adults and larvae, (11*Z*)-11,19-eicosadienyl acetate (C20:2-OAc), is a new natural product not

previously reported in animals or plants. Secretions of Thysanoptera species consist of different chemical constituents, such as aliphatic hydrocarbons, carboxylic acids, aliphatic esters, cyclic esters of hydroxyl carboxylic acids (lactones), pyranones, naphthoquinones, and terpenes (Terry, 1997). In this discussion, only aliphatic esters of acetic acid, found in 30% of the investigated species, are the focus of attention. The aliphatic ester with the longest chain known previously from Thysanoptera is octadecyl acetate (C18:0-OAc) from *Gynaikothrips uzeli* (Suzuki et al., 1989: only 2.2% of secretion). Hexadecyl acetate (C16:0-OAc) is reported from *Gynaikothrips ficorum* (Howard et al., 1987: in 1:1 mixture with pentadecane one of the main constituents), from *G. uzeli* (Suzuki et al., 1989: in 1:2 mixture with pentadecane one of the main constituents), from *Leeuwenia* (=*Varshneyia*) *pasaniae*, and from *Liothrips kuwanai* (Suzuki et al., 1988: only 3.0 and 4.7%). A small part of the *Li. kuwanai* secretion is probably hexadecenyl acetate (C16:1-OAc, double-bond position unknown) (Suzuki et al., 1988: 1.2%). The secretion of *L. pasaniae* contains mainly tetradecyl acetate (C14:0-OAc: 15.4%), tridecane (51.3%), and pentadecane (12.4%). In contrast to these species of Tubulifera, the only species of Terebrantia from which chemicals have been identified so far, *Frankliniella occidentalis*, produce a mixture of dodecyl (C12:0-OAc) and decyl acetate (C10:0-OAc) in the anal droplets of second instars, although only in traces in secretions by adults (Teerling et al., 1993a,b; Teerling, 1995; MacDonald et al., 2003). Tubulifera with gregarious and commonly social or gall-dwelling lifestyles may be more prolific producers of defensive secretions than solitary Tubulifera or Terebrantia. To maintain safety within a colony, a strong repellent allomone or alarm pheromone would be effective (Terry, 1997). In solitary Thysanoptera, defensive secretion is more important in larvae because adults are able to fly away if predators are near.

Most species of Tubulifera discharge the fluid from the apex of their tube. This allows them to apply the droplet to a predator, but to resorb the droplet if the application fails (Howard et al., 1987). For this reason, the secretion must be liquid down to the lowest temperature at which predators are active. If the secretion is to disable or irritate potential predators by coating them with the liquid, then a secretion with a low vapor pressure at all possible ambient temperatures would be best. This is necessary to avoid fast evaporation. To fulfill both requirements, the mixture should have a melting point (mp) near 0°C and a boiling point as high as possible. If one of the main constituents is an aliphatic acetate (discussed below), then there are several possible solutions: saturated acetates C12:0-OAc (mp 1°C) or C10:0-OAc (mp −15°C) singly or mixed; longer-chained saturated acetates (e.g., C16:0-OAc, mp 24°C) dissolved in a liquid hydrocarbon or other solvent; and unsaturated acetates with melting points near 0°C.

The first of these is used by larvae of *F. occidentalis*, and the second by the two species of *Gynaikothrips* mentioned above. Pentadecane has a melting point

of 10°C. The mixture has a melting point below 10°C because the acetate acts as an impurity of the pentadecane.

The third of these methods is used by *S. linguis*. Because of the lack of experimental data, estimations of melting points are necessary. Unfortunately, software such as MPBPWIN (version 1.41, part of EPI Suite from United States Environmental Protection Agency) is not suitable. For example, it estimates a melting point between 76 and 102°C for eicosyl acetate, whereas the measured melting point is approximately 41°C. Furthermore, this and other software that use the Joback algorithm, the Gold & Ogle algorithm, or similar methods do not consider the position of double-bonds or the stereochemistry. Kobayashi et al. (1978) found the following data for the 11-eicosenyl acetates (C20:1-OAc) and alcohols: (*Z*)-11-eicosenol (mp 26°C), (*E*)-11-eicosenol (mp 44°C), (*Z*)-11-eicosenyl acetate (liquid), and (*E*)-11-eicosenyl acetate (mp 20°C). The saturated eicosyl acetate has a melting point of 41°C. From data in Beilstein database (BS0402AE, Beilstein GmbH and MDL Information Systems GmbH 2004), it appears that acetates melt 26K (±3K) below the corresponding alcohol (data from nine pairs between C12:0 and C22:1). So the melting point of the (*Z*)-11-eicosenyl acetate can be estimated as below 0K. The change from single bond to ally group at the end of the alcohol lowers the melting point of fatty alcohols by 0–6K only (Duhamel, 1963; Stubbs and Smith, 1984). Thus, with an error of ±10K, it is possible to estimate the melting point of (11*Z*)-11,19-C20:2-OAc to be below −3°C and the melting point of (*E*)-11,19-C20:2-OAc to be around 17°C. The estimation for the *Z* isomer seems to be good because the synthesized reference substance remains liquid at 0°C in a refrigerator. As a single substance, the *Z* isomers of 11-C20:1-OAc and 11,19-C20:2-OAc acetate possess an appropriate melting point (around or below 0°C), but the *E* isomers do not.

The vapor pressure of 11,19-C20:2-OAc is lower than the vapor pressure of C10:0-OAc or C12:0-OAc because the boiling point rises with increasing chain length. In addition, saturated acetates possess lower boiling points than unsaturated acetates. In the secretion of *S. linguis*, the solid C18:0-OAc (mp 32°C) is dissolved in the main constituent, which leads to an additional vapor pressure lowering according to Raoult's law. If a potential predator is coated by such a mixture, the C18:0-OAc will remain on the surface after the unsaturated acetate has evaporated. However, it is also conceivable that modifications occur in the unsaturated acetate under the influence of oxygen and ultraviolet light [Panades et al., 1998: (7*E*,9*Z*)-7,9-C12:2-OAc] or ozone [Arndt et al., 1996: (*Z*)-11-C18:1-OAc and (*Z*)-9-C14:1-OAc]. The reaction products, e.g., epoxides and oligomers, are solid and would also stay for a longer time on the predator.

Acetates with 18, 20, and more C-atoms are well-known sex pheromones in Lepidoptera. In *Drosophila* species, (*Z*)-11-C18:1-OAc and (*Z*)-11-C20:

1-OAc act as aggregation pheromones. Acetates with defensive functions can be found in secretions of Coleoptera and Hymenoptera (Wheeler and Duffield, 1988). In Hymenoptera, larvae of a tenthridinid sawfly (Jonsson et al., 1988) and adults of social parasitic bumblebees (Zimma et al., 2003) use acetates such as C18:0-OAc in their defensive secretions. In the parasitic bumblebees, the main component C12:0-OAc is an effective repellent against host bumblebees. (*Z*)-11-C20:1-OAc can be found in the secretion of some chrysomelid beetles: in larvae of *Linaeidea aenea* [mixed with (*Z*)-9-C18:1-OAc and C16:0-OAc] (Sugawara et al., 1979), in adults of *Gastrophysa viridula* [mixed with (*Z*)-13-C22:1-OAc] (Eggenberger et al., 1994), and in *Gastrophysa atroceanea* [mixed with C18:0-OAc] (Sugawara et al., 1978). The last secretion was effective against *Lasius niger* ants and is similar to the secretion of *S. linguis* except that it has no allyl group at the end of the alcohol. There is thus strong evidence for long-chained acetates acting as repellents against predators, especially predatory ants.

Some other findings are also relevant. In a primitive Australian ant of the genus *Myrmecia*, (*Z*)-11-C20:1-OAc serves, together with (*Z*)-9-C18:1-OAc and some minor constituents, as an alarm pheromone produced by their Dufour gland (Jackson et al., 1989). C16:0-, C18:0-, C18:1 (9*Z*), and C20:0-OAc were found in the ovarioles of the weevil *Hylobius abietis* (Kalo, 1985; Kalo and Nederstrom, 1986), and the acetates may be useful in protecting the eggs against predators. An eicosadienal (double-bond positions unknown) has been described by Attygalle et al. (1998) as a trace component in the trail pheromone of the ant *Dolichoderus thoracicus*.

In Thysanoptera, Howard et al. (1987) tested the major constituents of *G. ficorum* secretions, C16:0-OAc and pentadecane, individually and in combination, and found that they were defensive allomones for predatory ants. C16:0-OAc individually is a better repellent than pentadecane, but a 1:1 mixture is more effective. They pointed out that the range of repellency is apparently short but effective. *G. ficorum* can disable potential predators by coating them with the irritating anal exudates.

Thus, many long-chained acetates serve as repellents, but why did they evolve to be repellents? Ester cocktails similar to the *S. linguis* defensive secretion are probably optimized to coat predators, especially jamming the chemosensory receptors. Acetates from C14:0 to C18:0 alcohols are known as water-insoluble surfactants able to spread quickly on surfaces (Karkare et al., 1993). In predators such as ants and mites that use mainly the olfactory sense, coating the sensory organs would make the animals "blind." They would not be able to find the prey again, and ants would have difficulty evaluating their own pheromones, such as trail pheromones to return to the nest and inform nest mates about potential prey. Further studies are needed to prove this and to find out why, in contrast to other insects, the allyl group is necessary in the main constituent of *S. linguis* secretions.

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PHYTOTOXIC AND ANTIFUNGAL COMPOUNDS FROM TWO *Apiaceae* SPECIES, *Lomatium californicum* AND *Ligusticum hultenii*, RICH SOURCES OF Z-LIGUSTILIDE AND APIOL, RESPECTIVELY

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Abstract—The seeds of two *Apiaceae* species, *Ligusticum hultenii* and *Lomatium californicum*, were investigated. Preliminary bioassays indicated that methylene chloride extracts of seeds of both species contained selective phytotoxic activity against monocots and antifungal activity against *Colletotrichum fragariae*. Active constituents were isolated by bioassay-guided fractionation, and the structures were elucidated by NMR and GC-MS as apiol and Z-ligustilide, isolated from *L. hultenii* and *L. californicum*, respectively. Apiol and Z-ligustilide had I_{50} values of about 80 and 600 μM, respectively, for inhibition of the growth of *Lemna paucicostata*. The methylene chloride (CH_2Cl_2) extracts of the seeds and the isolated and purified compounds were tested against the 2-methylisoborneol-producing cyanobacterium (blue-green alga) *Oscillatoria perornata*, and the green alga *Selenastrum capricornutum*. The CH_2Cl_2 extracts of both *Apiaceae* species and apiol were weakly toxic to both species of phytoplankton, while Z-ligustilide was toxic to both with a lowest complete inhibitory concentration (LCIC) of 53 μM. Seeds of *L. californicum* and *L. hultenii* were found to be rich sources of Z-ligustilide (97 mg/g of dry seed) and apiol (40 mg/g of dry seed), respectively.

Key Words—*Ligusticum hultenii*, *Lomatium californicum*, *Apiaceae*, Z-ligustilide, apiol, phytotoxic activity, antifungal activity, *Colletotrichum fragariae.*

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INTRODUCTION

The seeds of two *Apiaceae* species, *Lomatium californicum* (Torrey & A. Gray) Mathias & Constance, and *Ligusticum Hultenii* (Fernald) (Calder & Taylor), were investigated for their phytotoxic and antifungal activities. *Lomatium californicum*, also known as *California lomatium* or celery weed, is a perennial herb native to California and can be found in the southern part of Oregon on bushy slopes. *Ligusticum hultenii* is a perennial, common in the northwestern United States, particularly in Alaska. Seeds of both species have a distinct, strong aromatic odor similar to dill seeds. These two species have been previously categorized in the Umbelliferae family (renamed *Apiaceae*), but were recently reclassified in the subfamily Apioideae of the *Apiaceae* family (Watson, 2000). *Apiaceae* species possess compounds with many types of biological activities, such as apoptosis inducers, antibacterial, hepatoprotective, vasorelaxant, cyclooxygenase inhibitory, and antitumor compounds (Okuyama et al., 1991; Gonzalez et al., 1995; Liu et al., 1998; Matsuda et al., 2000; Ye et al., 2001; Pae et al., 2002). We reasoned that these species might have compounds with potential as pest management chemicals.

METHODS AND MATERIALS

Materials. Dried seeds of *L. hultenii*, and *L. californicum* were supplied by Aromagen (Albany, OR, USA). The plants were grown in Albany, OR, and the seeds were harvested in July 2002. All solvents were reagent grade and used without further purification. Extracts of the seeds were analyzed on silica gel thin-layer chromatography (TLC) plates (250 μm thickness, with fluorescent indicator; GF Uniplate Analtech, Newark, DE, USA) using 10% ethylacetate in hexane as the solvent system. Anisaldehyde spray reagent, iodine vapor, and UV light were used for the detection of compounds on the TLC plates. Column chromatography was carried out with kieselgel 60 (particle size 0.063–0.2 mm; Merck, Germany). Seeds of chewing fescue (*Festuca rubra* L., subsp. *commutata* Gaud.), lettuce (*Lactuca sativa* L., cv. Iceberg), and creeping bent grass (*Agrostis stolonifera* L., cv. Pencross) were purchased from Turf Management, Inc., Burpee Seeds, and Tee-2-Green Corp (USA), respectively.

Instrumentation. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer operating at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR. GC-MS analysis was carried out on an HP5790 MSD spectrometer (Hewlett Packard, USA) equipped with a GC 5890 using a 20 m × 0.2 mm i.d., 0.18 μm DB-1 column (J&W Scientific Inc., California, USA). The oven was temperature programmed from 60°C (5 min) to 280°C (20 min) at 5°C/min with helium as the carrier gas.

Extraction and Isolation of Bioactive Constituents. Dried seeds of *L. hultenii* (100 g) and *L. californicum* (100 g) were ground separately into a powder by macerating in a blender and then stirring the powdered seeds in CH_2Cl_2 (3 × 1 l each) for 1 hr at ambient temperature. The extracts were filtered through filter paper (Whatman #1), and the solvent was evaporated at 40°C to afford viscous oils (11.78 and 7.2 g, respectively).

*Isolation of Z-ligustilide (**1**).* The CH_2Cl_2 extract of *L. californicum* (5 g) was column chromatographed on silica gel (40 mm i.d., 200 mm length) using hexane and increasing amounts of CH_2Cl_2 (0–100%). Fractions of 125 ml were collected and concentrated at 40°C, and similar fractions according to TLC profiles were combined to yield eight fractions. Each fraction was tested with TLC bioautography and phytotoxicity bioassays (see below) to identify the bioactive fractions. Fractions that possessed antifungal activity and phytotoxicity, with similar TLC profiles, were pooled and further purified by silica gel column chromatography. The identity of the active compound eluted with 50% CH_2Cl_2 in hexane was confirmed by GC-MS and comparison of and ^1H and ^{13}C NMR data with those reported in the literature (Fischer and Gijbels, 1987; Miyazawa et al., 2004) as Z-ligustilide (**1**) (2.9 g).

*Isolation of Apiol (**2**).* The CH_2Cl_2 extract of *L. hultenii* (5 g) was column chromatographed on silica gel in a similar manner as that for the isolation of Z-ligustilide to afford 10 fractions from *L. hultenii*. Each fraction was tested on TLC bioautography and phytotoxicity bioassays to identify the bioactive fractions. Fractions that possessed antifungal activity and phytotoxicity with similar TLC profiles were pooled separately and further purified by silica gel column chromatography using ethyl acetate in hexane. The active compound was eluted with 20% ethyl acetate in hexane and was identified as apiol (**2**) (1.7 g) by GC-MS and comparison of ^1H and ^{13}C NMR data with those reported in the literature (Tyagi et al., 1993).

Phytotoxicity Assays. Phytotoxicity bioassays were carried out according to Dayan et al. (2000), using chewing fescue (*Festuca rubra* L., subsp. commutata Gaud.), bentgrass (*A. stolonifera*), and lettuce (*Lactuca sativa* cv L., Iceberg), in 24-well plates. The crude extracts and fractions were tested at 1 mg/ml in 10% acetone in water.

Phytotoxicity to *Lemna paucicostata* was determined according to the method of Michel et al. (2004). Plants were grown in non-pyrogenic polystyrene sterile 6-well plates (Costar 3506, Corning Incorporated) with a lid. Each well contained 4950 µl of the Hoagland's media plus 50 µl of apiol and Z-lugustilide in acetone, with the final concentration of acetone 1%. Each well was inoculated with two, three-frond colonies of approximately the same size. Total frond area per well was recorded by the image analysis system Scanalyser (LemnaTec, Würselen, Germany) once per day from d 0 to d 7. To test the potential influence of the acetone, an acetone control was included in each experiment.

Fungicidal Assays

Pathogen Production. Isolates of *Colletotrichum acutatum* Simmonds, *C. fragariae* Brooks, and *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. were obtained from B.J. Smith (USDA, ARS, Small Fruit Research Station, Poplarville, MS, USA). *Colletotrichum fragariae*, *C. acutatum*, and *C. gloeosporioides* were used for all pathogen and bioautography studies. The three *Colletotrichum* species were isolated from strawberry (*Fragaria* × *ananassa* Duchesne). *Botrytis cinerea* Pers.:Fr, was isolated from commercial grape (*Vitis vinifera* L.), and *Fusarium oxysporum* Schlechtend:Fr from orchid (*Cynoches* sp.) were used additionally in the microtiter assays of apiol and Z-ligustilide.

Bioautography. Bioautography on silica gel TLC plates with *Colletotrichum fragariae* was used as the preliminary bioassay to identify the antifungal activity according to a previously published method (Wedge and Nagle, 2000).

Microtiter Assay. A standardized 96-well microtiter plate assay developed for discovery of natural product fungicidal agents (Wedge and Kuhajek, 1998) was used to evaluate purified apiol and Z-ligustilide.

Algicidal Assays. Algicidal bioassays were carried out for CH_2Cl_2 extracts of *L. californicum* and *L. hultenii*, and the isolated, purified Z-ligustilide and apiol against the 2-methylisoborneol (MIB)-producing cyanobacterium *Oscillatoria perornata* and the green alga *Selenastrum capricornutum* according to previously published methods (Schrader et al., 1997) in order to determine the lowest observed effect concentration (LOEC) and the lowest complete inhibitiory concentration (LCIC).

Quantification of Active Components

Sample Preparation. For quantification, 5 g of dried seeds from each species were exhaustively extracted with CH_2Cl_2 (45 ml × 5) using a Dionex ASE 200 accelerated solvent extractor at 40°C and 68.9 bar pressure. Solvent in each sample was evaporated at 40°C under reduced pressure.

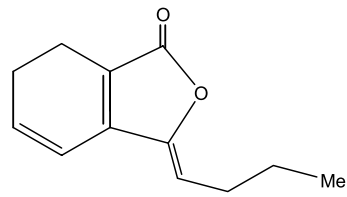
GC-MS Analysis. The concentrations of apiol and Z-ligustilide in the crude CH_2Cl_2 extracts were determined by GC-MS analysis. Standard curves were generated by linear regression, using pure apiol ($R^2 = 0.98$) and Z-ligustilide ($R^2 = 0.99$), which were isolated.

RESULTS AND DISCUSSION

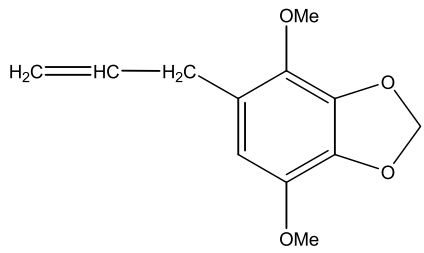
The CH_2Cl_2 extracts of *L. hultenii* and *L. californicum* on lettuce (dicot) and bentgrass (monocot) were selectively phytotoxic toward bentgrass at 1.0 mg/ml with a ranking of 5 in the scale of 0–5, where 0 indicated no effect and 5

indicated no germination. The *L. hultenii* extract gave a ranking of 1 on lettuce, whereas the *L. californicum* extract gave a ranking of 2. Through bioassay-guided fractionation, the active compounds were isolated and characterized by NMR and GC-MS as Z-ligustilide (**1**) from *L. californicum* and apiol (**2**) from *L. hultenii* (Figure 1). These two compounds, (**1**) and (**2**), were further tested in a dose-response manner, in the phytotoxicity bioassays (Table 1). Both compounds were much more toxic to the two monocots, bentgrass and fescue, than to lettuce.

Phytotoxicity was further evaluated in a *L. paucicostata* bioassay (Figure 2). Apiol caused about 75% reduction of growth of *L. paucicostata* at 80 μM, whereas Z-ligustilide caused similar effects at 1000 μM. At concentrations of 166 μM and above, apiol completely inhibited the growth of *L. paucicostata*. Apiol and Z-ligustilide had I_{50} values of about 80 and 600 μM, respectively, for inhibition of the growth of *Lemna paucicostata*. At doses of apiol between 25 and 50 μM, slight stimulation of the growth of *L. paucicostata* was observed. This type of stimulation by lower doses of phytotoxins (hormesis) occurs with some other phytotoxins (Schabenberger et al., 1999). The complete inhibition of *L. paucicostata* by Z-ligustilide was observed only at 1000 μM and above. These results suggest that apiol is a more potent growth inhibitor than Z-ligustilide. Aromatic compounds having allyl or isoallyl and methylenedioxy



Z-ligustilide (**1**)



Apiol (**2**)

FIG. 1. Structure of Z-ligustilide and apiol.

TABLE 1. EFFECTS OF Z-LIGUSTILIDE AND APIOL ON GROWTH OF THREE HIGHER PLANT SPECIES FOR 7 D

Compound	Concentration (μM)	Lettuce	Bentgrass	Fescue
Z-ligustilide	1	0	0	0
	2	0	0	0
	10	0	1	1
	33	0	2	2
	100	0	4	3
	333	1	4	4
	1000	1	4	4
Apiol	1	0	0	0
	2	0	0	0
	10	0	1	2
	33	1	1	3
	100	1	3	4
	333	1	4	4
	1000	1	5	5

0 = no effect compared to the control.
5 = complete mortality.

groups are plant growth inhibitors (Harada et al., 1985). Myristicin, apiol, and dillapiol inhibit growth of rice, affecting mostly the foliar part of the plants without inhibiting the growth of the roots (Harada et al., 1985).

Antifungal activity was observed using bioautography with *C. fragariae* (Figure 3). In order to obtain a quantitative evaluation of antifungal activity, a microtiter plate-based bioassay was carried out according to Wedge and Kuhajek (1998). Results indicated that both apiol and Z-ligustilide had antifungal activity at the concentrations that we tested (1, 10, and 100 μM). Z-Ligustilide and apiol showed the highest activity against *B. cinerea* (Figure 4) among all the pathogens tested. Benomyl at 1 μM was more active than at 100-μM concentrations of apiol and Z-ligustilide. Low antifungal activity was observed for apiol and Z-lingustilide against *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, and *F. oxysporium* in the microtiter assay (data not shown).

The cyanobacterium *O. perornata* is a pest in commercial catfish production ponds in the southeastern region of the USA. MIB is produced by *O. perornata* and accumulates in the flesh of pond-raised channel catfish (*Ictalurus punctatus*) causing a musty "off-flavor" that results in an unpalatable and unmarketable product. Green algae (Division Chlorophyta) such as *S. capricornutum* do not produce the off-flavor compounds commonly encountered in channel catfish aquaculture and are the preferred type of phytoplankton in catfish aquaculture ponds. Algicidal bioassay results indicated that CH_2Cl_2 extracts of both seeds had LOEC and LCIC values of 100 ppm and

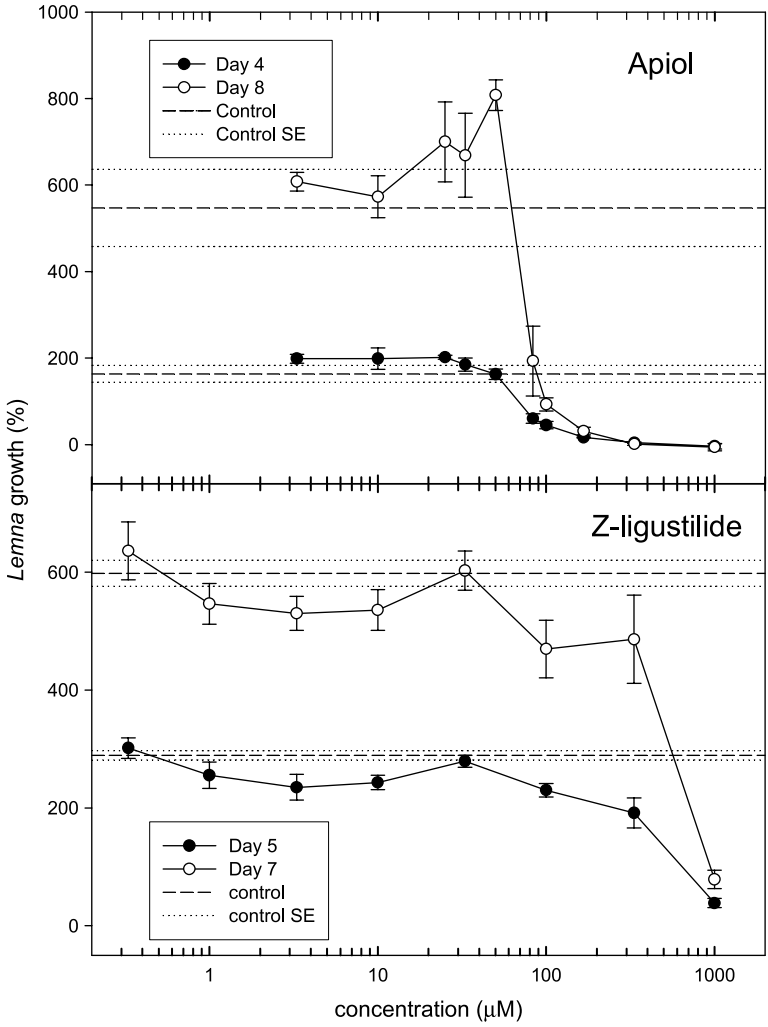


FIG. 2. Growth response of *L. paucicostata* at various concentrations of apiol and Z-ligustilide. Growth was monitored at d 4 and 8 after treatment for apiol and at d 5 and d 7 for Z-ligustilide.

higher (Table 2). Z-Ligustilide showed nonselective activity against *O. perornata* and the green alga *S. capricornutum* with LOEC and LCIC values of 53 μM (Table 2). Apiol showed no activity against either species of phytoplankton at the concentrations tested.

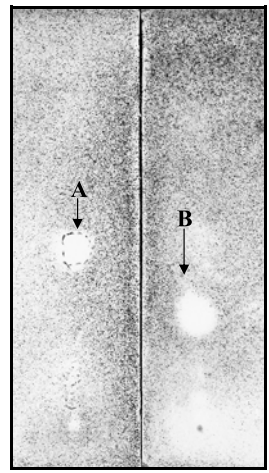


FIG. 3. Bioautography of apiol (A) and Z-ligustilide (B). The plate was sprayed with spores of *Colletotrichum fragariae*. Apiol and Z-ligustilide were chromatographed on silica gel TLC plate using 10% ethyl acetate in hexane.

Z-Ligustilide has been found previously in *Lomatium californicum* (Beauchamp et al., 1993), and is widely occurring with its *trans*-isomer in *Ligusticum* and *Lomatium* spp. (Kobayashi and Mitsuhashi, 1987; Bedrossian et al., 1998; Lu et al., 2004). Apiol can be found with ligustilides in other *Ligusticum* spp. (Brandt and Schultze, 1995).

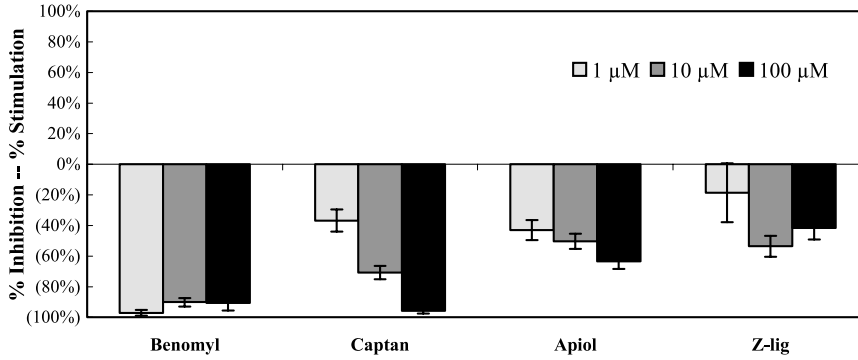


FIG. 4. Mean percent growth inhibition of *Botrytis cinerea* in response to 1, 10, and 100 μM concentrations of Z-ligustilide and apiol after 72 hr. Fungal growth showed that Z-ligustilide and apiol demonstrated less inhibition than the commercial fungicide standards benomyl and captan against *B. cinerea*.

TABLE 2. EFFECT OF CH_2CL_2 EXTRACTS OF *L. californicum* AND *L. hultenii* AND THE ISOLATED, PURIFIED Z-LIGUSTILIDE AND APIOL ON THE CYANOBACTERIUM *Oscillatoria perornata* AND *Selenastrum capricornutum*

	Test organism			
	Oscillatoria perornata		*Selenastrum capricornutum*	
Extract/compound	LOEC	LCIC	LOEC	LCIC
L. californicum	100 ppm	100 ppm	100 ppm	>100 ppm
Z-ligustilide	53 μM	53 μM	53 μM	53 μM
L. hultenii	100 ppm	>100 ppm	>100 ppm	>100 ppm
Apiol	>100 μM	>100 μM	>100 μM	>100 μM

LCIC: lowest complete inhibitory concentration.
LOEC: lowest observed effective concentration.

According to GC-MS analysis, the concentration of Z-ligustilide was 85% and that of apiol was 65% of the CH_2Cl_2 extracts of the seeds of *L. californicum* and *L. hultenii*, respectively (Figure 5). *L. californicum* and *L. hultenii* seeds were found to contain 97 mg of Z-ligustilide per g of dry seed and 40 mg of apiol per g of dry seed, respectively. These results indicated that both Z-ligustilide and apiol occur in high concentrations in these two seeds. The reason for the occurrence of these compounds in such high concentration is unclear. Insect antifeedant assays are ongoing for apiol and Z-ligustilide.

Z-Ligustilide is present in many other plants, and it is the active compound in the Chinese herbal drug *Angelica sinensis,* which is also known as Danggui or Female Gingseng (Zhao et al., 2003). This compound is chemically unstable at ambient temperature and will produce polymerized products when exposed to light and heat (Rios et al., 1998). The chemical instability is attributed by the presence of double bonds in the molecule that can undergo [2 + 2] Diels–Alder type cycloaddition, whereby one molecule serves as a diene and the other as a dienophile and resulting in insoluble complex molecules (Rios et al., 1998).

Our results show that apiol and Z-ligustilide are phytotoxic to the monocots tested in this study and are weakly antifungal. Apiol was not toxic to the MIB-producing cyanobacterium *O. perornata* and the green alga *S. capricornutum*, while Z-ligustilide was toxic, but not selective, to *O. perornata*. Z-Ligustilide is considered the active compound in the herbal drug *Angelica sinensis*, but there are no reports of the use of *L. californicum* as an herbal remedy. The possible uses of this plant or its seeds as herbal drugs as sources of Z-ligustilide are yet to be explored. Although the antimicrobial and phytotoxic activities are not sufficient for commercial use, these natural compounds could be used as templates to produce more active compounds. Furthermore, we have found Z-ligustilide to be a major compound in *L. californicum* seeds.

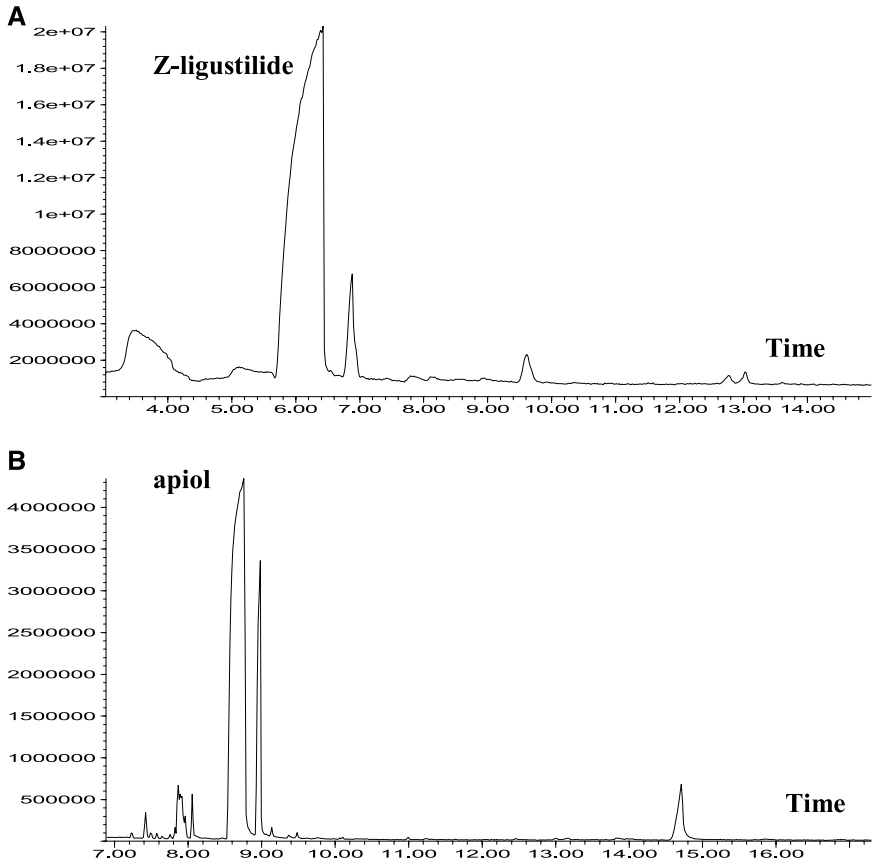


FIG. 5. Sample total ion chromatogram for the CH_2Cl_2 extracts of *L. californicum* (A) and *L. hultenii* (B).

The biological and/or ecological functions of these compounds in such high concentrations in the seeds of these species are topics worthy of further studies. Seeds of wild plants must have some resistance to herbivores and microbes in order to survive until dormancy is broken. The biological activities of the compounds that we report here suggest that they may play a role as antimicrobial compounds to prevent seed decay. Ligustilide has been reported to have activity against insects (Miyazawa et al., 2004). Whether the ecological function(s) of these two compounds also includes antifeedant or other properties should be investigated. Lastly, these compounds could have a role in the regulation of seed dormancy, a more challenging biological function to explore.

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INFLUENCE OF AUTOCLAVED FUNGAL MATERIALS ON SPEARMINT (*Mentha spicata* L.) GROWTH, MORPHOGENESIS, AND SECONDARY METABOLISM

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Abstract—The influence of autoclaved fungal materials such as culture filtrate, freeze-dried mycelium (FDM), mycelium suspension, and spore suspension (SS) on the growth, morphogenesis, and carvone production of spearmint (*Mentha spicata* L.) plants was studied. Fungal materials were either applied as a drench or spray on the plants. Spearmint plants (cv. "294099") drenched with SS (1×10^8 spores/ml) of *Trichoderma reesei* showed no significant differences in leaf numbers, root numbers, or shoot numbers compared with nontreated controls. However, significantly higher fresh weights and carvone levels were observed in plants drenched with *T. reesei* SS compared with the untreated controls. Fungal materials derived from *Aspergillus* sp., *Fusarium graminearum*, *F. sporotrichoides*, *Penicillium* sp., *P. acculeatum*, *Rhizopus oryzae*, and *T. reesei* were sprayed on spearmint foliage. *F. graminearum*, *F. sporotrichoides*, or *R. oryzae* elicited no enhanced growth, morphogenesis, or secondary metabolism responses. The best growth and morphogenesis responses were obtained employing *Aspergillus* sp., *Penicillium* sp., or *T. reesei* foliar sprays. For example, spearmint cv. "557807" plants sprayed with 100 mg/l FDM *T. reesei* isolate NRRL

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[4] Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

11460 C30 stimulated higher fresh weights (75%), shoot numbers (39%), leaf numbers (57%), and root numbers (108%) compared with untreated plants. This effect was not dose-dependent because similar growth and morphogenesis responses were obtained by testing 10, 100, or 1000 mg/l FDM concentrations. Carvone levels in fungal-treated foliar-sprayed plants were comparable to nontreated controls. However, total carvone levels per plant were higher in fungal-treated plants because of their increased fresh weight.

Key Words—*Aspergillus*, carvone, freeze-dried mycelium, *Fusarium*, mycelium, *Penicillium*, secondary metabolites, *Rhizopus*, spearmint, spores, *Trichoderma.*

INTRODUCTION

Some biocontrol microorganisms aside from combating pathogens may also cause plants to exhibit enhanced growth (Chang et al., 1986; Ryu et al., 1999, unpublished data; Raj et al., 2003). Microorganism preparations enhancing plant growth may be rhizobacteria (Ryu et al., 1999; Zehnder et al., 2000; Raj et al., 2003) or fungus (Elad, 2000). In these reports, biological preparations were composed of living microorganisms. In contrast, little research has been conducted studying the effects of dead microorganism fractions on growth (i.e., dry or fresh weights) and morphogenesis (i.e., leaf, root, and shoot numbers and sizes) responses in plants. Elad (2000) reported the effective biocontrol of powdery mildew on cucumber (*Cucumis sativus* L.) by application of foliar sprays containing either live or dead *Trichoderma harzianum* T39 cells. In addition, a slight increase in the leaf number occurred in sprayed plants employing either living or dead cells of T39 compared with untreated controls. This study suggested that dead fungal material may elicit both defense and growth and morphogenesis enhancement responses. Effective application of nonliving microorganism fractions to stimulate growth and/or combat pathogens would be of great benefit to the nursery and field grower. The drawbacks of using living microorganisms include their opportunistic human pathogenic nature (e.g., aspergillosis) and the permanent introduction of undesirable foreign fungal populations into soil (Anderson et al., 2002; Barkhage and Bernhard, 2002). These problems would be avoided by employing inert dead fungal materials.

In this study, we sought to determine the influence of applying dead fungal fractions on the growth, morphogenesis, and secondary metabolism responses in spearmint (*Mentha spicata* L.) plants. Secondary metabolites are constitutive chemicals that may represent a first-line defense to pathogen attack (Kuc, 1997). Treatments that stimulate secondary metabolite production could be useful to combat pathogens. The objectives of this study were to evaluate: (1) the effect of drenching the rhizosphere with spore suspensions (rhizosphere stimulation);

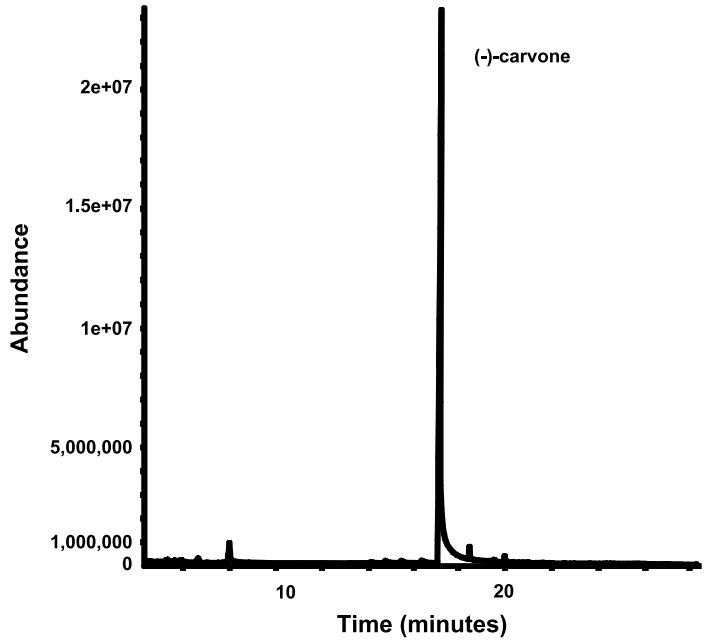

FIG. 1. Typical GC-MS profile of a spearmint plant showing the dominant monoterpene (–)-carvone in relationship to other monoterpenes present.

(2) the effect of applying suspensions of autoclaved mycelium suspension (MS), freeze-dried mycelium (FDM), spore suspensions (SS), or culture filtrate (CF) as foliar sprays (phylosphere stimulation); and (3) the effect of the concentration of FDM foliar sprays (dosage–response stimulation).

Spearmint was employed because it can be easily grown in the greenhouse as uniform-sized rooted shoots that readily manifest growth, morphogenesis, and secondary metabolism responses to various chemical and environmental treatments. The dominant essential oil component in spearmint oil is (−)-carvone, which constitutes >90% of the essential oil composition (Figure 1). Spearmint is commercially grown for its oils, which are employed as fragrance components in toothpastes, mouthwashes, soaps, detergents, lotions, insecticides, and perfumes (Leung, 1980).

METHODS AND MATERIALS

Fungal Cultures and Medium. For the production of SS, fungi isolates (*Aspergillus* sp. NRRL 32534 and *Trichoderma reesei* NRRL 11460 C30) were

grown on potato dextrose agar (PDA) medium at room temperature under fluorescent lighting (12 hr light/12 hr dark) for 7 d. For production of mycelium, fungi isolates (*Aspergillus* sp. NRRL 32534 and NRRL 363, *Fusarium graminearum* NRRL 23639, *F. sporotrichoides* NRRL 3299, *Penicillium* sp. NRRL 32532 and *P. acculeatum* NRRL 2129, *Rhizopus oryzae* NRRL 395, and *T. reesei* NRRL 11460 C30 and NRRL 3652) were grown in liquid potato dextrose broth (PDB) in Fernbach flasks inoculated with fungal cultures maintained on PDA and then placed on a shaker at 250 rpm and incubated at 25°C in the dark. MS were harvested after 72 hr by pouring the mycelium and medium through four layers of sterile cheesecloth followed by squeezing the cheesecloth to extract excess medium and then frozen at −20°C. FDM was prepared by freeze drying MS and then grinding first with mortar and pestle and then with a coffee grinder to produce a fine powder. CFs were collected from liquid PDB and kept in a sterile glass bottle at 0°C until use. All fungal sprays contained 0.025% Tween-80 as a surfactant and were autoclaved at 20 psi at 120°C for 15 min and allowed to cool to room temperature prior to application.

Plant Material. Spearmint stock plants cvs. "294099" and "557808" were maintained via shoot cuttings in soil under greenhouse conditions prior to testing with fungal treatments. For experiments, 4-cm tall plants established in Cone-tainers™ (RLC-4 Pine Cell, Stuewe and Sons Inc., Corvallis, OR, USA) containing 10 g of soil were employed. The soil mixture consisted of 1 peat moss/1 vermiculite (v/v) amended with 10.9 g/kg Micromax (Scotts Co., Marysville, OH, USA) and 62.3 g/kg Osmocote 14–14–14 (Scotts Co. USA). Plants were grown in a greenhouse at 20 ± 2°C under natural daylight.

Rhizosphere Stimulation. Spearmint (cv. "294099") plants were given the following drenching treatments (2 ml/cone): water only, 0.1% Tween-80, *Aspergillus* sp. NRRL 32534 SS (1×10^8 spores/ml), *T. reesei* NRRL 11460 C30 SS (1×10^8 spores/ml), 0.5% DMSO, or 1% DMSO. Treatments were administered once a week for two consecutive weeks. Following treatments, plants were grown on greenhouse benches for an additional 4 wk.

Phylosphere Stimulation. The following fungal species and isolates were employed in foliar spray testing: *Aspergillus* sp. NRRL 32534, *F. graminearum* NRRL Z3639, *F. sporotrichoides* NRRL 3299, *Penicillium* sp. NRRL 32532 and NRRL 32533 and *P. acculeatum* NRRL 2129, *R. oryzae* NRRL 395, or *T. reesei* NRRL 11460 C30. Spearmint (cvs. "294099" or "557808") plants were given the following fungal foliar sprays: water only, 0.025% Tween-80, 10% PDB, 10% CF, 100 mg/l FDM, 100 mg/l MS, or SS (1×10^8 spores/ml). Plants were sprayed until runoff twice with a 1-wk interval between sprays and then grown on the greenhouse benches for an additional 4 wk.

Dose Response Effect of FDM. FDM suspension from *Aspergillus* sp. NRRL 32534 or NRRL 363, *T. reesei* NRRL 11460 C30 or NRRL 3652, or

Penicillium sp. NRRL 32532 or NRRL 32533 or *P. acculeatum* NRRL 2129 were sprayed on spearmint plants at 10-, 100-, or 1000-mg/l concentrations. Plants were sprayed once a week for 2 consecutive wk and then grown on greenhouse benches for additional 4 wk prior to taking data.

Statistical Analysis. Whole seedlings fresh weight, leaf number per plant, root number per plant, and shoot number per plant were recorded from 5 to 10 plants with the remaining plants employed in essential oil analysis. Experiments were repeated at least twice. Data were analyzed by PC SAS using GLM or ANOVA procedures, and means were separated by Fisher's protected LSD at a 0.05 rejection level.

Essential Oil Analysis. Spearmint plants were randomly selected from each treatment. The top (4 cm from the apical tip) of each individual plant was excised to represent a replicate, and three to five replicates were mixed together per treatment. One gram of fresh plant tissue per treatment was incubated with 15 ml CH_2Cl_2 for 72 hr, allowing carvone levels to come to equilibrium in the solvent. Investigative studies have shown that this method is superior to multiple solvent extractions and subsequent roto-evaporation that result in losses of the volatile carvone (unpublished data). After filtering, samples were analyzed on an HP 5890 gas chromatograph (GC) equipped with a flame ionization detector (FID). GC–mass spectrometry was performed using an HP 6890 Series II gas chromatograph attached to an HP 5972A mass select detector. Columns used were fused silica HP-5MS capillaries (0.25-μm film thickness, 30 ml $\times$ 0.25 mm ID). The major compound found in the extracts, (−)-carvone, hereafter referred to simply as carvone, was identified by comparison with mass spectra of a library database, and concentration was calculated from a standard carvone curve. Carvone was measured at mg/g fresh weight.

RESULTS

Rhizosphere Stimulation. Spearmint cv. "294099" drenched with Tween-80, 0.5% DMSO, or 1.0% DMSO treatments did not show any significant differences for fresh weights, leaf numbers, root numbers, shoot numbers, or carvone levels compared with water-drenched controls. However, spearmint plants drenched with *T. reesei* spores showed higher fresh weights and carvone levels compared with controls, whereas the leaves, shoots, and roots were unchanged compared with controls (Figure 2). Spearmint plants drenched with *Aspergillus* sp. spores showed higher root numbers than control plants, although the other growth and morphogenesis responses were unchanged (Figure 2).

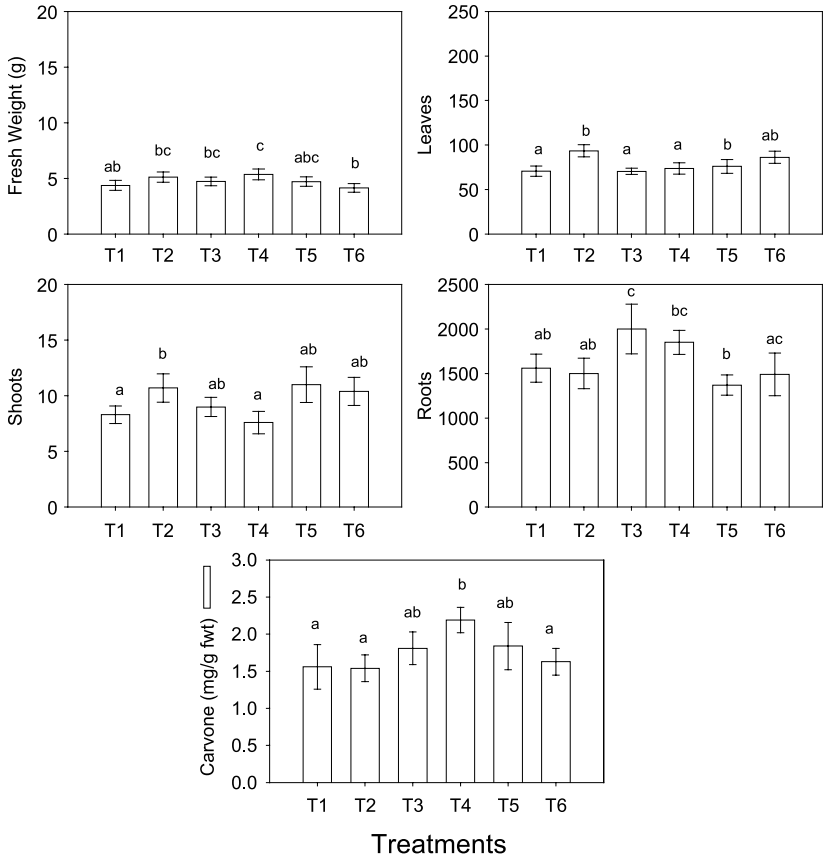

FIG. 2. Influence of various drench treatments on spearmint cv. "294099" carvone levels. T1 = Control; T2 = Tween-80; T3 = *Aspergillus* sp. NRRL 32534; T4 = *T. reesei* NRRL 11460 C30; T5 = 0.5% DMSO; and T6 = 1.0% DMSO. Columns having the same letters do not differ significantly ($P > 0.05$) in LSD test.

Phylosphere Stimulation. Plants sprayed with Tween-80, PDB, or DMSO did not exhibit any difference in growth or morphogenesis responses compared with untreated controls (Figures 3–6). Both spearmint cvs. sprayed with either *Aspergillus* sp. or *T. reesei* fungal materials had increased fresh weights, leaf numbers, root numbers, and shoot numbers compared with nontreated controls (Figures 3–6). For example, spearmint cv. "557808" plants sprayed with *T. reesei* NRRL 11460 C30 FDM or MS increased fresh weights (75 and 121%, respectively), shoot numbers (39 and 70%, respectively), leaf numbers (57 and 103%, respectively), and root numbers (108 and 108%, respectively) compared

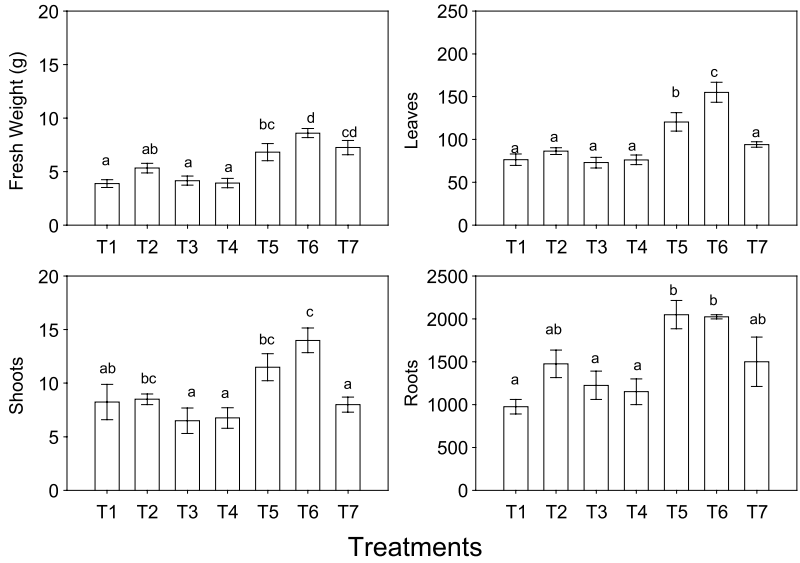

FIG. 3. Influence of *T. reesei* NRRL 11460 C30 foliar sprays on the growth and morphogenesis of spearmint cv. "557807." T1 = Control; T2 = Tween-80; T3 = potato dextrose broth; T4 = culture filtrate; T5 = freeze-dried mycelium; T6 = mycelium suspension; and T7 = spore suspension. Columns having the same letters do not differ significantly ($P > 0.05$) in LSD test.

with nontreated controls (Figure 2). In addition, fresh weights were also higher in plants sprayed with SS (87%) compared with controls (Figure 3). Nevertheless, regardless of the fungal material employed, no significant differences were observed among treatments for carvone levels compared with untreated controls ($P > 0.05$, data not shown).

Spearmint cv. "294099" plants sprayed with *T. reesei* NRRL 32534 CF, FDM, MS, or SS increased fresh weights (36, 53, 47, and 69%, respectively), leaf numbers (84, 91, 56, and 117%, respectively), root numbers (29, 69, 100, and 147%, respectively), and shoots (95, 129, 33, and 67%, respectively) compared with nontreated controls (Figure 4).

Spraying spearmint cv. "557807" plants with *Aspergillus* sp. NRRL 32534 CF, FDM, MS, or SS increased in fresh weights (88, 81, 108, and 85%, respectively) over nontreated controls (Figure 5). Significant increases in leaf numbers were observed in plants sprayed with FDM, MS, and SS (59, 129, and 69%, respectively) over those of nontreated controls. Significant increases in root numbers in plants sprayed with MS or SS were observed (69 and 69%, respectively) over those of nontreated controls. Plants sprayed with *Aspergillus*

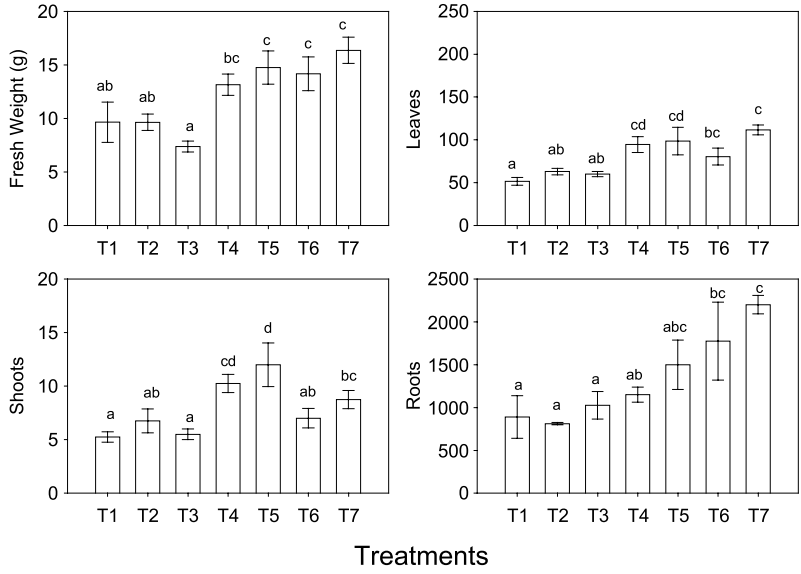

FIG. 4. Influence of *T. reesei* NRRL 11460 C30 foliar sprays on the growth and morphogenesis of spearmint cv. "294099." T1 = Control; T2 = Tween-80; T3 = potato dextrose broth; T4 = culture filtrate; T5 = freeze-dried mycelium; T6 = mycelium suspension; and T7 = spore suspension. Columns having the same letters do not differ significantly ($P > 0.05$) in LSD test.

sp. MS also showed significant increases in shoot number (73%) over controls (Figure 5). Spearmint cv. 294099 plants sprayed with *Aspergillus* sp. CF, FDM, MS, or SS caused higher fresh weights (68, 86, 78, and 56%, respectively), leaves (100, 90, 145, and 131%, respectively), roots (139, 55, 98, and 145%, respectively), and shoots (71, 71, 124, and 90%, respectively) compared with those of nontreated controls (Figure 6).

Dosage Response Effect of FDM. Plants sprayed with 10, 100, or 1000 mg/l FDM from isolates of *T. reesei* NRRL 11460 C30 or NRRL 3652 exhibited significant increases in all fresh weights (>41%) and shoot numbers (>54%) (Table 1). Similarly, for all concentrations tested, higher but not always significant increases occurred for root numbers and leaf numbers compared with untreated control plants. No change in carvone levels were observed in any plants treated with any isolate concentration tested (Table 1). Both isolates of *Aspergillus* sp. NRRL 32534 or NRRL 363 administered at 10, 100, and 1000 mg/l caused spearmint plants to exhibit higher (but not always significantly higher) fresh weights, root numbers, shoot numbers, and leaf numbers compared with untreated controls (Table 2). Only spearmint plants treated with *Asper-*

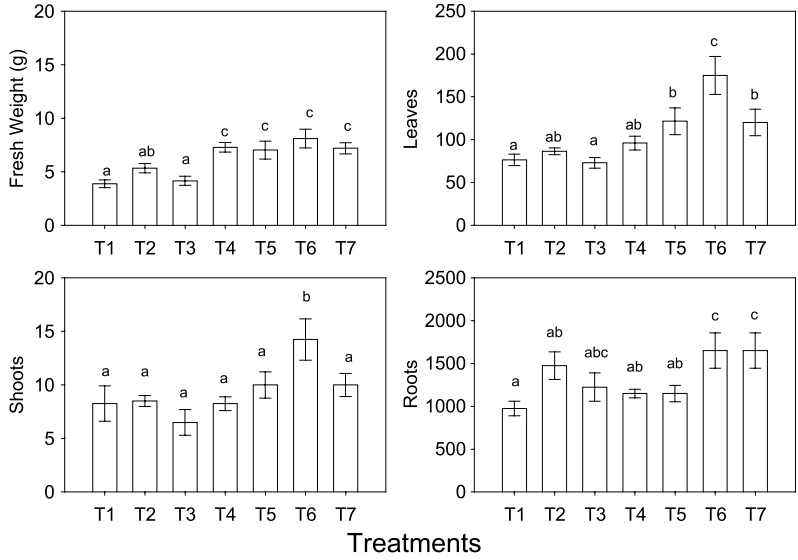

FIG. 5. Influence of *Aspergillus* sp. NRRL 32534 foliar sprays on the growth and morphogenesis of spearmint cv. "557807." T1 = Control; T2 = Tween-80; T3 = potato dextrose broth; T4 = culture filtrate; T5 = freeze-dried mycelium; T6 = mycelium suspension; and T7 = spore suspension. Columns having the same letters do not differ significantly ($P > 0.05$) in LSD test.

gillus sp. NRRL 32534 at 100 and 1000 mg/l gave significantly higher fresh weights, leaf numbers, shoot numbers, and root numbers compared with untreated plants. Carvone levels were similar for all plants treated with *Aspergillus* sp. isolates compared with untreated controls. All *Penicillium* sp. isolates NRRL 32532, NRRL 32533, and *P. acculeatum* NRRL 2129 increased fresh weights, shoot numbers, leaf numbers (except 10 mg/l NRRL 32532), and root numbers compared with untreated controls (Table 3). Overall, *Penicillium* sp. isolates were less effective for the induction of significant increases in growth and morphogenesis responses compared with other fungal species isolates tested. Nevertheless, higher and sometimes significant increases in growth and morphogenesis occurred in plants sprayed with the 100-mg/l concentrations (except for fresh weights and root numbers with isolate NRRL 32532) compared with that occurring in plants sprayed with the 10-mg/l concentration regardless of the *Penicillium* sp. isolates tested. Generally, carvone levels were similar for all isolates, and concentrations tested, except 100 mg/l NRRL 32533, caused a significant increase in carvone production to occur from treated spearmint plants.

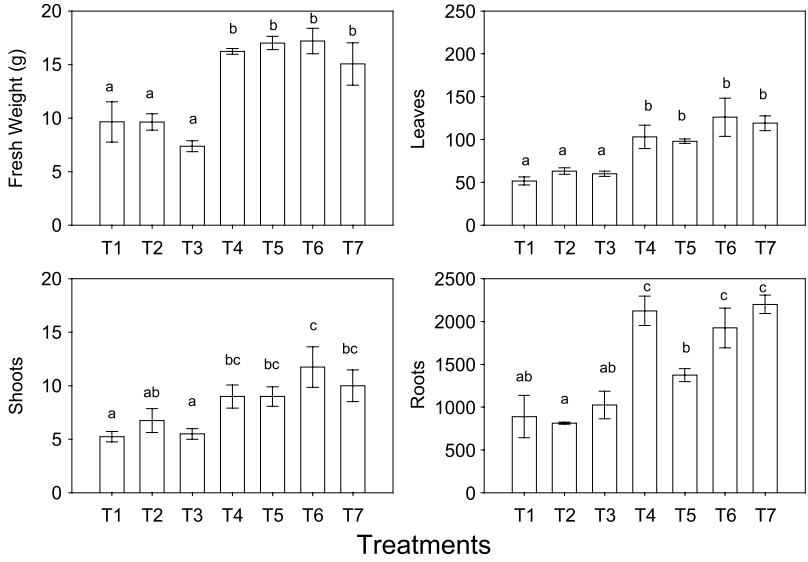

FIG. 6. Influence of *Aspergillus* sp. NRRL 32534 foliar sprays on the growth and morphogenesis of spearmint cv. "294099." T1 = Control; T2 = Tween-80; T3 = potato dextrose broth; T4 = culture filtrate; T5 = freeze-dried mycelium; T6 = mycelium suspension; and T7 = spore suspension. Columns having the same letters do not differ significantly ($P > 0.05$) in LSD test.

DISCUSSION

In this study, we have identified isolates of dead fungi that enhance spearmint growth, morphogenesis, and secondary metabolism. Aside from the enhanced leaf production noted by Elad (2000), the application of dead fungal cells to plants to enhance growth has not been reported in the literature. Drenching soil with fungal spores was found to be a less effective method of promoting growth and morphogenesis responses on spearmint plants than foliar sprays. For some fungal species (i.e., *F. graminearum*, *F. sporotrichoides*, and *R. oryzae*), foliar sprays failed to elicit any response whatsoever in terms of growth, morphogenesis, or secondary metabolism in spearmint, whereas other fungal species foliar sprays such as from *Aspergillus* sp. and *T. reesei* elicited strong growth and morphogenesis responses. Clearly, the species and isolate type appear important in obtaining growth and morphogenesis responses. The method of application (foliar spray vs. drench) and type of fungal material (CF, FDM, MS, or SS) employed are critical to obtaining increased growth and morphogenesis responses. We also found that growth and morphogenesis were

TABLE 1. EFFECT OF FOLIAR SPRAYS OF *T. reesei* NRRL 11460 C30 OR NRRL 3652 ON THE GROWTH, MORPHOGENESIS AND SECONDARY METABOLISM OF SPEARMINT CV. "557807"

Treatments[a]	Percentage increase over control/mean ± standard error				
	Root number	Shoot number	Leaf number	Fresh weight (g)	Carvone (mg/g fwt)
T1	0	0	0	0	0
	1650.0 ± 125.83	6.5 ± 0.65	112.25 ± 8.17	9.66 ± 0.92	2.34 ± 0.12
T2	37.88	12.0	3.79	9.97	−16.7
	2275.0 ± 103.1	7.25 ± 0.75	116.5 ± 6.6	10.62 ± 0.91	1.95 ± 0.08
T3	42.42	88.46	29.18	54.17	−11.5
	2350.0 ± 236.29	12.25 ± 1.49	145.0 ± 11.1	14.89 ± 0.88	2.07 ± 0.1
T4	30.3	88.46	37.42	58.28	18.5
	2150.0 ± 95.74	12.25 ± 0.85	154.25 ± 8.37	15.29 ± 0.62	2.77 ± 0.14
T5	45.45	53.85	16.04	50.56	−7.3
	2400.0 ± 158.1	10.0 ± 1.41	130.25 ± 10.28	14.54 ± 1.07	2.17 ± 0.09
T6	62.12	119.24	68.82	58.07	−2.1
	2675.0 ± 137.69	14.25 ± 0.75	189.5 ± 14.97	15.27 ± 1.76	2.29 ± 0.27
T7	36.36	80.77	38.08	41.19	0.9
	2250.0 ± 221.74	11.75 ± 1.38	155.0 ± 17.87	13.64 ± 0.57	2.36 ± 0.15
T8	54.54	57.69	24.28	45.14	−8.5
	2550.0 ± 275.38	10.25 ± 0.75	139.5 ± 11.29	14.02 ± 0.8	2.14 ± 0.26
LSD[b]	526.61	3.1	33.92	2.93	0.5

[a]T1 = Control; T2 = Tween-80; T3 = 10 mg/l NRRL 11460 C30; T4 = 100 mg/l NRRL 11460 C30; T5 = 1000 mg/l NRRL 11460 C30; T6 = 10 mg/l NRRL 3652; T7 = 100 mg/l NRRL 3652; T8 = 1000 mg/l NRRL 3652.
[b]Means were separated by Fisher's protected LSD test ($P > 0.05$).

enhanced more by FDM, MS, or SS than by using CF. We usually did not observe any differences between employing FDM, MS, or SS on the growth and morphogenesis responses obtained (Figures 3–6). CF activity was inconsistent and may or may not enhance growth and morphogenesis in spearmint depending on the isolate employed. Both clones of spearmint reacted similarly in the presence of fungal isolates, types, and concentrations. Our results confirm those by Elad (2000) in which leaf number in cucumber was enhanced by the application of dead *T. harzianum* T39 cells. In addition, we noted strong positive correlations between fresh weight, shoot number, leaf number, and root number responses when growth or morphogenesis is stimulated in the presence of dead fungal materials.

We did not promote any significant increases in absolute mint carvone levels by using any of the fungal foliar spray studies. Apparently, enhanced growth and morphogenesis is not associated with enhanced secondary metabolism. However, inert dead fungal material applications did not reduce

TABLE 2. EFFECT OF FOLIAR SPRAY OF *Aspergillus* sp. NRRL 32534 OR NRRL 363 ON GROWTH, MORPHOGENESIS AND SECONDARY METABOLISM OF SPEARMINT CV. "557807"

Treatments[a]	Percentage increase over control/mean ± standard error				
	Root number	Shoot number	Leaf number	Fresh weight (g)	Carvone (mg/g fwt)
T1	0	0	0	0	0
	1650.0 ± 125.83	6.5 ± 0.65	112.25 ± 8.17	9.66 ± 0.92	2.34 ± 0.12
T2	37.88	11.54	3.78	9.97	−16.7
	2275.0 ± 103.1	7.25 ± 0.75	116.5 ± 6.6	10.62 ± 0.91	1.95 ± 0.08
T3	60.6	76.92	4.09	16.63	−22.2
	2650.0 ± 347.61	11.5 ± 3.33	157.25 ± 32.25	11.27 ± 0.52	1.82 ± 0.19
T4	57.58	115.38	71.05	34.42	−15.0
	2600.0 ± 70.71	14.0 ± 1.47	192.0 ± 23.71	12.98 ± 1.59	1.99 ± 0.14
T5	59.09	130.77	58.13	50.68	−9.0
	2625.0 ± 278.01	15.0 ± 2.04	177.5 ± 25.17	14.55 ± 2.1	2.13 ± 0.15
T6	16.67	69.23	38.31	29.4	12.4
	1925.0 ± 188.75	11.0 ± 0.71	155.25 ± 8.4	12.5 ± 0.68	2.63 ± 0.18
T7	56.06	57.69	42.98	44.97	−6.0
	2575.0 ± 356.78	10.25 ± 0.85	160.5 ± 21.88	14.0 ± 1.45	2.2 ± 0.24
T8	24.24	80.77	33.63	23.03	5.6
	2050.0 ± 95.74	11.75 ± 0.95	150.0 ± 6.68	11.88 ± 0.9	2.47 ± 0.07
LSD[b]	654.02	4.67	55.96	3.61	0.47

[a]T1 = Control; T2 = Tween-80; T3 = 10 mg/l NRRL 32534; T4 = 100 mg/l NRRL 32534; T5 = 1000 mg/l NRRL 32534; T6 = 10 mg/l NRRL 363; T7 = 100 mg/l NRRL 363; T8 = 1000 mg/l NRRL 363.

[b]Means were separated by Fisher's protected LSD test ($P > 0.05$).

carvone levels compared with untreated control plants. Secondary metabolites have been found to increase in plants in response to microorganism infection and stress (Bell, 1981; Cheniclet et al., 1988; Dalkin et al., 1990; Dixon and Lamb, 1990). For example, terpene content increased when maritime pine (*Pinus pinaster*) was attacked by *Tomicus piniperda* beetle (Cheniclet et al., 1988). Inclusion of dead microorganisms into nutrient medium is commonly employed to enhance secondary metabolite production in plant cell suspension cultures (Heinstein, 1985; Eilert and Contabel, 1986; Eilert et al., 1986; Dalkin et al., 1990; Mahady and Beecher, 1994). For example, Mahady and Beecher (1994) reported that addition of *Penicillium expansum* to a culture of *Sanguinaria canadensis* induced the production of benzophenanthridine alkaloids such as sanguinarine and chelerythrine in a dose-dependent manner. Similarly, the addition of fungal elicitors to cultures of *Papaver somniferum* stimulated sanguinarine production (Eilert et al., 1986). It is interesting that these fungal additions were administered typically at the end of the cell

TABLE 3. EFFECT OF FOLIAR SPRAY OF *Penicillium* sp NRRL 3232 AND NRRL 32533 OR *P. acculeatum* NRRL 2129 ON GROWTH, MORPHOGENESIS AND SECONDARY METABOLISM OF SPEARMINT CV. "557807"

	Percentage increase over control/mean ± standard error				
Treatments[a]	Root number	Shoot number	Leaf number	Fresh weight (g)	Carvone (mg/g fwt)
T1	0	0	0	0	0
	1650.0 ± 125.83	6.5 ± 0.65	112.25 ± 8.17	9.66 ± 0.92	2.34 ± 0.12
T2	37.88	11.54	3.79	9.97	–16.7
	2275.0 ± 103.1	7.25 ± 0.75	116.5 ± 6.6	10.62 ± 0.91	1.95 ± 0.08
T3	54.55	30.77	0	46.12	3.0
	2550.0 ± 165.83	8.5 ± 1.26	111.0 ± 5.12	14.11 ± 1.02	2.41 ± 0.24
T4	31.82	73.08	24.94	44.42	–28.2
	2175.0 ± 118.15	11.25 ± 2.36	140.25 ± 20.95	13.95 ± 1.24	1.68 ± 0.16
T5	18.18	26.92	7.35	15.63	25.6
	1950.0 ± 210.16	8.25 ± 1.31	120.5 ± 10.96	11.17 ± 0.55	2.94 ± 0.09
T6	21.21	103.85	41.87	34.39	7.3
	2000.0 ± 204.12	12.98 ± 2.19	159.25 ± 4.42	12.98 ± 2.19	2.51 ± 0.08
T7	18.18	26.92	21.38	18.49	–13.2
	1950.0 ± 50.0	8.25 ± 0.75	136.25 ± 6.56	11.44 ± 0. 7	2.03 ± 0.12
T8	66.67	69.23	28.51	21.38	–17.5
	2550.0 ± 275.38	11.0 ± 1.08	144.25 ± 5.14	11.72 ± 0.78	1.93 ± 0.08
LSD[b]	434.26	3.55	28.9	3.34	0.4

[a]T1 = Control; T2 = Tween-80; T3= 10 mg/l NRRL 32532; T4 = 100 mg/l NRRL 32532; T5 = 10 mg/l NRRL 32533; T6 = 100 mg/l NRRL 32533; T7 = 10 mg/l NRRL 2129; T8 = 100 mg/l NRRL 2129.

[b]Means were separated by Fisher's protected LSD test ($P > 0.05$).

suspension growth cycle. It is not reported if the fungal additives stimulated or depressed cell suspension growth.

Under favorable environmental conditions, plants prioritize their resources towards growth and differentiation over secondary metabolism (Bazzaz et al., 1987; Chapin et al., 1990; Dickson and Isebrands, 1991). Plant growth processes require high amounts of primary carbon resources that may come at the cost of secondary metabolite production (Gulmon and Mooney, 1986; Williams et al., 1987; Margna et al., 1989; Lambers and Rychter, 1990; Chapin, 1991). Generally, in our study, there was no significant change in carvone levels in spearmint plants that showed significant increases in growth and morphogenesis over untreated controls. However, because of the increases in growth, the total carvone levels per plant were significantly higher in fungal-treated plants than untreated spearmint plants.

Dead fungal cell stimulation of plant growth and differentiation may be either chemical or physical or both. If the phenomenon is physical, the plants

may perceive themselves under attack by fungal cells and in response initiate rapid growth. If chemical, we speculate that dead fungal cells may emit a stimulatory chemical substance that then encourages plant growth.

Currently, we are extending our studies into this phenomenon by examining various fungal species and isolates with different plant species. Thus far, the results confirm those presented in this paper. These studies may lead to industrial products that can be used as growth stimulators for a variety of nursery and greenhouse plants. For two of the fungi (*T. reesei* and *Aspergillus* sp.) examined in this study, both are employed in the paper pulp processing (Toyama et al., 2002) and enzyme production (Chen and Reese, 2002) industries. Perhaps mycelium suspension, which is currently obtained as a waste product, may have potential as a plant growth promoter.

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THE FATE OF *Lyngbya majuscula* TOXINS IN THREE POTENTIAL CONSUMERS

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Abstract—Blooms of *Lyngbya majuscula* have been reported with increasing frequency and severity in the last decade in Moreton Bay, Australia. A number of grazers have been observed feeding upon this toxic cyanobacterium. Differences in sequestration of toxic compounds from *L. majuscula* were investigated in two anaspideans, *Stylocheilus striatus*, *Bursatella leachii*, and the cephalaspidean *Diniatys dentifer*. Species fed a monospecific diet of *L. majuscula* had different toxin distribution in their tissues and excretions. A high concentration of lyngbyatoxin-a was observed in the body of *S. striatus* (3.94 mg/kg^{-1}) compared to bodily secretions (ink 0.12 mg/kg^{-1}; fecal matter 0.56 mg/kg^{-1}; eggs 0.05 mg/kg^{-1}). In contrast, *B. leachii* secreted greater concentrations of lyngbyatoxin-a (ink 5.41 mg/kg^{-1}; fecal matter 6.71 mg/kg^{-1}) than that stored in the body (2.24 mg/kg^{-1}). The major internal repository of lyngbyatoxin-a and debromoaplysiatoxin was the digestive gland for both *S. striatus* (6.31 ± 0.31 mg/kg^{-1}) and *B. leachii* (156.39 ± 46.92 mg/kg^{-1}). *D. dentifer* showed high variability in the distribution of sequestered compounds. Lyngbyatoxin-a was detected in the digestive gland (3.56 ± 3.56 mg/kg^{-1}) but not in the head and foot, while debromoaplysiatoxin was detected in the head and foot (133.73 ± 129.82 mg/kg^{-1}) but not in the digestive gland. The concentrations of sequestered secondary metabolites in these animals did not correspond to the concentrations found in *L. majuscula* used as food for these experiments, suggesting it may have been from previous dietary exposure. Trophic transfer of debromoaplysiatoxin from *L. majuscula* into *S. striatus* is well established; however, a lack of knowledge

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exists for other grazers. The high levels of secondary metabolites observed in both the anaspidean and the cephalapsidean species suggest that these toxins may bioaccumulate through marine food chains.

Key Words—*Stylocheilus striatus*, *Bursatella leachii*, *Diniatys dentifer*, lyngbyatoxin-a, debromoaplysiatoxin, bioaccumulation, Opisthobranchia, secondary metabolites.

INTRODUCTION

Opisthobranch mollusks are renowned for their ability to sequester a range of dietary-derived compounds (Avila, 1995; Johnson and Willows, 1999). Pennings and Paul (1993a) suggest that sea hares have a generic mechanism for sequestering algal metabolites rather than mechanisms that are tightly linked to different compounds. If this is the case, then the physiology of sequestration would not be different among species. Study of the storage, secretion, and egestion of these compounds by marine mollusks helps us to understand defensive strategies and transfer of toxins within food webs.

Stylocheilus striatus Quoy and Gaimard 1832 (formerly *longicauda*; Rudman, 1999) is an anaspidean species that can sequester, store, and transform marine plant secondary metabolites (Faulkner, 1984; Avila, 1995), including those found in the toxic cyanobacterium *Lyngbya majuscula* (Watson and Rayner, 1973; Kato and Scheuer, 1975; Pennings and Paul, 1993a,b; Pennings et al., 1996). *S. striatus* adults preferentially feed and grow well upon a monospecific diet of *L. majuscula* (Paul and Pennings, 1991). Some *L. majuscula* compounds (malyngamides and majusculamides) stimulate feeding by *S. striatus* at low concentrations (Nagle et al., 1998; Nagle and Paul, 1999). *S. striatus* sequester secondary metabolites in the digestive gland where transformation to less harmful acetates could reduce negative postingestive consequences (Pennings et al., 1996). This hypothesis is supported by the isolation of lyngbyatoxin-a and debromoaplysiatoxin acetates from *S. striatus* (Kato and Scheuer, 1976). Both compounds are less toxic than their precursors in mice bioassays (Kato and Scheuer, 1975; Gallimore et al., 2000).

The fate of *L. majuscula* secondary metabolites is known for *S. striatus*, but other grazers of *L. majuscula* have received little attention. Cruz-Rivera and Paul (2002) identified 43 invertebrate species in *L. majuscula* mats in Guam including 14 species of opisthobranchs. The cephalaspidean *Diniatys dentifer* (Adams, 1850) is one of the most abundant organisms observed in *L. majuscula* blooms in Guam (Cruz-Rivera and Paul, unpublished data). *D. dentifer* was observed feeding on *L. majuscula*, but this has not yet been quantified (Cruz-Rivera, personal communication), nor its dietary repertoire established. *Bursatella leachii* de Blainville, 1817, is a "generalist" grazer of cyanobacteria

(Ramos et al., 1995) and is a common component of shallow water subtropical fauna (Lowe and Turner, 1976; Paige, 1988). Like *S. striatus*, this anaspidean species appears to use chemical cues from *L. majuscula* to induce settlement and development of larvae (Switzer-Dunlap and Hadfield, 1977; Paige, 1988). To date, there are no comprehensive studies to determine the fate of *L. majuscula* compounds in *D. dentifer* and *B. leachii*.

In this study, we examined intraspecific differences in internal partitioning of lyngbyatoxin-a and debromoaplysiatoxin between two populations of *S. striatus*: (1) those previously feeding on *L. majuscula*; and (2) those naïve to *L. majuscula*. Interspecific differences in internal compartmentalization, secretion, and egestion of lyngbyatoxin-a were investigated between *S. striatus*, *B. leachii*, and *D. dentifer* over a 10-d-feeding experiment. The strategies for storage and excretion of *Lyngbya* compounds by these species have important implications for understanding the transfer of toxins through marine food chains.

METHODS AND MATERIALS

Study Sites. Two areas were chosen because they had persistent blooms of *L. majuscula*: Deception Bay (DB), on the Western shore of Moreton Bay (27°05′S, 153°09′E); and Adams Beach (AB), a sheltered area on the Eastern side of Moreton Bay (27°51′S, 153°41′E). Both sites have extensive sea grass beds with macroalgae locally dominating in places (Abal et al., 2001). *L. majuscula* was collected from these sites and maintained in aquaria at Moreton Bay Research Station (MBRS).

Study Organisms. S. striatus (*Lyngbya* exposed) were collected from *L. majuscula* blooms at the sites described above in 2002. A second group of *S. striatus* was obtained from a prawn pond at Bribie Island Aquaculture Research Centre (BIARC). Sea hares had been recruited in the pond as larvae and as there was no apparent *L. majuscula* growth, they were considered "naïve" to *L. majuscula* (*Lyngbya* naïve). In the laboratory, the *Lyngbya*-naïve group of *S. striatus* was fed a diet of blanched lettuce before our feeding experiments. *B. leachii* and *D. dentifer* were collected from a *L. majuscula* bloom at Adams Beach. All opisthobranchs were maintained in separate 50-l tanks in a closed aquarium system at MBRS. Salinities were kept between 34 and 36 ppt and temperature consistent with ambient (24°C) with 12-hr light and dark cycles.

Sample Preparation and Chemical Analyses. To quantify *L. majuscula* secondary metabolites in mesograzers, animals were starved for 24 hr to allow evacuation of gut contents before being euthanized in iced salt water. Shells

were removed from *D. dentifer* and discarded. Organisms were dissected into head and foot (HF) to represent external body parts, and digestive gland (DG) to represent total viscera (Avila, 1995). These samples were exhaustively extracted (five times) using 1:1 dichloromethane:methanol. Extracts were rotary evaporated and dried under pure nitrogen before being resolubilized in 1:1 methanol:distilled water and then analyzed by high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS).

To determine if *L. majuscula* secondary metabolites were secreted or egested by *S. striatus* and *B. leachii*, samples of ink, feces, and eggs (where available) were analyzed. Ink was collected on absorbent filter paper after gently squeezing an emersed animal between the fingers. Ink was extracted three times in 1:1 dichloromethane:methanol (Pennings and Paul, 1993a). Replicates were pooled and dried by rotary evaporation. Fecal samples were collected, frozen, freeze-dried, and then extracted in acetone three times. Acetone was used, as it extracts organics from plant-derived matrices due to its hydrophilic properties. Replicates were pooled and dried by rotary evaporation. The extract was resolubilized in 1:1 methanol:distilled water and analyzed by HPLC–MS/MS.

To quantify secondary metabolites in *L. majuscula*, voucher samples from all assays were frozen at −20°C and then freeze-dried. Plant material was extracted three times in acetone. Three replicate extracts were dried by rotary evaporation. Concentrations of secondary metabolites (in milligrams per kilogram) were determined by HPLC–MS/MS using a PE/Sciex API 300 mass spectrometer equipped with a high-flow electrospray interface (TurboIonspray) coupled to a Perkin Elmer series 200 HPLC system. Separation was achieved using a 150 × 4.6-mm Altima C_{18} column (Alltech) run at 35°C, with a mobile phase consisting of 80/20 acetonitrile/hi-pure water containing 0.1% formic acid and 2 mM ammonium formate at a flow rate of 0.8 ml min^{-1}. The flow was split post column such that the flow to the mass spectrometer interface was 250 µl min^{-1}. Under these conditions, the retention times were 11.7 and 9.3 min for lyngbyatoxin-a and debromoaplysiatoxin, respectively. The mass spectrometer was operated in the positive ion, multiple-ion monitoring mode. Ions monitored with dwell times of 300 msec were 438.3 $(M+H)^+$ and 410.3 for lyngbyatoxin-a and 543.3 for debromoaplysiatoxin. Quantification was achieved by comparison to standards of DAT (kindly provided by Dr. R.E. Moore, Department of Chemistry, University of Hawaii at Manoa) and LTA (Calbiochem) run under the same conditions. Using a 20-µl injection, the detection limit for both toxins was typically 0.01 mg kg^{-1} in the extracted solution.

Partitioning of Secondary Metabolites. Lyngbya-exposed *S. striatus* (1.31 g_{wwt} ± 0.10 SE, $N = 9$) were housed individually in 1-l glass jars filled with unfiltered aerated seawater and allowed to acclimate for 48 hr. Seawater was changed every 2 d. Each sea hare was offered *L. majuscula* from the Deception Bay bloom and left to feed for a 10-d-period (Table 1). Voucher samples of

TABLE 1. SECONDARY METABOLITE CONCENTRATIONS DETECTED IN *L. Majuscula* USED IN FEEDING ASSAYS

Assay	Food type and source[a]	Food weight (blotted g_{wwt})[b]	Day of test	Lyngyatoxin-a concentration[b,c] (mg kg^{-1})	Debromoaplysiatoxin concentration[b,c] (mg kg^{-1})
Internal partitioning assays					
S. striatus	*L. majuscula* (DB)	10.0 (±0.01)	1	n/d	0.410 (±0.022)
(*Lyngbya* exposed)			10	n/d	0.048 (±0.020)
S. striatus	Lettuce	9.9 (±0.01)	1	n/d	n/d
(*Lyngbya* naive)			10	n/d	n/d
B. leachii	*L. majuscula* (AB)	2.0 (±0.01)	1	0.362 (±0.04)	n/d
		3.0 (±0.02)	3	7.981 (±0.16)	n/d
		3.0 (±0.01)	5	8.003 (±2.13)	n/d
		3.0 (±0.01)	7	1.619 (±0.15)	n/d
		5.0 (±0.02)	9	1.296 (±0.45)	n/d
D. dentifer	*L. majuscula* (AB)	2.0 (±0.01)	1	11.77 (±0.66)	n/d
			10	40.66 (±5.43)	n/d
Whole animal, body secretions and egested material assays					
S. striatus	*L. majuscula* (AB)	2.0 (±0.02)	1	1.215 (±0.11)	n/d
		2.0 (±0.02)	3	0.053 (±0.01)	n/d
		5.3 (±0.02)	5	n/d	n/d
		5.0 (±0.03)	7	0.258 (±0.02)	n/d
		9.9 (±0.02)	9	0.013 (±0.00)	n/d
B. leachii	*L. majuscula* (AB)	5.1 (±0.11)	1	4.561 (±0.18)	n/d
			10	3.493 (±0.26)	n/d

[a] Letters in parentheses refer to location of *L. majuscula* collected for use in feeding assays: AB, Adams Beach; DB, Deception Bay.
[b] Numbers in parentheses refer to standard error values.
[c] n/d = not detected by HPLC–MS/MS.

L. majuscula were retained on d 1 and d 10 to quantify secondary metabolite concentrations (Table 1). The second group of *S. striatus* (0.79 g_{wwt} ± 0.06 SE, $N = 9$), *Lyngbya* naïve, was only fed blanched lettuce (Table 1).

B. leachii (0.26 g_{wwt} ± 0.03 SE, $N = 9$) and *D. dentifer* (0.23 g_{wwt} ± 0.03 SE, $N = 9$) were maintained as described above. *B. leachii* were fed with fresh *L. majuscula* collected from blooms at Adams Beach every 2 d (Table 1), while *D. dentifer* received fresh *L. majuscula* only on d 1 since they only consumed a small amount during our study. Voucher samples of *L. majuscula* from each food change were retained for secondary metabolite quantification (Table 1).

S. striatus (0.11 g_{wwt} ± 0.01 SE, $N = 9$) and *B. leachii* (1.05 g_{wwt} ± 0.09 SE, $N = 9$) were collected from the Adams Beach bloom of *L. majuscula*. *S. striatus* were maintained as described above and fed with fresh *L. majuscula* collected from Adams Beach every 2 d since they rapidly consumed their food. *B. leachii* individuals consumed less *L. majuscula* and so they were only fed on d 1 (Table 1). Body secretions including ink, eggs (where available), and fecal matter were collected from each animal at the end of our experiment. Nine whole animals were pooled to form three replicates for HPLC–MS/MS analysis. Secretions were processed in the same manner.

The mean concentration of secondary metabolites in whole animal, secretions, and egested material were analyzed using single-factor analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) pairwise comparison tests. Data sets were subjected to Cochran and Levene's homogeneity of variance tests. Data that deviated from a normal distribution were transformed using √mean concentration.

RESULTS

Internal Partitioning of Secondary Metabolites. Significant differences were observed between the sequestration of secondary metabolites in the digestive gland (DG), and head and foot (HF) of *S. striatus* fed *L. majuscula* from Deception Bay (Figure 1A, ANOVA, df_1, $F_{318.36}$, $P < 0.001$). Both secondary metabolites (lyngbyatoxin-a and debromoaplysiatoxin) were sequestered at equivalent rates (ANOVA, df_1, $F_{1.95}$, $P = 0.20$). No secondary metabolites were detected in *S. striatus* fed blanched lettuce. Significant differences were also observed in the concentrations of lyngbyatoxin-a sequestered between the DG and HF of *B. leachii* (Figure 1B, ANOVA, df_1, $F_{10.77}$, $P = 0.030$). *Diniatys denifer* exhibited high individual variability in concentrations of secondary metabolites sequestered in both DG and HF (Figure 1C) with lyngbyatoxin-a detected in the DG but not the HF, and debromoaplysiatoxin in the HF and not in the DG.

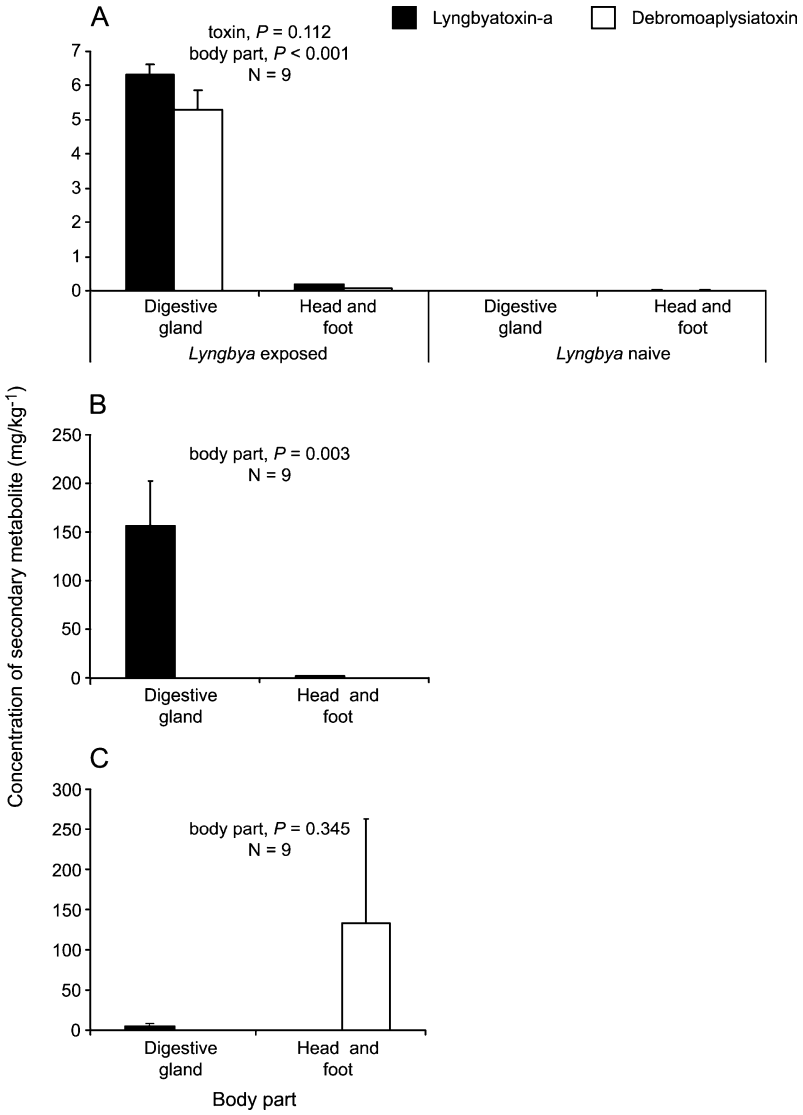

FIG. 1. Concentrations of lyngyatoxin-a and debromoaplysiatoxin in digestive gland and body tissue of (A) *S. striatus* previously exposed to *L. majuscula* fed *L. majuscula* from Deception Bay and *S. striatus* not knowingly exposed to *L. majuscula* fed blanched lettuce. (B) *B. leachii* fed *L. majuscula* from Adams Beach; (C) *D. dentifer* fed *L. majuscula* from Adams Beach. Bars are mean concentration of secondary metabolite (+SE). Body parts are significantly different from each other in (A) and (B) (single-factor ANOVA), but not (C). Scales differ between vertical axes.

Partitioning of Secondary Metabolites between Whole Animal and Secretions. Significant differences were observed between whole animal, secretions, and egested material for both *S. striatus* and *B. leachii* (ANOVA, df_1, $F_{441.89}$, $P < 0.001$) (Figure 2A,B). Lyngbyatoxin-a concentrations in *S. striatus* whole animal (3.94 mg/kg^{-1}) was much greater than secretions or egested material (ink 0.12 mg/kg^{-1}, fecal matter 0.56 mg/kg^{-1}, and eggs 0.05 mg/kg^{-1}) (ANOVA, df_3, $F_{188.93}$, $P < 0.001$, Figure 2A). *B. leachii*

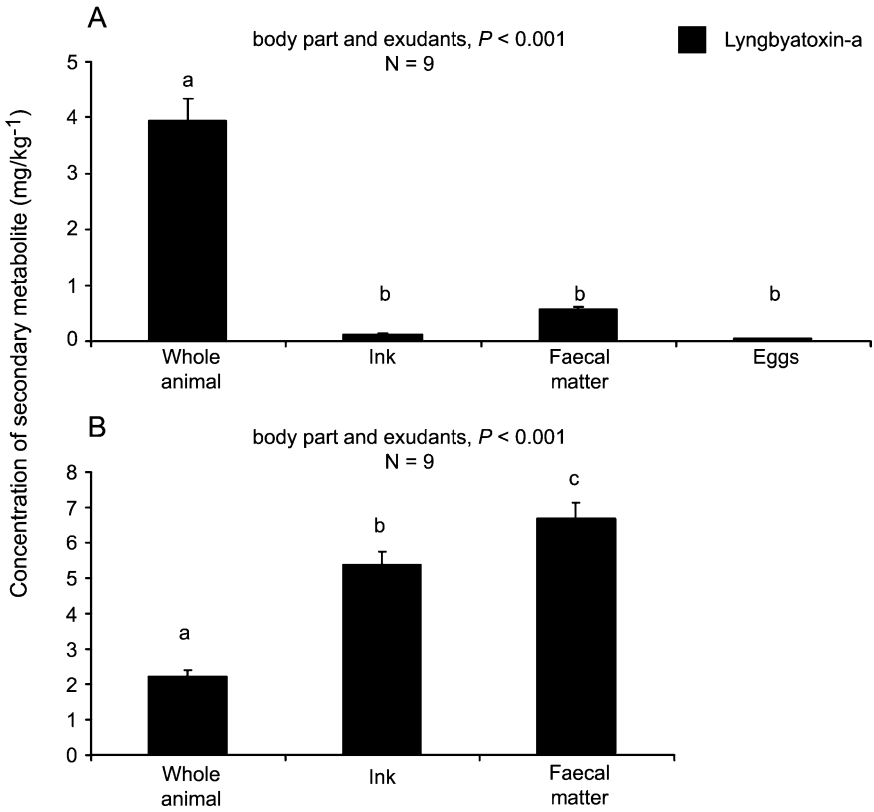

FIG. 2. Concentrations of lyngyatoxin-a (LTA) in whole animal, secretions, and egested material of (A) *S. striatus* and (B) *B. leachii* fed *L. majuscula* from Adams Beach. Bars are mean concentrations of LTA (+SE). Significant differences between whole animal and their bodily secretions are shown (single factor ANOVA). Different letters above the bars indicate significant differences between tissues, secretions and egested material ($P < 0.05$) using Fisher's LSD pairwise comparison tests. Bars with the same letter are not significantly different. (Eggs were not available from *B. leachii* during the trial period). Scales differ between vertical axes.

had lower concentrations of lyngbyatoxin-a (ANOVA, df_2, $F_{57.62}$, $P < 0.001$, Figure 2B) in whole animal (2.24 mg/kg^{-1}) compared to that of its ink (5.41 mg/kg^{-1}) and fecal matter (6.71 mg/kg^{-1}) (Figure 2B).

DISCUSSION

Our results show that *S. striatus* can sequester and compartmentalize both lyngbyatoxin-a and debromoaplysiatoxin. The different concentrations of lyngbyatoxin-a and debromoaplysiatoxin in the digestive gland were lower than those detected in whole *L. majuscula*, which may indicate bioaccumulation from feeding before our experiments. This highlights the importance of diet history when examining secondary metabolite dynamics in sea hares. Pennings and Paul (1993a) found that over a 3 wk period the concentration of malyngamides in *S. longicauda* did not change.

B. leachii has a different strategy for sequestering *L. majuscula* secondary metabolites. The digestive gland, ink, and the fecal matter had high concentrations of lyngbyatoxin-a. Transfer of lyngbyatoxin-a from body tissue to body secretions is one potential mechanism for toxin excretion. *S. striatus* seems to transform lyngbyatoxin-a and debromoaplysiatoxin into less harmful acetates (Pennings et al., 1996), but *B. leachii* may lack the digestive and physiological ability for transformation and, therefore, excretes these compounds. Lyngbyatoxin-a in the ink could also provide *B. leachii* with a defense mechanism against predation (Johnson and Willows, 1999).

The question of whether opisthobranch mollusks use sequestered secondary metabolites for defensive purposes has sparked some debate (Paul and Pennings, 1991; Pennings et al., 1999, 2001; Rogers et al., 2000). Compartmentalization of compounds in the digestive gland offers little protection if the sea hare must be eaten before the predator encounters the deterrent (Pennings et al., 2001). Pennings et al. (1999) argue that accumulation of extremely high concentrations of dietary-derived metabolites in the digestive gland are more likely to assist detoxification of a chemically rich diet than deter predators, whereas deployment of secondary metabolites to the skin or ink may afford some defensive protection (Pennings and Paul, 1993a; Rogers et al., 2000).

Ink containing high concentrations of lyngbyatoxin-a excreted by *B. leachii* might provide a defensive role; however, we suggest that this transfer might be an excretory mechanism employed by *B. leachii* to avoid toxicity. *B. leachii* demonstrated increasing growth rates over a 10-d period in the laboratory when fed a monospecific diet of *L. majuscula*. This reached a plateau between 10 and 20 d (Capper et al., unpublished data) with the death of the animal following soon after (personal observation). While morbidity may be related to the

toxicity of *L. majuscula*, it could also be related to incomplete nutritional requirements. Dense aggregations of *B. leachii* are found in local *L. majuscula* blooms. This may reflect exploitation of an abundant food source rather than dietary specialization. It is more likely that *B. leachii* consumes a mixed diet in the field (Paige, 1988), possibly getting more nutrients and preventing harmful additive accumulation of secondary metabolites in the digestive gland. The consequences of debromoaplysiatoxin consumption in *B. leachii* are unknown, as none was detected in *L. majuscula* used in these feeding trials.

Lyngbyatoxin-a and debromoaplysiatoxin found in *D. dentifer* after it ate *L. majuscula* for 10 d showed high individual variability. This may be related to the palatability of *L. majuscula* used in our feeding trials. However, a lack of uniformity in the distribution of secondary metabolites among test animals may suggest *D. dentifer* are not preferential consumers of *L. majuscula*. *D. dentifer* may use *L. majuscula* as a source of refuge from predation rather than a food source (Cruz-Rivera and Paul, 2002). Little is known of the dietary repertoire in this species and fate of *L. majuscula* secondary metabolites.

Debromoaplysiatoxin was the predominant toxin in *L. majuscula* blooms at Deception Bay during our experiments, but lyngbyatoxin-a was also detected. Lyngbyatoxin-a and debromoaplysiatoxin had previously been detected at Adams Beach, however, only lyngbyatoxin-a was detected during these experiments. This variation in *L. majuscula* secondary metabolites highlights their temporal and spatial variability.

Our results show distinct differences in sequestration mechanisms utilized by three species of opisthobranchs fed *L. majuscula*. This suggests that the generic sequestration hypothesis may not be applicable to all opisthobranch mollusks. Implications for bioaccumulation or biomagnification of lyngbyatoxin-a and debromoaplysiatoxin through the marine food web warrant further investigation in both *B. leachii* and *S. striatus*, particularly following mass mortalities after the disappearance of *L. majuscula* blooms. High quantities of lyngbyatoxin-a egested via fecal matter in *B. leachii* may also result in the introduction of *L. majuscula* secondary metabolites to phagotrophic or detrital food webs.

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COMPARISON OF THE VOLATILE ORGANIC COMPOUNDS PRESENT IN HUMAN ODOR USING SPME-GC/MS

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Abstract—We evaluated the volatile organic compounds (VOCs) present in human odor by using headspace solid phase microextraction gas chromatography–mass spectrometry for the extraction, separation, and analysis of the collected samples. Volatile organic compounds present in the headspace above axillary sweat samples collected from different individuals showed the presence of various types of compounds including organic fatty acids, ketones, aldehydes, esters, and alcohols. Qualitative differences and similarities noted between the males and females studied along with differences in chemical ratio patterns among the common compounds demonstrated the ability to differentiate between individuals through the examination of VOCs.

Key Words—Human scent, odor, uniqueness of odor profiles, axillary sweat, absorbent material, cotton absorbers, VOCs, SFE, headspace SPME, SPME-GC/MS.

INTRODUCTION

The idea that people can be distinguished based on their odors is not a new concept; tracking, trailing, and scent identification lineup canines have been used successfully for over 100 yr. Until recently, technological limitations have restricted the ability of researchers to identify the chemical components that comprise human odor or to use the information to chemically distinguish individuals.

Individual body odors of humans are determined by several factors that are either stable over time (genetic factors) or vary with environmental or internal conditions. We have developed distinguishing terminology for these factors: the

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"primary odor" of an individual contains constituents that are stable over time regardless of diet or environmental factors; the "secondary odor" contains constituents that are present because of diet and environmental factors; and the "tertiary odor" contains constituents that are present because of the influence of outside sources (i.e., lotions, soaps, perfumes, etc.) (Curran et al., 2005).

There is a limited understanding of how the body produces the volatile organic compounds present in human odor. Most of the relevant scientific research pertaining to human scent has evaluated the contents of axillary (armpit) and plantar (foot) sweat. Compounds present in both male (Zeng et al., 1991) and female (Zeng et al., 1996) pH-adjusted axillary secretion extracts have been isolated and identified through preparative gas chromatography, analysis by gas chromatography/mass spectrometry (GC/MS) and by gas chromatography/Fourier transform infrared spectroscopy (GC/FTIR). The analysis showed the presence of several C_6–C_{10} straight chains, branched, and unsaturated acids. Important odor contributors were terminally unsaturated acids, 2-methyl C_6–C_{10} acids, and 4-ethyl C_5–C_{11} acids, along with (*E*)-3-methyl-2-hexenoic acid, which was said to be the major odor-causing compound. Male and female extracts were qualitatively similar with minor quantitative differences. Short-chain fatty acids have also been extracted from sweat samples obtained from feet (Kanda et al., 1990). Extracts from socks were obtained through a 6-hr Soxhlet extraction with diethyl ether and analyzed by GC/MS. Short-chain fatty acids were found in all of the samples, but in greater amounts in subjects that claimed to have strong foot odor. Isovaleric acid was found only in the subjects who claimed to have strong foot odor.

Investigations into compounds emitted by humans that attract the yellow-fever mosquito have also provided insight into compounds present in human secretions (Bernier et al., 1999, 2000, 2002). Samples were collected using glass beads rolled between the fingers. The beads were loaded into a GC and cryofocused by liquid nitrogen at the head of the column before analysis by GC/MS. A total of 277 compounds were identified as components of human skin emanations. The results showed qualitative similarities in compounds among the individuals studied; however, quantitative differences were also noted.

Studies pertaining to residual axillary odor in clothing have shown the absence of organic acids and the presence of other odiferous compound classes. Laundry soiled with human sweat and then washed with a color laundry detergent has been analyzed for the residual presence of human odor (Munk et al., 2000). Esters, ketones, and aldehydes were identified as primary odorants in the swatches postwashing. However, organic acids, which are considered to be the dominant characteristic odorants in human axillary sweat, were not present in the extracts of residual odor.

Although the components of human sweat have been studied extensively, comparatively little work has been carried out to determine the volatile organic

compounds (VOCs) present in human odor. Knowing the contents of human sweat may not accurately represent the nature of what volatile compounds are present in the headspace above such samples that would comprise the odor.

Human odor components have been studied through headspace GC-MS for compounds specific to age (Haze et al., 2001). Compound classes such as hydrocarbons, alcohols, acids, ketones, and aldehydes were present in human odor. This study also presented 2-nonenal as a compound that is only present in the odor of individuals over 40 yr of age. 2-Nonenal, as well as other aldehydes, was produced through oxidative degradation of monosaturated fatty acids, such as palmitoleic acid and vaccenic acid.

Solid phase microextraction in conjunction with GC/MS has been used to identify volatile components from the skin of females (Ostrovskaya et al., 2002). Several different classes of compounds, including short- and long-chain hydrocarbons, short-chain aldehydes, and a branched ketone, were identified in human skin headspace analysis. The abundances of these compounds varied among individuals, and some subjects exhibited specific volatiles, such as 6-methyl-5-hepten-2-one, and hydrocarbons of shorter-chain lengths including decane.

We have previously shown human scent to be a combination of various compounds differing in ratio from person to person along with some compounds that differ among individuals (Curran et al., 2005). The chromatographic distinction among the headspace analysis of axillary sweat samples from males is possible based on a combination of the relative ratios of the common compounds among individuals. With the presence of some differing compounds, this combination of relative ratios supports the individual odor theory that has been anecdotally demonstrated by the ability of canines to discriminate among individuals based on their odor. Although there is some variation present within the same individual when sampled on multiple occasions, the ratio pattern of common compounds has been shown to be distinguishable among individuals. There were significantly greater variations in the ratio of components observed among individuals tested than for one individual.

We have defined human scent to be the most abundant VOCs identified in the headspace above scent samples. Other substances, however, may make contributions to human odor. Here, we discuss the use of headspace solid phase microextraction gas chromatography/mass spectrometry (SPME-GC/MS) as a method for the extraction, separation, and identification of the volatile components of human odor and how to use the information to differentiate among individuals.

METHODS AND MATERIALS

Materials. In previous studies by the authors, the absorbent material used to collect the odor samples was biologically sterile (meaning that it had been

sterilized utilizing an autoclave); however, it was determined that biologically sterile does not equate to "analytically clean," resulting in the exclusion of several key odor compounds including decanal and nonanal because of their initial presence in the headspace of the absorbent material. In this study, supercritical fluid extraction (SFE) using methanol modified carbon dioxide has been introduced as a pretreatment for the absorbent material that allows for an "analytically clean" collection medium. Gauze pads were Dukal brand, sterile, 2×2, 8-ply, gauze sponges (Dukal Corporation, Syosset, NY, USA). The vials used to hold the gauze were 10-ml glass, clear, screw top vials with PTFE/silicone septa (Supelco, Bellefonte, PA, USA). The extraction solvent for the pretreatment of the gauze pads by SFE was supercritical grade carbon dioxide (Air Products, Allentown, PA, USA). The methanol used as the modifier for the pretreatment of the gauze pads was HPLC grade (Fisher Scientific, Pittsburgh, PA, USA).

The neutrality of the soap chosen for this study was not a determining factor for use; rather the presence of commonly reported compounds in humans in the headspace of soaps provided the criteria for exclusion. Headspace analysis of different soap types revealed that soaps made from animal fat show the presence of some compounds previously reported in humans. The soap used by the subjects to wash all areas of the body was Natural, Clear Olive Oil Soap from Life of the Party (North Brunswick, NJ, USA). The headspace analysis of this brand of olive oil soap did not reveal the presence of these types of compounds and, thus, was chosen for use in this study.

Pretreatment of Gauze Pads by SFE. The equipment used was an ISCO Model 260D Syringe Pump with an SFX 2-10 Supercritical Fluid Extractor. Each round of SFE began by filling the plastic extraction vessel with two pieces of sterile gauze pads (~0.6 g). The optimum SFE conditions developed to extract organic volatile compounds from sterile absorbers included a 30-min static extraction time followed by a 10-min dynamic extraction time at an extraction temperature of 130°C, a pressure of 4500 psi, and the direct spiking of 500 μl HPLC grade methanol into the 10-ml extraction vessel. These samples were analyzed by a similar SPME-GC/MS procedure for compound identification, and quantification was employed to analyze scent samples described later in the text.

Method for Armpit Sampling. Eight subjects were evaluated: four males (M1, M2, M3, and M4) and four females (F1, F2, F3, and F4). Male 2 was 17 yr of age and male 4 was 22 yr. Female 1 was 21, and female 3 was 23 yr. Male 1, male 3, female 2, and female 4 were 24 yr. It is relevant to note that F3 and F4 are sisters who live in the same household. Subjects were required to use the olive oil soap and directed to shower at least twice by using the provided soap during the 48-hr period prior to sampling. Subjects were also instructed to discontinue the use of deodorants, lotions, and perfumes for at least 48 hr before

sampling to minimize the potential influence of "tertiary odors." No attempt was made to control the diet of the subjects. Each subject exercised outdoors for a period of 30 min while wearing a tank top to minimize the influence of compounds present because of the influence of clothing. Subjects sampled themselves with a pretreated 2×2 sterile gauze pad. They were instructed to wipe the armpit area to collect their sweat and then reseal the sample back into the 10-ml glass vial. All samples were stored in the vials at room temperature and allowed to sit for approximately 24 hr prior to extraction. These storage conditions were chosen to simulate the conditions under which odor is collected for canine evaluation purposes, and no attempt was made to control microbial interactions with the substrate as it may make contributions to the overall odor profile. The climatic conditions present during the samplings included an average temperature of 22.8°C and an average humidity of 77%.

Extraction and Analysis of Armpit Samples (SPME-GC/MS). Divinylbenzene/carboxen on polydimethylsiloxane (DVB/CAR on PDMS) 50-/30-μm fibers (Supelco) were used to extract the volatile organic compounds from the headspace of the vials containing the gauze. Exposure was conducted at room temperature for 15 hr, which was previously determined to be the optimal extraction time based on a combination of the number and abundance of compounds recorded (Curran and Furton, 2004). An Agilent 6970 gas chromatograph (GC) was used with a 5973 mass selective detector (MSD), and the column was an HP5-MS, 30 m, 0.25 μm, 0.25 mm with helium as the carrier gas (flow rate: 1.0 ml/min). The analytes were desorbed in the injection port of the GC with an inlet temperature of 250°C. The GC method was initiated with an initial oven temperature of 40°C for 5 min. The temperature was then ramped at 10°C/min until it reached 300°C, and then was held at 300°C for 2 min (total run time: 33 min). The mass spectrometer used was an HP 5973 MSD with a quadrupole analyzer in full scan mode (range: 50–550).

RESULTS

Pretreatment of Gauze by SFE. Headspace evaluation of initially sterile gauze revealed the presence of many different compounds including those listed

TABLE 1. COMPOUNDS PRESENT IN DUKAL GAUZE PRIOR TO SFE

Examples of compounds extracted from DUKAL Gauze		
Nonanal	Tetradecane	Undecanal
Decanal	Heptadecane	Cyclotetradecane
Hexadecane	Phenol	Nonadecane
2-Ethyl-hexanoic acid	Benzyl alcohol	2-Butoxy-ethanol

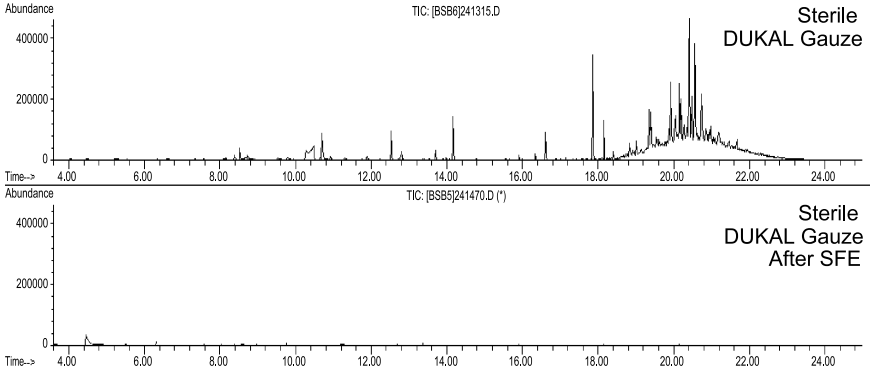

FIG. 1. Chromatographic comparison of sterile gauze before and after SFE treatment.

in Table 1. It is important to remove these compounds prior to sampling because some have been reported as components of human emanations, such as nonanal, decanal, and tridecane. Figure 1 shows the chromatographic difference between the gauze as purchased and after SFE extraction. It is shown with the siloxane peaks attributed to the SPME fiber coating and the column removed. The resulting headspace of the gauze is "chromatographically clean" meaning that all compounds have been removed.

Comparison of Chromatograms Produced from Different Individuals. Figure 2 shows two chromatograms produced from samplings of male 2 collected 7 d apart. It is shown with the siloxane peaks removed; these peaks are attributed to the SPME fiber coating and the column. Differences in abundance between the chromatograms are noted and may be due to concentration

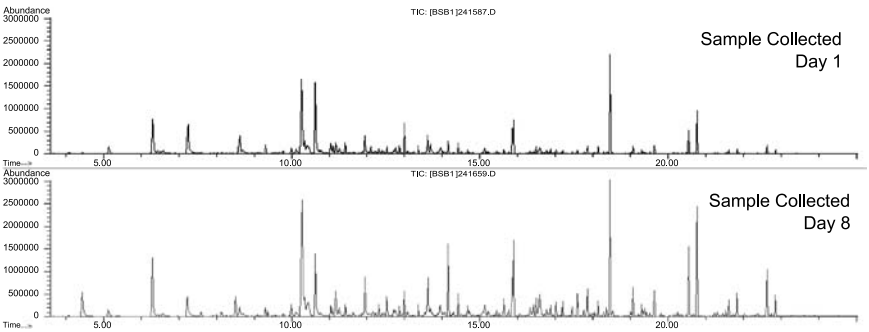

FIG. 2. Chromatograms from 3.5 to 25 min, comparison of male 2 odor profiles produced from samples collected 7 d apart.

differences among the samples; however, similarities in the ratio patterns of the peaks are evident between the two chromatograms. There are 23 common compounds extracted within the two samplings of male 2. We have previously shown the ratio patterns for an individual to be reproducible over time (Curran et al., 2005).

Figure 3 shows the chromatograms produced from the four different male subjects, and Figure 4 demonstrates the chromatograms produced from the different females. Figure 3 shows the chromatograms expanded to highlight the profiles produced among the male subjects, which results in off-scale decanal, dodecanoic acid-methyl ester, and dodecanoic acid peaks in the chromatogram for male 3. Figure 4 shows the chromatograms expanded to highlight the profiles produced among the female subjects, which results in off-scale nonanal and decanal peaks in the chromatogram for female 3. As can be seen from Figures 3 and 4, there are some common compounds present among the subjects and also some compounds present that differ. Table 2 lists the compounds found in the odor profiles of the eight individuals, with superscripts noting where they have been previously reported. The types of compounds determined to be in the odor

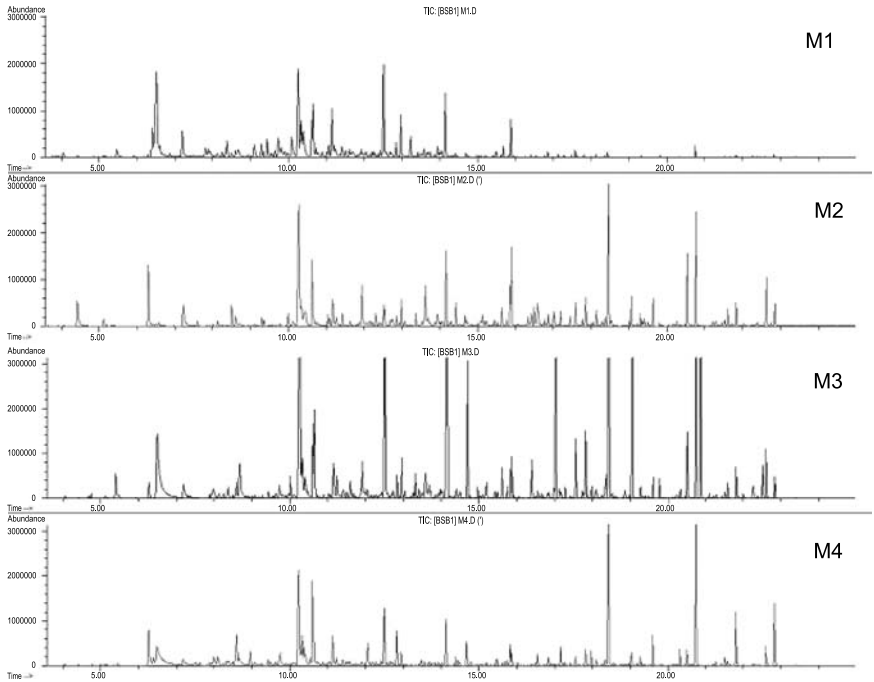

FIG. 3. Chromatograms from 3.5 to 24 min, comparison of odor profiles from male 1, male 2, male 3, and male 4 after 30 min of exercise.

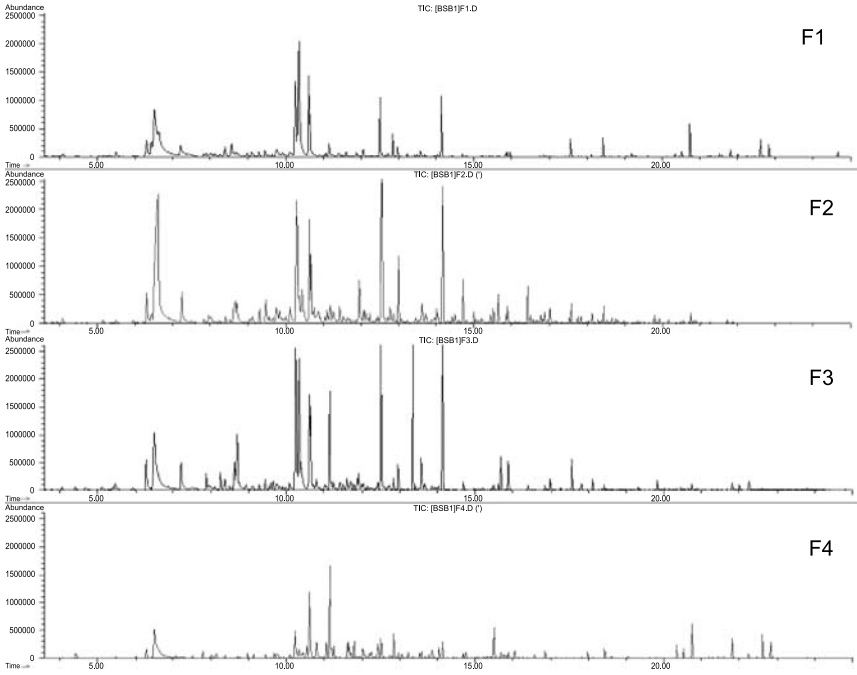

FIG. 4. Chromatograms from 3.5 to 24 min, comparison of odor profiles from female 1, female 2, female 3, and female 4 after 30 min of exercise.

profile include alcohols, aldehydes, alkanes, carboxylic acids, esters, and ketones. The compounds were identified by spectral library comparisons or by standard comparison. Seven of the compounds were present in all of the subjects studied: decanal, dodecanoic acid-methyl ester, nonanal, octanoic acid-methyl ester, phenol, tetradecane, and tetradecanoic acid-methyl ester. Hexanal and 1-chloro-nonane were extracted in only one of the subjects. Dodecanoic acid, propanedioic acid-methyl ester, octanal, and tetradecanoic acid were extracted in some of the males studied, yet were not present in any of the female profiles.

Table 3 shows the ratios of the 23 common compounds extracted from the samplings of male 2 across the eight subjects relative to decanal, which was chosen because it has been extracted in the profiles of every individual studied through this method. The resulting ratio patterns of the common compounds differ among the eight individuals studied. The differences in profiles determined for female 3 and female 4 who live in the same household suggest that the odor profiles produced from individuals with similar genetic makeup and residing in the same environmental conditions can produce distinguishable odor profiles.

TABLE 2. COMPOUNDS EXTRACTED FROM MALE 1 (M1), MALE 2 (M2), MALE 3 (M3), MALE 4 (M4), FEMALE 1 (F1), FEMALE 2 (F2), FEMALE (F3), AND FEMALE 4 (F4) AND REFERENCED FOR PREVIOUS REPORTS

Compound (by functional group)	M1	M2	M3	M4	F1	F2	F3	F4
Aldehydes								
2-Furancarboxaldehyde	X		X	X	X	X	X	X
(*E*)-2-Nonenal[a,b,c]			X		X	X	X	
Benzaldehyde[c,d,e,f]	X		X			X		
Decanal[b,d,g,h,i]	X	X	X	X	X	X	X	X
Hexanal[a,b,c,f,h,j,k]					X			
Heptanal[b,c,d,e,f,j,k]			X				X	
Nonanal[b,d,e,g,h,j]	X	X	X	X	X	X	X	X
Octanal[a,b,d,g,h]	X		X					
Tetradecanal	X	X	X			X		
Undecanal[c,h]		X	X			X	X	
Carboxylic acids								
Dodecanoic acid[c,d,e,h,l]			X					
Tetradecanoic acid[d,h,i]			X					
Ketones								
6,10-Dimethyl-5,9-Undecadien-2-one[c,d]	X	X	X	X	X	X	X	
6-Methyl-5-hepten-2-one[b,c,d,e,g]	X			X	X		X	
Alcohols								
2-Furanmethanol[d]	X	X	X		X	X	X	
Benzyl Alcohol[c,d,m]			X					
Phenol[c,d,l,m]	X	X	X	X	X	X	X	X
Aliphatic/aromatic								
α-Pinene[f]							X	X
Dodecane[b,j]	X	X			X	X	X	X
Heptadecane[c,d]		X	X	X		X	X	
Hexadecane[d,g]	X	X				X	X	
Naphthalene[a]				X			X	X
Nonane[c,d,j]				X				X
Nonane, 1-chloro-[d,e]					X			
Tetradecane[d,g]	X	X	X	X	X	X	X	X
Toluene[c,d,e,f]		X		X			X	X
Tridecane	X	X		X		X	X	
Undecane[b,j,k]				X		X	X	
Esters								
7-Hexadecenoic acid–methyl ester[m]		X	X	X	X			X
Acetic acid-phenylmethyl ester			X					
Cyclopentanetridecanoic acid–methyl ester	X							
Decanoic acid–methyl ester		X	X	X	X	X		X
Dodecanoic acid, 10-methyl-, methyl ester		X	X	X	X			X
Dodecanoic acid–methyl ester	X	X	X	X	X	X	X	X
Furancarboxylic acid–methyl ester	X				X	X		
Hexadecanoic acid–methyl ester[d]	X	X	X	X	X		X	X
Hexanedioic acid–dimethyl ester[d]	X			X	X	X		
Hexanoic acid–methyl ester	X			X	X			X

TABLE 2. CONTINUED

Compound (by functional group)	M1	M2	M3	M4	F1	F2	F3	F4
Methyl 9-methyltetradecanoate	X			X		X		X
Nonanoic acid–methyl ester[d]	X	X	X	X	X	X	X	
Octanoic acid–methyl ester	X	X	X	X	X	X	X	X
Pentadecanoic acid–methyl ester		X		X	X			
Propanedioic acid–dimethyl ester		X	X					
Tetradecanoic acid–methyl ester	X	X	X	X	X	X	X	X
Tridecanoic acid–methyl ester[d]		X	X	X	X	X		X
Undecanoic acid–methyl ester		X	X	X		X		
Amines/amides								
Pyridine[d,e]	X				X			

[a] Component of residual armpit odor (Munk et al., 2000).
[b] Component of human body odor (Haze et al., 2001).
[c] Component of human odor (Curran et al., 2005).
[d] Component in human skin emanation (Bernier et al., 2000).
[e] Component of skin emanations (Bernier et al., 2002).
[f] Volatile component of blood (Deng et al., 2004).
[g] Volatile component of the skin (Ostrovskaya et al., 2002).
[h] Component of skin emanations (Bernier et al., 1999).
[i] Component of fingerprint residue (Asano et al., 2002).
[j] Component of human breath (Philips, 1997).
[k] Component of human breath (Philips et al., 1999).
[l] Component of armpit odor (Zeng et al., 1991).
[m] Component of armpit odor (Zeng et al., 1996).
[n] Component of fingerprint residue (Ramotowski, 2001).

DISCUSSION

The SPME-GC/MS headspace analysis method proved to be an effective route for obtaining odor profiles from both males and females. The use of SFE as a pretreatment for the absorber material used as the collection medium allows for the inclusion of compounds that were previously excluded because of their background presence, such as decanal and nonanal. Both qualitative and quantitative differences were observed among the males and females studied. Various types of compounds were extracted from males and females, including a variety of aldehydes, organic fatty acids, ketones, and alkanes, which agrees with Haze et al. (2001); Ostrovskaya et al. (2002); Curran et al. (2005) and our previous findings. Compounds including 2-furancarboxaldehyde, tetradecanal, tridecane, along with various esters are reported here for the first time as components of human odor. A majority of the compounds in the odor profiles of both males and females were esters and aldehydes, and this is in agreement with research previously conducted on the compounds responsible for the residual presence of human odor in washed clothing by Munk et al. (2000). The common

TABLE 3. COMPARISON OF THE RELATIVE RATIOS OF 23 COMPOUNDS EXTRACTED FROM SAMPLINGS OF MALE 2 ACROSS THE EIGHT SUBJECTS

Compound name	M1	M2	M3	M4	F1	F2	F3	F4
2-Furanmethanol	0.8123	0.6587	0.0629	0.0000	0.3270	0.4442	0.4495	0.0000
6,10-Dimethyl-5,9-undecadien-2-one	0.1317	0.3298	0.1301	0.2555	0.3203	0.1184	0.1881	0.0000
7-Hexadecenoic acid–methyl ester	0.0000	0.7508	0.1230	0.1322	0.3782	0.0000	0.0000	0.1903
Decanal	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Decanoic acid–methyl ester	0.0000	0.5549	0.0981	0.4836	0.0570	0.0494	0.0000	0.2717
10-Methyl dodecanoic acid–methyl ester	0.0000	0.2161	0.0438	0.2596	0.0212	0.0000	0.0000	0.0644
Dodecanoic acid–methyl ester	0.1443	1.7986	0.8422	3.5507	0.3677	0.1143	0.0635	0.8293
Heptadecane	0.0000	0.0782	0.0117	0.0968	0.0000	0.0189	0.0266	0.0000
Hexadecane	0.0487	0.1129	0.0000	0.0000	0.0000	0.0249	0.0277	0.0000
Hexadecanoic acid–methyl ester	0.0937	0.3257	0.0746	1.3404	0.2626	0.0000	0.0222	1.3264
Nonanal	1.4722	0.3687	0.6401	1.2663	1.0102	1.3343	1.0494	1.4757
Nonanoic acid–methyl ester	0.1700	0.4556	0.0444	0.2976	0.0496	0.0585	0.0199	0.0000
Octanoic acid–methyl ester	0.3707	0.2343	0.0764	0.8153	0.4346	0.1017	0.1255	1.6473
Pentadecanoic acid–methyl ester	0.0000	0.3573	0.0000	1.2698	0.1624	0.0000	0.0000	0.0000
Phenol	2.1319	2.9416	1.0536	3.7101	1.9029	1.5757	1.2160	2.4801
Propanedioic acid–dimethyl ester	0.0000	0.1291	0.0233	0.0000	0.0000	0.0000	0.0000	0.0000
Tetradecanal	0.0585	0.3002	0.0112	0.0000	0.0000	0.0943	0.0000	0.0000
Tetradecane	0.1264	0.2141	0.0215	0.2300	0.0275	0.0747	0.0425	0.5113
Tetradecanoic acid–methyl ester	0.2199	1.4487	0.3543	3.5937	0.5599	0.1004	0.0727	2.0648
Toluene	0.0000	0.7099	0.0000	0.0604	0.0000	0.0000	0.0401	0.5343
Tridecane	0.1408	0.1421	0.0000	0.0967	0.0000	0.0849	0.0619	0.0000
Tridecanoic acid–methyl ester	0.0000	0.4404	0.0886	0.7546	0.0385	0.0144	0.0000	0.1619
Undecanal	0.0000	0.3395	0.0699	0.0000	0.0000	0.1555	0.0449	0.0000

compounds determined among the individuals studied included aldehydes, an alcohol, alkanes, and esters. The common compounds were present in differing ratio patterns between the males and females, indicating qualitative similarities among individuals with quantitative differences.

It is worth noting that (*E*)-3-methyl-2-hexenoic acid was not detected in the headspace of the axillary samples studied, which was reported to be the major odor-causing compound by Zeng et al. (1991, 1996). Ostrovskaya et al. (2002) suggested that 6-methyl-5-hepten-2-one was a volatile compound specific to some individuals, and it was detected in male 1, male 4, female 1, and female 3. The compound 2-nonenal was also present in the odor profiles of three of the females and one of the males studied, and we have previously reported it in the odor profile of males (Curran et al., 2005). The presence of 2-nonenal in human odor has been reported by Haze et al. (2001) to be an age-specific compound that is only seen in individuals ≥40 yr of age. The presence of 2-nonenal in the odor profiles of individuals under 25 yr of age in the current and previous study does not support the conclusions reached by Haze et al. (2001). Further study into the effect of race, age, and sex on odor profiles is necessary to determine whether there are compounds present that are group specific.

Overall, the SPME-GC/MS headspace method developed for the analysis of human odor profiles allows for identification of the volatile organic compounds present in human scent samples. Through this method, a combination of the relative ratios of common compounds and the presence of differing compounds allows for the chromatographic distinction among individuals. The authors have defined human scent to be the most abundant VOCs present in the headspace above scent samples; however, other substances that have a relatively low volatility or are present in low concentrations may also make contributions to human odor. Ongoing studies include large scale sampling of population groups in an attempt to determine the compounds that lead to the uniqueness of human scent.

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MANDIBULAR GLAND SECRETION OF *Melipona beecheii*: CHEMISTRY AND BEHAVIOR

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Abstract—The mandibular gland secretion of *Melipona beecheii* contains a rich mixture of terpenoid and oxygenated compounds and unsaturated and saturated hydrocarbons. However, it differs markedly from the 11 other Brazilian species examined in previous studies, both by the absence of 2-heptanol and the presence of rose oxides. The most abundant compound was geranyl hexanoate, whereas the most volatile compounds were *cis*- and *trans*-rose oxide and geraniol. The complete blend and five individual components found in the gland secretion were tested by electroantennography (EAG) and behavioral assays. The complete mandibular gland extract and geraniol elicited the strongest EAG responses, whereas these and farnesyl acetate induced the strongest attack response from workers. The role of the rose oxides remains to be elucidated, as they do not appear to play a major role as an alarm pheromone of this species.

Key Words—Stingless bees, mandibular glands, *cis*-rose oxide, *trans*-rose oxide, geraniol, alarm response, electroantennography.

INTRODUCTION

Stingless bees employ a diversity of recruitment strategies ranging from odor communication to auditory signals produced inside the nest (Lindauer and Kerr,

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1958; Esch et al., 1965; Biesmeijer et al., 1998; Nieh and Roubik, 1998; Aguilar and Briceño, 2002). Mandibular gland volatiles serve as alarm pheromones in honeybees and many species of stingless bees (Cruz-Landim, 1967; Collins et al., 1989). In others, such as *Trigona hyalinata*, the mandibular gland secretion is used as an odor trail to guide nest mates (Nieh et al., 2003). The mandibular gland secretion of *Melipona* species appears to be used for alarm recruitment and defense, as seen in *M. fasciata* and *M. interrupta triplaridis* (Smith and Roubik, 1983). Both species have 2-heptanol as the major component with a small amount of undecane, whereas *M. interrupta triplaridis* also contains skatole and nerol. Francke et al. (1987) found that the cephalic secretions of both *M. marginata* and *M. quadrifasciata* contained 2-heptanol as the major component. Gracioli-Vitti et al. (2004) recently studied the mandibular glands of different *M. bicolor* castes and sexes and found a blend of hydrocarbons, alcohols, esters, and carboxylic acids, with 2-heptanol present in both workers and virgin queens. However, in neither study was 2-heptanol tested in behavioral assays. In contrast, Nieh (1998) reported that workers of *M. panamica* exhibited typical alarm behavior towards conspecific mandibular gland extracts, but no chemical information was provided.

Melipona beecheii, a species distributed throughout much of South and Central America (Roubik, 1990) is of potential economic and ecological importance in Mexico (Quezada-Euán and González-Acereto, 1994; Ayala, 1999; Moo-Valle et al., 2000), both for honey production and as a pollinator of crops. Some evidence suggest that *Melipona* species do not use odor trails, but returned foragers produce a series of bursts of sounds to indicate the distance of the food source (Wille, 1983). Greater knowledge of the organization and communication of *M. beecheii* is required, so the aim of this work was to chemically identify the mandibular gland secretions and to learn something of their function.

METHODS AND MATERIALS

Extracts. Preliminary experiments were made using a colony from Costa Rica through the kind cooperation of M. J. Sommeijer, University of Utrecht, The Netherlands. For the main experiments, workers of *M. beecheii* were obtained from an established colony in the El Colegio de la Frontera Sur (ECOSUR) meliponary, located in Tapachula, Chiapas, Mexico. Foraging workers were collected outside of the nest, cooled, and then the mandibles, together with the mandibular glands, were dissected out under distilled water. Six glands were cleanly detached from the mandibles and sealed in individual small soft glass capillaries for analysis. Another series was extracted

in a small tissue grinder with HPLC-grade hexane (200 μl) for testing in bioassays.

Chemical Analysis. Glands were introduced directly into the gas chromatograph (GC), without solvent, as described by Morgan (1990). Gas chromatographic separation was performed on a Varian Star model 3400 CX GC (Palo Alto, CA, USA). A DB-5 column (30 m × 0.25 mm ID) was temperature programmed from 50°C (held for 2 min) to 250°C at 15°C min^{-1}. The injection port temperature was held at 200°C. The sample was heated in the injector to 200°C for 5 min before crushing the glass capillary. The GC was coupled to a Varian Saturn 4D mass spectrometer and integrated data system (Palo Alto, CA, USA). Ionization was by electron impact at 70 eV, 230°C. The amount of each compound was calculated from the peak area, whereas the relative percentage of the components was calculated from the sum of areas of all recorded peaks. Mass spectral identifications were confirmed, wherever possible, by comparison of retention times and mass spectra with those of synthetic standards. Where pure standards were not available, tentative identification was based on comparison with spectra from the computer library NIST 2001.

Chemicals. Synthetic geraniol, farnesyl acetate, (−)-*cis*-rose oxide, (−)-rose oxide, and (+)-rose oxide were obtained from Fluka Sigma-Aldrich (Toluca, Mexico). Both (−)-rose oxide, and (+)-rose oxide contained more of the *cis*-isomer, with a *cis*/*trans* ratio of 2:1. The purity of these compounds was >95% by GC. Geranyl hexanoate and geranyl octanoate were synthesized in our laboratory with a purity >95% using the method of Attygalle and Morgan (1986).

Bioassays. Tests were carried out using protocols similar to those of Smith and Roubik (1983). One established colony of *M. beecheii* in the ECOSUR meliponary was used. In a first experiment, the response of *M. beecheii* workers to different concentrations of mandibular gland extract was evaluated. In a second experiment, the effect of selected synthetic compounds found in the mandibular gland was tested at different doses. Test materials were applied to a 7-mm diam Whatman No. 2 filter paper disk, on an entomological pin, 2.5 cm above the control stimulus, a dark-blue nylon-cloth ball of approximately 8-cm diam. The number of bees biting the ball was recorded for 1 min when it was held 5 cm below the nest entrance. Ten microliters of extract (0.1, 0.5, or 1 gland equivalent) or 10 μl of an HPLC-grade hexane solution of individual synthetic compounds (1 μg, 100 ng, 10 ng) were applied to the filter paper disk. Controls consisted of 10 μl of HPLC-grade hexane, and in all cases, the solvent was allowed to evaporate for at least 20 sec before each test. After presenting the control stimulus alone, extracts, synthetics, and controls were tested in random order between 0700 and 0900 hr local time, with 20-min intervals between stimuli to allow the nest to calm down. The complete assay was repeated on eight dry days with a similar temperature (ca. 30°C).

TABLE 1. CHEMICAL COMPOSITION OF THE MANDIBULAR GLAND SECRETION OF *Melipona beecheii* WORKERS

Peak number	Compound	Percentage	±SD	Mol. mass
1	*cis*-Rose oxide	t		154
2	*trans*-Rose oxide	t		154
3	Geraniol	t		154
4	Unidentified	0.45	0.52	
5	Rose oxide homologue ($C_{15}H_{26}O$)	0.55	0.63	222
6	Rose oxide homologue ($C_{15}H_{26}O$)	0.60	0.65	222
7	Rose oxide homologue ($C_{15}H_{26}O$)	0.25	0.21	222
8	Ethyl laurate	0.34	0.28	228
9	Methyl 2,3-dihydrofarnesoate	0.98	0.34	252
10	Methyl myristate	t		256
11	Geranyl hexanoate	15.10	3.49	252
12	Terpene ester, possibly structure 3	4.47	0.38	266
13	Ethyl myristate	0.34	0.10	256
14	Isopropyl 2,3-dihydrofarnesoate ?	0.38	0.23	280
15	Farnesyl acetate	0.28	1.94	264
16	Propyl 2,3-dihydrofarnesoate ?	1.60	0.08	280
17	Nonadecene	0.14	1.49	266
18	Nonadecane	1.43	0.22	268
19	β-Springene	0.38	0.75	272
20	Methyl palmitate	1.85	0.75	270
21	Geranyl octanoate	1.21	0.80	280
22	Possible springene isomer ?	1.12	0.20	272
23	Possible springene isomer ?	1.30	0.68	272 ?
24	Ethyl palmitate	1.58	1.18	284
25	A dihydrogeranylgeraniol	1.58	2.26	292
26	Ester of dihydrofarnesoic acid ?	4.18	2.82	306
27	Ester of dihydrofarnesoic acid ?	5.16	6.69	306
28	Heneicosene	5.16	4.18	294
29	Methyl oleate	1.23	2.23	296
30	Methyl stearate	3.61	0.39	298
31	Geranyl decanoate	2.56	3.03	308
32	Ethyl oleate	5.62	3.03	310
33	Unidentified and ethyl stearate ?	1.67	1.60	312
34	Terpenoid	1.28	0.68	
35	Terpenoid	0.91	0.69	
36	Ester of dihydrofarnesoic acid ?	0.38	0.34	
37	Tricosene	0.59	0.31	322
38	Terpenoid	0.74	0.30	
39	Tricosane	0.60	0.22	324
40	Terpenoid	0.26	0.20	
41	Unidentified	0.62	0.46	
42	Terpenoid	0.76	8.42	
43	Docosanoic acid	9.72	2.85	
44	Terpenoid	3.01	1.87	
45	Pentacosadiene	2.89	0.44	348

TABLE 1. CONTINUED

Peak number	Compound	Percentage	±SD	Mol. mass
46	Pentacosene	0.90	2.34	350
47	Pentacosane	2.67	0.48	352
48	Terpenoid	3.28	2.78	
49	Terpenoid	1.89	0.81	
50	Terpenoid	2.04	0.39	
51	Terpenoid	3.05	2.01	
52	Terpenoid	2.39	1.67	

t = Trace, $N = 6$. Unconfirmed identifications are marked with ?.

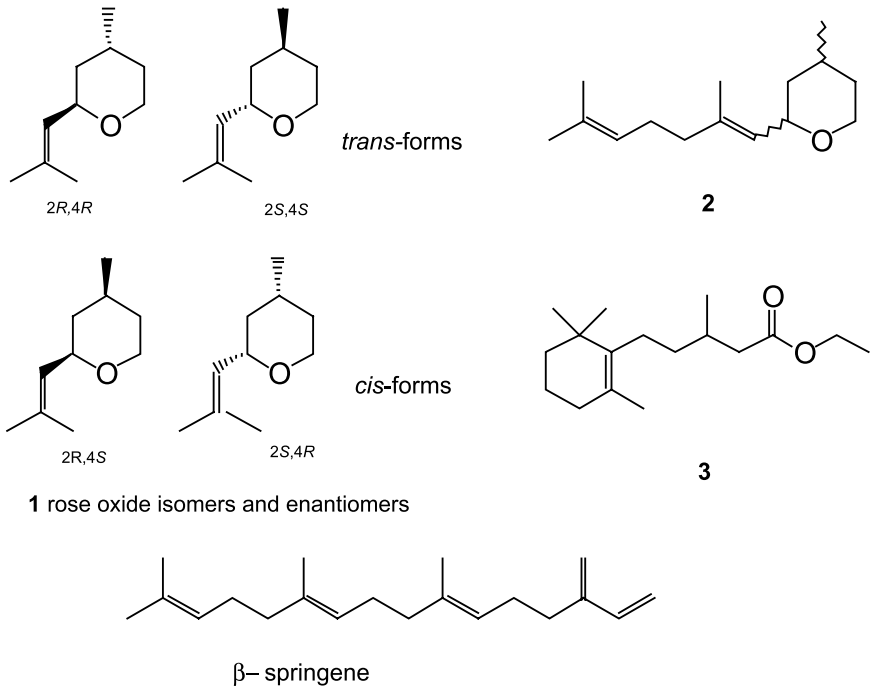

FIG. 1. Structures, and proposed structures, of compounds found in the mandibular gland of *Melipona beecheii* workers. **1**, The rose oxide enantiomers; **2**, the proposed structure of compounds 5–7 in Table 1, homologues of rose oxide; **3**, the suggested structure of compound 12 in Table 1, and the structure of β-springene.

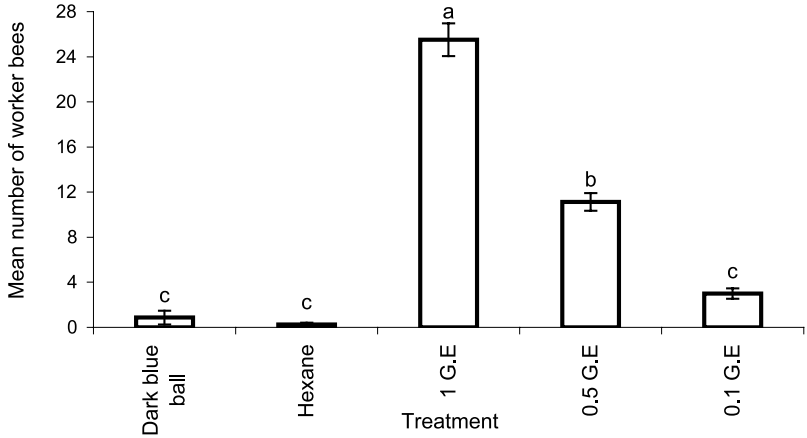

FIG. 2. Response of *M. beecheii* workers to different concentrations of mandibular gland extracts.

Electroantennography. Antennal receptivity of the *M. beecheii* workers to the selected chemical compounds was determined by electroantennography (EAG). The head of a worker was cut off carefully, and the reference glass capillary electrode was inserted into its base. The distal end of the antenna was inserted into the tip of the recording glass capillary electrode. The capillaries

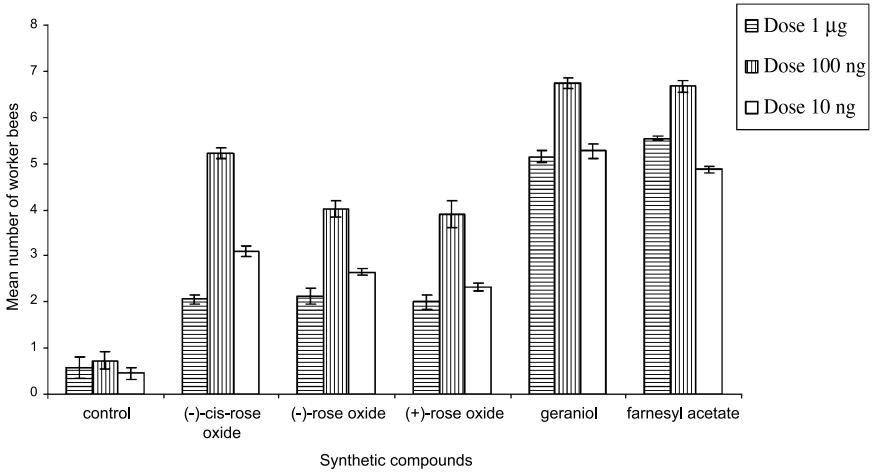

FIG. 3. Response of *M. beecheii* workers at different concentrations of synthetic compounds known to occur in the mandibular gland secretions.

were filled with saline solution (Malo et al., 2002). The signals generated by the antenna were passed through a high-impedance amplifier (Syntech NL 1200, Hilversum, The Netherlands) and displayed on a monitor using Syntech software for processing EAG signals.

Dilutions of the synthetic compounds and extracted mandibular glands were prepared in HPLC-grade hexane to make 1-μg/μl solutions. For each solution, 1 μl was applied to a filter paper (0.5 × 3.0 cm, Whatman no. 1), left 20 sec to allow the solvent to evaporate, and then inserted into a glass Pasteur pipette or sample cartridge 40 sec before testing. New cartridges were prepared for every insect tested. A stimulus controller (CS-05, Syntech) was used to generate stimuli at 1-min intervals. A current of humidified pure air (0.7 l/min) was constantly directed onto the antenna through a glass tube of 10-mm diam to ensure that odors were removed immediately from the vicinity. To present a stimulus, the pipette tip containing the test compounds or the extracts was inserted through a side hole located at the midpoint of the glass tube (10-mm diam) through which humidified pure air flowed at 0.5 l/min. The duration of the stimulus was 1 sec. Control stimulus (hexane) was presented at the beginning, followed by stimuli of the chemical products and gland extracts in

TABLE 2. THE RESPONSE OF *M. beecheii* WORKERS TO SYNTHETIC COMPOUNDS, FOUND IN THE MANDIBULAR GLAND SECRETIONS, AS A FUNCTION OF CONCENTRATION

Dose	Synthetic compounds	Mean	SD	Comparisons
1 μg	Farnesyl acetate	5.5	0.04	A
	Geraniol	5.1	0.1	A
	(−)-Rose oxide	2.1	0.1	B
	(−)-*cis*-Rose oxide	2.0	0.09	B
	(+)-Rose oxide	1.9	0.1	B
	Control	0.5	0.2	C
100 ng	Farnesyl acetate	6.6	0.1	A
	Geraniol	6.7	0.1	A
	(−)-Rose oxide	4.0	0.1	C
	(−)-*cis*-Rose oxide	5.2	0.1	B
	(+)-Rose oxide	3.8	0.2	C
	Control	0.7	0.1	D
10 ng	Farnesyl acetate	4.8	0.07	A
	Geraniol	5.2	0.1	A
	(−)-Rose oxide	2.6	0.08	BC
	(−)-*cis*-Rose oxide	3.0	0.1	B
	(+)-Rose oxide	2.3	0.09	C
	control	0.4	0.1	D

Means followed by different letters in the same column are significantly different at the 5% level by Tukey test.

random order. At the end, a second control stimulus was applied. For analysis of the EAG recorded, we used only the amplitude value in mV. We used one antenna of the bee for each series of the chemical products and the extracts tested, and at least 10 bees were used.

Data Analysis. The workers dose response to the gland extracts and EAG data were analyzed by one-way analysis of variance (ANOVA) (program GLM, SAS Institute, 2001). In some cases, before ANOVA, data were $\sqrt{x}$ transformed to stabilize variances. Significant differences were separated by a Tukey test. Dose response to synthetic compounds was analyzed by a two-way ANOVA (Statistica, Statsoft, Inc., 2004). Data were $(\sqrt{x} + 0.1)^{0.6}$ transformed to stabilize variances. Means were separated by a Tukey test. In all cases, $P = 0.05$.

RESULTS

Chemical Analysis. The mandibular gland secretion of *M. beecheii* contains a rich mixture of terpenoids, fatty acid esters, and unsaturated and saturated hydrocarbons (Table 1). The major component geranyl hexanoate, as well as geranyl octanoate, farnesyl acetate, and *cis*- and *trans*-rose oxide (Figure 1),

TABLE 3. THE RESPONSE OF *M. beecheii* TO COMPOUNDS FOUND IN THE MANDIBULAR GLAND SECRETIONS AS A FUNCTION OF CONCENTRATION

Synthetic compounds	Dose	Mean	SD	Comparisons
Farnesyl acetate	1 μg	5.5	0.04	B
	100 ng	6.6	0.1	A
	10 ng	4.8	0.07	C
Geraniol	1 μg	5.1	0.1	B
	100 ng	6.7	0.1	A
	10 ng	5.2	0.1	B
(−)-Rose oxide	1 μg	2.1	0.1	C
	100 ng	4.0	0.1	A
	10 ng	2.6	0.08	B
(−)-*cis*-Rose oxide	1 μg	2.0	0.09	C
	100 ng	5.2	0.1	A
	10 ng	3.0	0.1	B
(+)-Rose oxide	1 μg	1.9	0.1	B
	100 ng	3.8	0.2	A
	10 ng	2.3	0.09	B
Control	1 μg	0.5	0.2	A
	100 ng	0.7	0.1	A
	10 ng	0.4	0.1	A

Means followed by different letters in the same column are significantly different at the 5% level by Tukey test.

was identified by using spectra and retention times of synthetic compounds. Other rose oxide homologues (peaks 5–7 in Table 1) have higher masses but all the spectral characteristics of rose oxides, with base peaks at *m*/*z* 139. We suggest they share structure **2** shown in Figure 1. Methyl 2,3-dihydrofarnesoate, as were the methyl and ethyl esters of common fatty acids, was tentatively identified based on good matches with the spectra in the NIST spectral library. β-Springene, identified by its mass spectrum, has already been identified in other stingless bees. The structure of an abundant ethyl ester, related to but not a homologue of methyl 2,3-dihydrofarnesoate, was tentatively deduced from its spectrum (*m*/*z* 109 as base peak) to be similar to structure **3** in Figure 1. There were also smaller amounts of hydrocarbons and higher unidentified terpenes. Some of the hydrocarbons were most likely of cuticular origin as they were found in an analysis of a small fragment of cuticle.

Bioassays. M. beecheii workers exhibited a significant dose-dependent response to mandibular extracts ($F = 168.87$, $df = 4, 35$; $P < 0.001$) (Figure 2). In the case of the individual synthetic compounds, *M. beecheii* workers exhibited a significant interaction between dose and synthetic compounds (two-way ANOVA, $F = 12.89$, $df = 10, 126$, $P < 0.001$) (Figure 3). At each concentration tested, all synthetic compounds resulted in significantly higher responses than controls, with geraniol and farnesyl acetate eliciting the highest

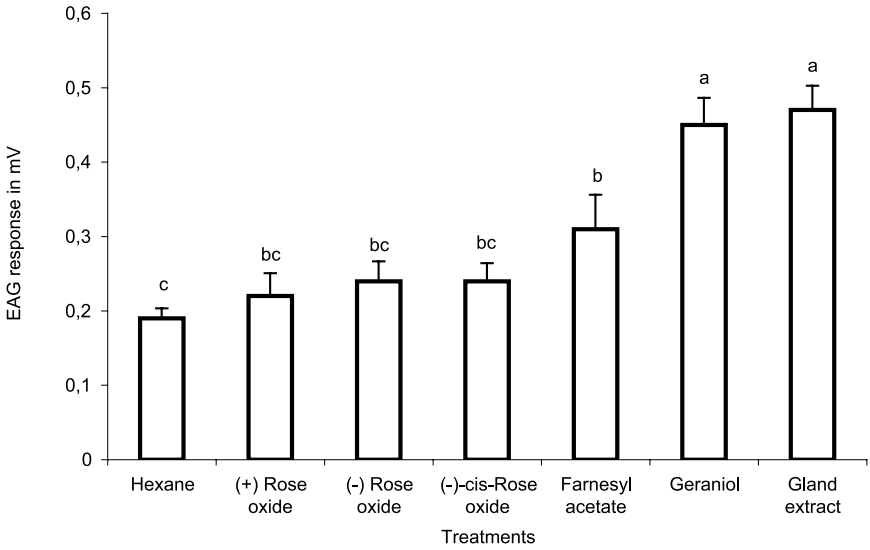

FIG. 4. Antennal response of *M. beecheii* to a 1 gland equivalent extract of mandibular gland secretions and to 1 μg of different synthetic compounds.

and similar responses (Table 2). Furthermore, for all compounds tested, the workers showed a significantly higher response to 100-ng dose than 1 μg and 10 ng (Table 3).

Electroantennography. There were significant differences in the antennal response to the different compounds tested ($F = 15.7$, $df = 6, 72$; $P < 0.001$). The mandibular gland extract, geraniol, and farnesyl acetate elicited significantly higher antennal responses than the control, whereas (+)-rose oxide, (−)-rose oxide, and (−)-*cis*-rose oxide did not (Figure 4).

DISCUSSION

A number of compounds found in the mandibular gland secretion of *M. beecheii* are common to other stingless bees, including geraniol, farnesyl acetate, and β-springene (Francke et al., 2000; Cruz-Lopez et al., 2001). Furthermore, geranyl hexanoate has been identified from the mandibular gland secretions of *Geotrigona mombuca* (Francke et al., 2000). In contrast, 2-heptanol, a common substance in previously studied *Melipona* species (Smith and Roubik, 1983; Francke et al., 1987), was not found. Our finding of two rose oxide isomers in *M. beecheii* is noteworthy, as these compounds were not found in 11 species from four different genera of Brazilian stingless bees (Francke et al., 2000). While these compounds are quite common in plant odors (de Maris and Moreira, 2003), in insects, they have only been previously reported from the secretions of two coleopteran species, and are thought to function as defensive substances (Vidari et al., 1973).

While rose oxide isomers do not give significantly greater EAG responses than hexane (Figure 4), they do elicit higher levels of defense behavior (Figure 3). There are two enantiomeric forms, the rose oxide *cis* and *trans* isomers (Figure 1), but we do not know which ones are present in the secretions, as we did not have a chiral column to separate the enantiomers. The names of the commercially available products tested are confusing. The (2*S*,4*R*)-enantiomer is available as (−)-*cis*-rose oxide, whereas (−)-rose oxide and (+)-rose oxide are 2:1 mixtures of (2*S*,4*R*):(2*R*,4*R*)-isomers and (2*R*,4*S*):(2*S*,4*S*)-isomers. The results of both EAGs and bioassays suggest that (2*S*,4*R*)-rose oxide [or (−)-*cis*-rose oxide] is responsible for all or most of the activity in (−)-rose oxide. However, geraniol and farnesyl acetate, at levels similar to a mandibular extract, elicit the most pronounced defense reactions from workers, which suggests that rose oxide isomers are of minor importance as an alarm pheromone. Clearly, more work is needed to determine what role, if any, these rose oxides play. The presence of the rose oxides, together with the lack of 2-alcohols, in the mandibular secretion of *M. beecheii* workers demarcates this

species from the other four *Melipona* species previously analyzed. These findings suggest that there may be different assemblages within the genus *Melipona*, but the mandibular secretions of more species need to be studied to clarify the situation.

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SEX PHEROMONE OF THE CITRUS FLOWER MOTH *Prays nephelomima*: PHEROMONE IDENTIFICATION, FIELD TRAPPING TRIALS, AND PHENOLOGY

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Abstract—Analysis of sex pheromone gland extract of the citrus flower moth, *Prays nephelomima* (Lepidoptera: Yponomeutidae) by coupled gas chromatography–electroantennogram detection, revealed one electrophysiologically active compound. Structural analysis using gas chromatography, gas chromatography–mass spectrometry, and dimethyldisulfide derivatization identified this as the monounsaturated aldehyde (*Z*)-7-tetradecenal. Field trials in commercial citrus orchards on the North Island of New Zealand showed that (*Z*)-7-tetradecenal was highly attractive to male *P. nephelomima*. Phenology data, collected over 19 months in three commercial orchards, from traps baited with the sex pheromone at a lure loading of 300 μg on a red rubber septum, indicated that male moths may be present throughout the year, with numbers peaking in late summer and autumn.

Key Words—Citrus flower moth, *Prays nephelomima*, Yponomeutidae, Lepidoptera, (*Z*)-7-tetradecenal, GC-EAD, GC-MS, DMDS derivatization, trapping trials, phenology.

INTRODUCTION

The citrus flower moth, *Prays nephelomima* (Meyrick), is a major pest of citrus in New Zealand (Sale, 2003). The larvae feed within a shelter formed by webbing flower or leaf parts together with a little silk and then pupate in a

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flimsy open network cocoon. This feeding severely damages the flower buds, flowers, and young leaves of many citrus species. While citrus flower moths are regarded as thinning agents for flowers, and fruitlets in citrus, heavy infestations can result in excessive fruit loss and low yields.

We, therefore, aimed to identify the sex pheromone of *P. nephelomima*. Development of an effective attractant was considered to be the first step towards successful management of this pest, and may also allow alternative control techniques to insecticide use, such as mating disruption, lure and kill, and/or mass trapping (Sternlicht et al., 1990; Suckling and Karg, 2000) to be adopted by the industry, as part of an IPM strategy. Furthermore, the identification of the sex pheromone will help in understanding the biology, ecology, and behavior of this pest. For example, the yearly adult flight phenology of *P. nephelomima* in New Zealand citrus orchards is not known.

METHODS AND MATERIALS

Insects. Approximately 200 *P. nephelomima* larvae were collected from a citrus orchard (c.v. Yen Ben lemons) in Kerikeri, Northland, New Zealand (lat. 35.22°S, 173.95°E), by harvesting citrus flowers with visible feeding damage. The flowers were kept in tissue-lined plastic containers in the laboratory at room temperature. As the mature larvae exited the flowers, they were transferred to individual 50-cm petri dishes to pupate.

Pheromone Gland Extracts. Four batches (10, 19, 14, and 22) of pheromone glands from 24- to 48-hr-old female *P. nephelomima* were excised into liquid nitrogen-cooled 0.5-ml V-vials (Wheaton, Millville, NJ, USA) containing 20–30 μl of *n*-hexane. After all glands had been excised, each vial and its contents were brought to room temperature, and the glands were extracted for 5 min. The gland extract was then transferred to a 1.1-ml tapered-bottom clear glass vial (Alltech, IL, USA) and reduced to ca. 10 μl using a stream of argon before being stored at −18°C.

Chemicals. (*Z*)-7-Tetradecenal (>97% isomerically pure) was purchased from Pherobank, Wageningen, The Netherlands. Dimethyldisulfide (DMDS) was obtained from Merck, Darmstadt, Germany, and *n*-hexane (99%), iodine, sodium sulfate, and sodium thiosulfate were from BDH Laboratory Supplies, Poole, England.

Dimethyldisulfide Derivatizations. Dimethyldisulfide derivatizations of (*Z*)-7-tetradecenal (10 ng in 20 μl of *n*-hexane) and a pooled pheromone gland extract (the remnants of the 10 and 19 female gland extracts used initially for gas chromatography–mass spectrometry (GC-MS) and gas chromatography and electroantennographic detection (GC-EAD) analysis combined with those of 14 and 22 other females) were carried out based on the methods of Buser et al.

(1983) and Leonhardt and DeVilbiss (1985). In each case, approximately 50 μl of DMDS and 5 μl of iodine solution (60 mg of I_2 in 1 ml of diethyl ether) were added to the solution in a 1.8-ml glass vial, sealed with a Teflon-lined cap, and reacted at 40°C for 15 hr. The reaction was stopped with 50 μl of a 5% aqueous solution of sodium thiosulfate, and the aqueous layer was dried with anhydrous sodium sulfate. The remaining organic layer was then carefully removed to a clean 1.5-ml tapered-bottom vial (Alltech, Glenfield, New Zealand), blown down with a stream of argon to less then 5 μl, and a 1-μl aliquot immediately was analyzed by GC-MS.

Gas Chromatography and Electroantennographic Detection. A pheromone gland extract of 19 *P. nephelomima* females was analyzed by GC-EAD using a Varian 3800 gas chromatograph equipped with both polar and nonpolar columns, coupled to an EAD Recording Unit (Syntech Research and Equipment, Hilversum, Netherlands). A 1-μl aliquot of the extract [1.9 FE (female equivalent)] was first run on a DB-5 (Agilent Technologies, CA, USA) capillary column (30 m × 0.25 mm ID × 0.5-μm film thickness) and then a polar BPX-70 capillary column (SGE, Melbourne, Australia) (30 m × 0.25 mm ID × 0.25-μm film thickness). Both capillary columns had 1:1 split outlets. Helium was used as the carrier gas at a flow rate of 1 ml/min, and injections were in splitless mode. The injector temperature was set at 250°C, the detector at 300°C, and the GC oven temperature programmed from 80°C (1-min hold) to 240°C at 10°C/min and held for 15 min.

A male *P. nephelomima* antenna was excised and positioned between two glass electrodes, containing BE Ringer's solution with 10% polyvinylpyrrolidone (molecular weight 360,000) (Sigma Chemical Co., New South Wales, Australia). Each glass electrode held a length of 1-mm silver wire that electrically connected the preparation to the recording unit's preamplifier. The EAD exit port temperature was maintained at 200°C, and the antennal preparation was placed in a charcoal-filtered and humidified air stream (400 ml/min). Kováts retention indexes (KIs) (Kováts, 1965; Marques et al., 2000) were calculated for the antennally active compound and the (*Z*)-7-tetradecenal standard on both the polar and nonpolar columns.

Gas Chromatography–Mass Spectrometry. A 1-μl aliquot (1 FE) of a 10 female gland extract of *P. nephelomima*, (*Z*)-7-tetradecenal, and DMDS derivatizations of the pooled gland extract and (*Z*)-7-tetradecenal, were analyzed by GC-MS on a VF-5ms capillary column (30 m × 0.25 mm ID × 0.25-μm film thickness) (Varian Inc., CA, USA) using a Varian 3800 gas chromatograph in splitless injection mode coupled to a Varian 2200 MS. The injector temperature was set at 250°C, and the GC oven temperature was programmed from 80 to 240°C at 10°C/min and held for 15 min. Preliminary gas chromatography indicated that the (*Z*)-7-tetradecenal standard contained ca. 2% of (*E*)- 7-tetradecenal, but that separation was incomplete on the VF-5ms capillary

column. However, after derivatization with DMDS, the resulting isomeric adducts had identical spectra but were easily separated. KIs for these adducts were calculated and comparisons were made to an adduct formed in the derivatized gland extract of *P. nephelomima*.

Trapping Trials—Trial 1. A preliminary trapping trial was undertaken in a c.v. Yen Ben lemon orchard (Clevedon, Auckland, New Zealand) from 31st October 2002 until 31st January 2003 to ascertain if *P. nephelomima* males were attracted to (*Z*)-7-tetradecenal and to determine whether any dose response was evident. Red rubber septa (Thomas Scientific Inc., Philadelphia, PA, USA) pheromone lures were prepared with three replicates of six loadings (0, 10, 30, 100, 300, and 1000 μg) of (*Z*)-7-tetradecenal and tested using green delta pheromone traps (Clare et al., 2000). A randomized complete block design was used. Traps were placed at a height of 1–2 m in the crop canopy, with 15-m spacing between treatments and 50-m spacing between replicates. At fortnightly intervals, trap positions were rotated within each replicate and sticky bases changed and sent to HortResearch, Auckland, for identification and collation of *P. nephelomima* numbers.

Trial 2. A fully replicated dose response trapping trial was set up on three commercial citrus orchards in the Auckland and Northland areas to determine the optimum loading of (*Z*)-7-tetradecenal for use in monitoring traps. Properties were situated in Clevedon, Auckland (c.v. Yen Ben lemons), Kerikeri, Northland (c.v. Yen Ben lemons), and Houhora, Northland, New Zealand (c.v. Satsuma mandarins). The trial at the Clevedon orchard ran from 31st of January 2003 to 4th of April 2003. At the Kerikeri and Houhora orchards, the trials ran from 4th of February 2003 until 15th April 2003. Trap layout, replication, loading, and data handling were as in trial 1.

Phenology. After establishing the optimal pheromone loading for trapping *P. nephelomima* (see Results), three traps baited with septa loaded with 300 μg of (*Z*)-7-tetradecenal were maintained at each of the three trial orchards to monitor weekly catch of *P. nephelomima* moths from mid-April 2003 to late September 2004. Sticky bases were collected weekly if any moths were observed, coded, stored in a fridge/freezer, and sent to HortResearch, Auckland, for identification and collation of *P. nephelomima* numbers. Data were presented as males caught per trap per day. Pheromone lures were replaced every 6 wk.

Statistical Analyses. For all trapping experiments, a mixed model was fitted to the data using GenStat Release 7.2 (VSN International Ltd., United Kingdom) with sampling interval, loading, and its interaction as fixed effects, and replicates as random effects. The experimental model could be expressed as follows: $y_{ijkl} = \mu + \tau_i + \gamma_j + \tau\gamma_{ij} + \beta_k + \varepsilon_{ijkl}$ where y_{ijkl} is the response variable, τ_i is the effect of the ith sampling interval, γ_j is the effect of the jth loading, β_k is the effect of the kth replicate; and ε_{ijkl} is the error term

associated with the treatments. The model assumed that the error terms were normally and independently distributed with zero mean and constant variances. Moth counts were transformed to their respective $\log_{10}(x + 1)$ values prior to statistical analyses to ensure that the model assumptions were met.

To determine the amount of variability in the data that could be attributed to each of the model terms, an ANOVA was performed on the transformed data. Mean separation tests were carried out for different levels of the treatment factors using Fisher's protected LSD at the 5% significance level, and model adequacy checks were performed by examining various plots (histograms, normal probability plots, scatter plots) of the residuals. These showed no evidence of violation of the independence or constant variance assumptions of the model.

RESULTS

Gas chromatography and electroantennographic detection analyses of *P. nephelomima* pheromone extract identified one small peak that repeatedly elicited a response from male antennae (Figure 1). The elution time of this active

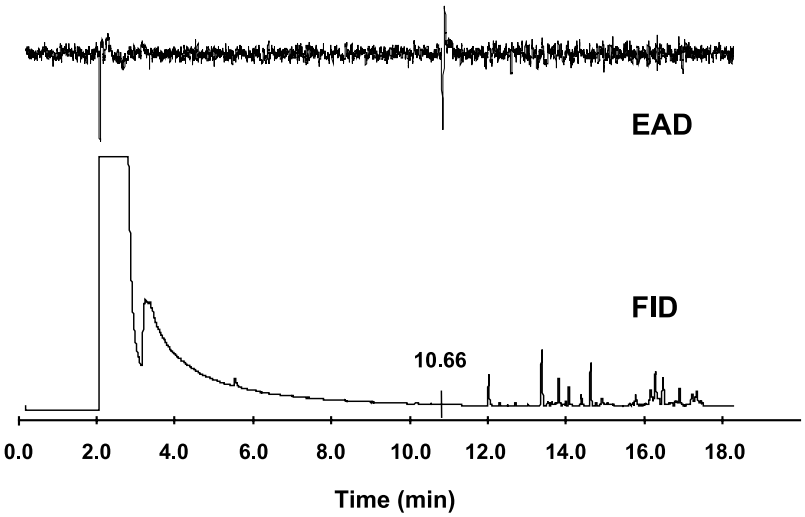

FIG. 1. GC-EAD of the response of a male *Prays nephelomima* antenna (EAD) to the active compound (*Z*)-7-tetradecenal (retention time of 10.66 min) in female moth pheromone extract eluting from a BPX-70 gas chromatography column (FID).

compound was identical with that of the (*Z*)-7-tetradecenal standard, on both the DB-5 (nonpolar) and the BPX-70 (polar) columns [KIs of 1602 and 2139, respectively, for the active compound and the (*Z*)-7-tetradecenal].

Analysis of *P. nephelomima* gland extract by GC-MS revealed a small peak with a spectrum suggestive of a monounsaturated aldehyde. The base peak was at *m*/*z* 67, and significant fragments were evident at *m*/*z* (relative intensity) 39 (63), 55 (93), 81 (81), 93 (50), 121 (41), and 149 (29). A peak at *m*/*z* 192 (7) suggested loss of water (M-18) from a molecule having a molecular weight of *m*/*z* 210. Furthermore, the fragmentation pattern and the compound's retention time were identical to that of a (*Z*)-7-tetradecenal standard. In addition, GC-MS analysis of the DMDS adduct formed in the derivatized *P. nephelomima* gland extract revealed a small peak with an identical spectrum to that of the (*Z*)-7-tetradecenal standard DMDS adduct. The molecular weight at *m*/*z* 304 (22.5) was the result of addition of *m*/*z* 94 from inclusion of two SCH_3 in one double bond of the parent compound, whereas the location of diagnostic peaks at *m*/*z* 145 (72) and *m*/*z* 159 (16) was indicative of a double bond at position 7. A KI of 2245 for this adduct confirmed the active compound as (*Z*)-7-tetradecenal rather than (*E*)-7-tetradecenal, as the (*E*)-7-tetradecenal DMDS adduct had a KI of 2256.

(*Z*)-7-Tetradecenal was highly attractive to male *P. nephelomima* capturing a total of 1579 moths, whereas control traps only captured seven (Table 1 and Figure 2). There were effects of both pheromone concentration ($P < 0.001$) and sampling interval ($P < 0.001$), as well as their resulting interaction ($P < 0.001$).

Similar results were obtained in full-scale trials in three different orchards (Tables 2–4), with 30,403 moths trapped in pheromone-baited traps compared

TABLE 1. MEAN BIWEEKLY CATCHES OF *Prays nephelomima* MALE MOTHS IN TRAPS BAITED WITH VARIOUS LOADINGS OF (Z)-7-TETRADECENAL IN A LEMON ORCHARD AT CLEVEDON, AUCKLAND, NEW ZEALAND, OCTOBER 31, 2002–JANUARY 31, 2003

Loading	11/14/2002	11/28/2002	12/12/2002	1/3/2003	1/14/2003	1/31/2003
Blank	0.26[a] (0.10)	0.26 (0.10)	0.26 (0.10)	0.26 (0.10)	0.82 (0.26)	0.00 (0.00)
10 μg	0.00 (0.00)	1.00 (0.30)	4.24 (0.72)	0.59 (0.20)	22.12 (1.36)	0.00 (0.00)
30 μg	0.82 (0.26)	0.82 (0.26)	8.77 (0.99)	3.16 (0.62)	67.87 (1.84)	0.91 (0.28)
100 μg	1.88 (0.46)	1.47 (0.39)	24.12 (1.40)	18.32 (1.29)	49.70 (1.71)	0.82 (0.26)
300 μg	1.62 (0.42)	1.29 (0.36)	51.48 (1.72)	25.30 (1.42)	67.08 (1.83)	3.16 (0.62)
1000 μg	0.44 (0.16)	2.42 (0.53)	23.83 (1.40)	26.61 (1.44)	64.31 (1.82)	3.16 (0.62)
5% LSD[†]	(0.39)					

[a] Values given in the table are the back-transformed means with the associated transformed $\log_{10}$(moth counts + 1) in parentheses.

[†] The indicated LSD gives the minimum difference required between two transformed means in order for them to be statistically different at the 5% level of significance.

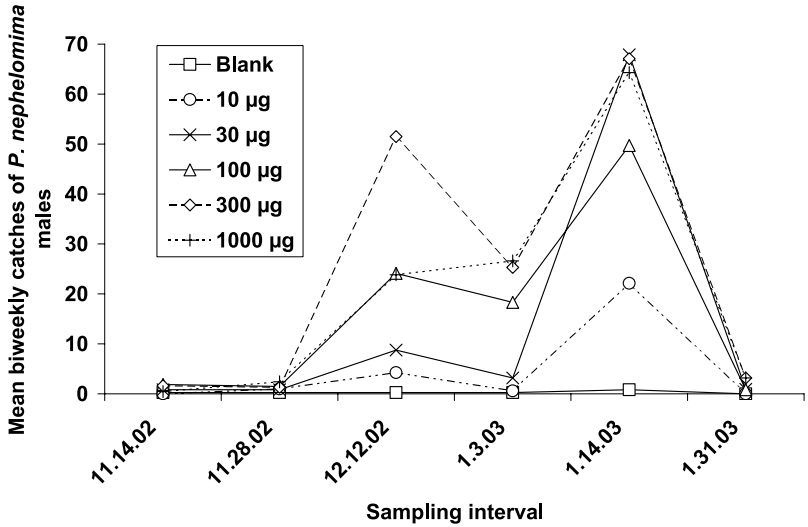

FIG. 2. Mean biweekly catches (back-transformed data) of *P. nephelomima* male moths in green delta traps baited with various loadings of (*Z*)-7-tetradecenal on six sampling occasions (from October 2002 to January 2003) in a commercial lemon orchard at Clevedon, Auckland, New Zealand.

with only 14 in the blank controls. At each site, effects of pheromone concentration ($P < 0.001$), sampling interval ($P < 0.001$), and their resulting interaction ($P < 0.001$) were observed. The results of both trials suggest that the 300-µg loading of (*Z*)-7-tetradecenal would be the most appropriate for routine

TABLE 2. MEAN BIWEEKLY CATCHES OF *P. nephelomima* MALE MOTHS IN TRAPS BAITED WITH VARIOUS LOADINGS OF (Z)-7-TETRADECENAL IN A LEMON ORCHARD AT CLEVEDON, AUCKLAND, NEW ZEALAND, JANUARY 2003–APRIL 2003

Loading	2/15/2003	3/5/2003	3/21/2003	4/4/2003
Blank	0.26[a] (0.10)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
10 µg	4.19 (0.72)	26.73 (1.44)	22.12 (1.36)	3.76 (0.68)
30 µg	33.36 (1.54)	51.48 (1.72)	24.70 (1.41)	14.10 (1.18)
100 µg	17.79 (1.27)	86.50 (1.94)	46.86 (1.68)	50.76 (1.71)
300 µg	14.70 (1.20)	79.17 (1.90)	129.02 (2.11)	38.99 (1.60)
1000 µg	17.49 (1.27)	77.16 (1.89)	50.52 (1.71)	25.18 (1.42)
5% LSD[†]	(0.30)			

[a] Values given in the table are the back-transformed means with the associated transformed $\log_{10}$(moth counts + 1) in parentheses.

[†] The indicated LSD gives the minimum difference required between two transformed means in order for them to be statistically different at the 5% level of significance.

TABLE 3. MEAN BIWEEKLY CATCHES OF *P. nephelomima* MALE MOTHS IN TRAPS BAITED WITH VARIOUS LOADINGS OF (Z)-7-TETRADECENAL IN A LEMON ORCHARD AT KERIKERI, NORTHLAND, NEW ZEALAND, FEBRUARY 2003–APRIL 2003

Loading	2/18/2003	3/4/2003	3/18/2003	4/4/2003	4/15/2003
Blank	0.00[a] (0.00)	0.26 (0.10)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
10 μg	184.35 (2.27)	152.82 (2.19)	97.40 (1.99)	52.21 (1.73)	22.93 (1.38)
30 μg	281.49 (2.45)	288.07 (2.46)	197.61 (2.30)	143.54 (2.16)	60.94 (1.79)
100 μg	307.32 (2.49)	277.61 (2.45)	281.49 (2.45)	204.12 (2.31)	160.81 (2.21)
300 μg	322.59 (2.51)	347.34 (2.54)	352.18 (2.55)	260.22 (2.42)	214.28 (2.33)
1000 μg	206.49 (2.32)	296.85 (2.47)	266.30 (2.43)	307.32 (2.49)	313.05 (2.50)
5% LSD[†]	(0.29)				

[a] Values given in the table are the back-transformed means with the associated transformed $\log_{10}$(moth counts + 1) in parentheses.
[†] The indicated LSD gives the minimum difference required between two transformed means in order for them to be statistically different at the 5% level of significance.

monitoring of male *P. nephelomima*. Many more male *P. nephelomima* were captured at the Kerikeri lemon and Houhora mandarin orchards than at the Clevedon lemon orchard. While the reasons for this observed difference in moth density are unknown, differing management practices (e.g., insecticide applications) and citrus type were likely to be factors.

The major activity period at all three sites extended from November through June, with lower activity but intersite variability in other months (Figure 3). Higher numbers of moths were trapped at the Kerikeri and Clevedon lemon orchards (18,295 and 13,005 moths, respectively) than at the Houhora mandarin orchard (4,545 moths) during the 19-mo period. This difference re-

TABLE 4. MEAN BIWEEKLY CATCHES OF *P. nephelomima* MALE MOTHS IN TRAPS BAITED WITH VARIOUS LOADINGS OF (Z)-7-TETRADECENAL IN A MANDARIN ORCHARD AT HOUHORA, NORTHLAND, NEW ZEALAND, FEBRUARY 2003–APRIL 2003

Loading	2/17/2003	3/4/2003	3/19/2003	4/1/2003	4/15/2003
Blank	0.00[a] (0.00)	1.08 (0.32)	0.00 (0.00)	0.82 (0.26)	0.82 (0.26)
10 μg	11.74 (1.11)	122.88 (2.09)	62.97 (1.81)	41.56 (1.63)	1.62 (0.42)
30 μg	67.71 (1.84)	155.68 (2.20)	133.28 (2.13)	88.33 (1.95)	15.33 (1.21)
100 μg	75.91 (1.89)	162.31 (2.21)	261.42 (2.42)	133.90 (2.13)	53.95 (1.74)
300 μg	89.16 (1.96)	229.67 (2.36)	203.64 (2.31)	341.77 (2.54)	31.21 (1.51)
1000 μg	44.81 (1.66)	214.77 (2.33)	255.45 (2.41)	271.90 (2.44)	44.50 (1.66)
5% LSD[†]	(0.42)				

[a] Values given in the table are the back-transformed means with the associated transformed $\log_{10}$(moth counts + 1) in parentheses.
[†] The indicated LSD gives the minimum difference required between two transformed means in order for them to be statistically different at the 5% level of significance.

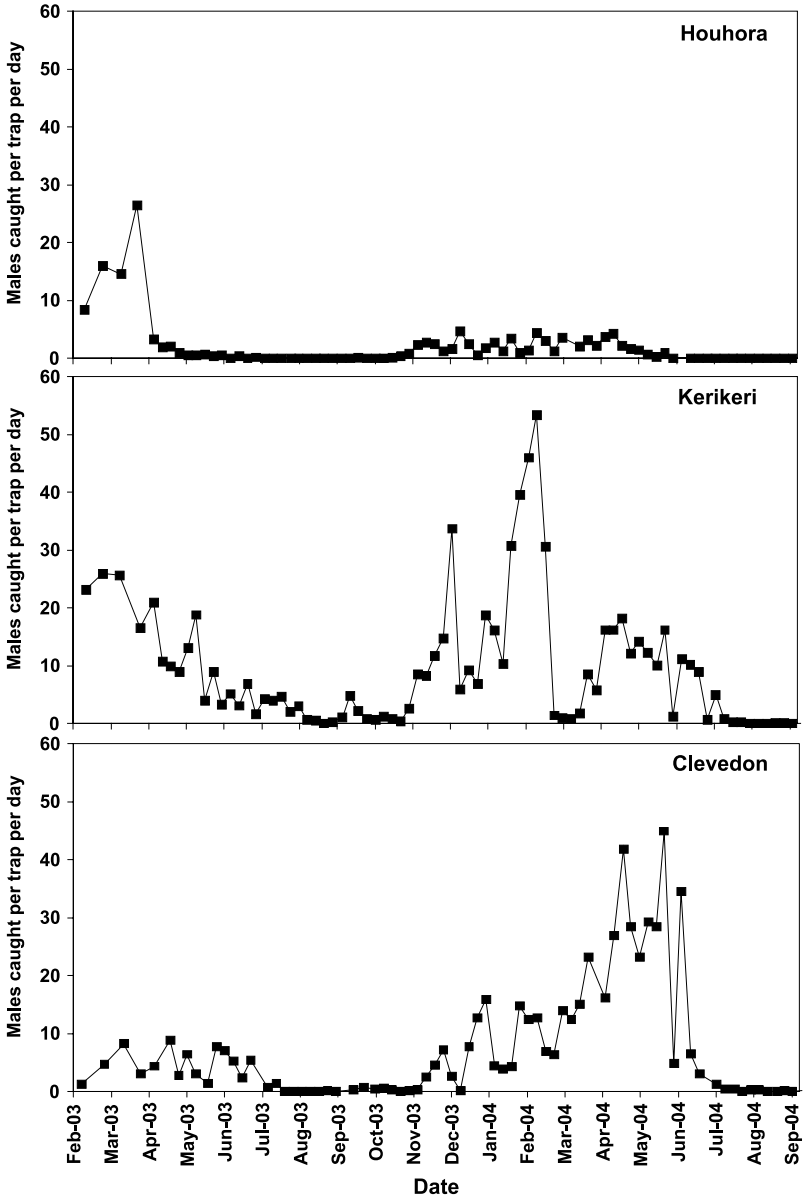

FIG. 3. Phenology of male *P. nephelomima* (using a 300-μg loading of (*Z*)-7-tetradecenal in sticky traps) from February 2003 to September 2004, in three commercial citrus orchards in the North Island of New Zealand.

flects the approximately threefold greater number of *P. nephelomima* larvae and pupae on lemons than mandarins in North Island citrus orchards during 2003–2004 (Jamieson et al., 2004).

DISCUSSION

We identified the sex pheromone of *P. nephelomima* as the monounsaturated aldehyde, (*Z*)-7-tetradecenal, which has proven to be highly attractive to male *P. nephelomima* moths in replicated field trials. The identification of a pheromone attractant for *P. nephelomima* has provided New Zealand citrus growers with a tool to monitor levels of this moth. While upwards of 300 moths per week were captured on the sticky base of a trap baited with a 300-μg lure during the summer months, given the small size of the adult male moth (ca. 5 mm in length), trap saturation is unlikely to be a major problem at this catch level. In addition, the pheromone may be useful as part of an IPM program to control or at least suppress levels of infestation in commercial citrus crops using mating disruption or lure and kill technologies. Trials are underway to investigate the potential of the pheromone for use in male annihilation of *P. nephelomima*.

(*Z*)-7-Tetradecenal has been identified as a pheromone component or sex attractant in a number of moths (Steck et al., 1982; El-Sayed, 2005) and the pheromone of three *Prays* species: *P. nephelomima* (this study), *P. citri* (Nesbitt et al., 1977), and *P. oleae* (Bernard) (Campion et al., 1979; Renou et al., 1979). Both *P. nephelomima* and *P. citri* share (like their common hosts in the *Citrus* genus) an Oriental/Indo-Malayan origin (Dugdale, personal communication). Although *P. nephelomima* superficially resembles the type (and topotypic) specimens of *P. citri*, there are differences in genitalia (Dugdale, 1988), which could play a role in reproductive isolation if the species are sympatric. However, it is unclear as to whether *P. nephelomima* and *P. citri* are allopatric or sympatric. In contrast, *P. citri* and *P. oleae* are sympatric (Campion et al., 1979; Renou et al., 1979), but *P. oleae* attacks olives rather than citrus, so, even if both species use (*Z*)-7-tetradecenal as their sex pheromone, then temporal/spatial differences in activity could insure reproductive isolation. However, one cannot rule out additional and as yet unidentified pheromone components in one or all of the *Prays* species. For example, Campion et al. (1979) and Renou et al. (1979) found that the addition of (*Z*)-9-tetradecenal to a synthetic lure containing (*Z*)-7-tetradecenal markedly reduced the attractancy for *P. oleae*, but not for *P. citri* males. Additional research is required to resolve this question, and an in-depth comparison of the seven *Prays* species attacking the Rutaceae in Australia (Nielsen and Common, 1991) would provide an excellent point of departure.

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EFFECTS OF SUBLETHAL DOSES OF MALATHION ON RESPONSES TO SEX PHEROMONES BY MALE ASIAN CORN BORER MOTHS, *Ostrinia furnacalis* (GUENÉE)

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Abstract—We evaluated the sublethal effects of malathion treatment on the behavioral responses of male Asian corn borers, *Ostrinia furnacalis* (Guenée), to their sex pheromones. Doses of malathion causing 1–50% mortality were topically applied to Asian corn borer male moths. The survivors were tested for behavioral and electroantennogram (EAG) responses to their sex pheromones. Sublethal doses of malathion affected males' ability to locate a pheromone source. In wind tunnel tests, male moths treated with sublethal doses of malathion took 2.5 to 12 times as long to take flight as control moths. Malathion-treated male moths were 60–96% less likely to locate a sex pheromone source than control moths. Multiple linear regression analysis revealed that the proportions of males performing each key stage were significantly correlated with the doses of malathion, but not with the time since treatment. Sensitivity and specificity of perception to pheromones in male moths were also disrupted by sublethal doses of malathion. The EAG responses of males treated with malathion decreased 0.13 mV on average compared with control moths. In addition, the ratios of pheromone components that elicited the largest responses shifted from approximately 5:5 to 1:9 (*E*/*Z*) and 9:1. We conclude that treatment with sublethal doses of malathion significantly reduced the likelihood that male Asian corn borers could successfully locate a normal female releasing sex pheromones.

Key Words—*Ostrinia furnacalis*, malathion, sublethal effects, sex pheromone response.

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INTRODUCTION

In recent years, much attention has been paid to the sublethal effects of insecticides and herbicides (Pinckney et al., 2002; Shofer and Tjeerdema, 2002; Yang and Du, 2003; Bain et al., 2004). These chemicals have been shown to affect not only target pests (Yang and Du, 2003), but also nontarget organisms such as estuarine phytoplankton (Pinckney et al., 2002), salamanders (Rohr et al., 2003), and fish (Shofer and Tjeerdema, 2002; Dutta and Meijer, 2003). Sublethal effects of pesticides are many and varied. For example, mosquitofish, *Gambusia affinis*, exposed to sublethal concentrations of malathion for several days showed a variety of histopathological effects under light microscopy (Cengiz and Unlu, 2003). Wang et al. (2003) found that the eggs of Asian ladybird beetles (*Harmonia axyridis*) treated with sublethal concentrations of imidacloprid, rotenone, fenvalerate, and abamectin were less likely to hatch than controls, and time to hatching was significantly prolonged.

The Asian corn borer, *Ostrinia furnacalis* (Guenée), is an important pest of corn and other crops in China and Southeast Asia. Its pheromone blend is a mixture of (*Z*)-12-tetradecenyl acetate and (*E*)-12-tetradecenyl acetate (Klun et al., 1980; Cheng et al., 1981). Because the insect lives inside its host plants throughout most of its life cycle, control of Asian corn borer has proven difficult. Pesticides are widely used against the corn borer, and it is not surprising that it has developed resistance to most insecticides applied. Management of resistance has thus become an important issue in pest control (Onstad et al., 2002).

Because female Asian corn borers exposed to sublethal doses of insecticide produced less sex pheromone (Yang and Du, 2003), sublethal treatment with specific doses of insecticide may also modify male behavioral responses. Previously, our laboratory demonstrated that female moths and larvae treated with doses of neurotoxicants reduced pheromone emissions (Yang and Du, 2003) or produced altered ratios of sex pheromone components (Wei et al., 2004). However, data on male responses were lacking. We report here the effects of sublethal doses of malathion on responses of Asian corn borer male moths to sex pheromones, and on the sensitivity and specificity of responses to different doses and ratios of sex pheromones.

METHODS AND MATERIALS

Insects. Mature larvae of *O. furnacalis* were collected from a corn field in Meng county, Henan Province, China, and maintained in our laboratory for 2 yr prior to our study. Larvae were fed on artificial diet as described by Zhou et al. (1980). Pupae were separated by sex. Male pupae were kept in $40 \times 20 \times 15$ cm^3

wooden boxes with screen covers at 23–25°C, 60–70% relative humidity and 14L:10D photoperiod until emergence. Adults were separated daily and fed with 5% sugar water.

Chemicals. The synthetic pheromone components, *E*12-14:OAc and *Z*12-14:OAc, were purchased from the Research Institute for Plant Protection (Wageningen, Netherlands), and the isomeric purity of these compounds was greater than 99% as determined by GC. Stock solutions of the single components were made up in redistilled hexane (10 μg/μl). Blends of components to be tested were prepared by diluting various amounts of the stock solutions with redistilled hexane to the desired concentrations. All blends were checked on a Hewlett-Packard (HP) 5890 GC fitted with a splitless injector and an HP-5 capillary column (25 m × 0.2 mm ID). The oven temperature was maintained at 100°C for 2 min, programmed at 10°C/min to 250°C. The carrier gas was nitrogen. Malathion (99% active ingredient, Ningbo Factory of Agricultural Chemicals) was dissolved in redistilled acetone, and a dose series of 30–150 ng/μl was prepared. Fresh solutions of all chemicals were prepared weekly.

Procedure for Treating Insects. Two-d-old Asian corn borer male moths were cold-anesthetized 2 hr before the onset of scotophase, and were dosed topically on the back of the thorax with 1 μl of insecticide solution (range of concentrations 30–150 ng/μl), using a Burkard microapplicator (Shanghai Institute of Entomology). Control moths were treated with 1 μl acetone. After treatment, moths were held as described above.

Determination of Sublethal Doses. A method based on Floyd and Crowder (1981) was used to determine the sublethal doses. Two-d-old male moths were treated with 1 μ1 of solution containing 10–500 ng of malathion ($N = 90$ moths/dosage). Mortality was recorded at 24-hr intervals for at least 96 hr until no additional mortality was observed. Doses resulting in mortality less than 50% were calculated with a probit line for lethal effects at 96 hr after treatment (the formula of the probit line: $y = 2.3988\ x - 0.2084$, $r = 0.8304$, $LD_{50} = 148.3$ ng/male). As a result, we used 30, 60, 90, 120, and 150 ng/male as sublethal doses in our experiments. Mortality ranging from 1% to 50% was observed within 96 hr after treatment. Only those survivors that did not show any abnormal behaviors were used for bioassays.

Procedure for Testing Insect Behavior. The behavioral responses of male moths to sex pheromones were observed in a wind tunnel by using the method of Zhou and Du (1999). For all tests, the wind tunnel conditions were 0.3 lux, 25 ± 2°C, 60–80% RH, and 0.3 m/sec wind speed. Males for testing were placed into individual screen cages [5 cm (diam.) × 10 cm (height)] with screen lids. Caged males were transferred 1 hr before the assay from their rearing room to the darkened room housing the wind tunnel. Assays were run 6–8 hr after the onset of scotophase, and each male was used once. Individual males were

released from a steel screen platform 28 cm above the floor of the wind tunnel and 2 m directly downwind from the pheromone source. Males were allowed 2 min to respond and were scored for the following behaviors: taking flight (TF), stationary orientation (OR), upwind flight (UF), and source contact (SC). Responses of at least 20 males were recorded for each test. The experiment was repeated three times. The pheromone source was a rubber septum impregnated with 100 μg of a 1:1 (*E*/*Z*) blend of the pheromone components, which was the most effective blend in previous tests (Zhou and Du, 1999).

Electroantennogram (EAG) Responses. EAGs were recorded using the methods of Du (1988), except that a block of hollow metal was used to hold the electrodes. All male moths tested were 2-d-old and were 6–7 hr into scotophase. The antennal flagellum was cut off with microscissors. The distal one to two segments of the antenna were removed and the antenna was rapidly mounted on the electrodes. Air (2 l/min) was delivered from a stream blown constantly over the antenna during the test period. The distance from air stream to antenna was 2 cm. Compounds tested were placed on 6-mm-diam circles of filter paper. After the solvent evaporated, the filter paper was held for 2 min, and then placed into the air stream 1 cm above the air stream outlet. A paper treated with solvent only was used as control. Each treatment was tested on nine antennae.

Data Analysis. Microsoft Excel 97 and Statistica for Windows™ 4.2 were used for ANOVA and regression analysis. Levels of statistical significance were estimated by arcsine square root permutation of the percent data to normalize the distribution.

RESULTS

Doses of 30 and 60, 90 and 120, and 150 ng/moth corresponded to 96 hr mortality of 1–20%, 20–40%, and 40–50%, respectively. The time required for *O. furnacalis* males to take flight was significantly increased by all doses of malathion tested, and the increase was positively related to malathion dose (Figure 1). At the LD_{50} of 150 ng/moth, it took 12 times longer for treated males to take flight than controls.

Sublethal doses of malathion adversely affected several other stages in the responses of Asian corn borer males to sex pheromones (Table 1). The TF, OR, and UF behaviors of males treated with lower dosages (30 and 60 ng/moth) were slightly affected, whereas males treated with the higher dosages (90 ng/moth or above) showed significantly lower response rates in these three stages than those in the control. Given that the TF rate was 100% in the control, males treated with 150 ng/moth were 84% less likely to take flight, 89% less likely to orient to the pheromone source, 90% less likely to fly upwind, and 96%

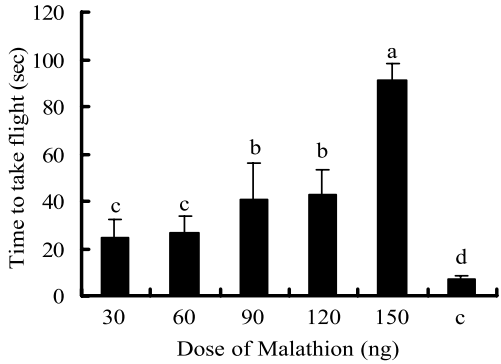

FIG. 1. Mean time required for male Asian corn borers to take flight towards a pheromone source after treatment with different doses of malathion. Males treated with 1 μl of acetone were used as control (C). Bars labeled with different letters differ significantly (LSD test, $P < 0.05$). Error bars represent 95% confidence intervals.

less likely to contact the pheromone source. In the tested dosage series, even at the dose of 30 ng/moth, SC behavior was 60% less likely than in the control. No significant differences in male responses to sex pheromone were found in males at different times after treatment with sublethal doses of malathion (Table 2).

A multiple linear regression was used to test the coeffect of the doses and time since treatment with sublethal malathion on male response to pheromone (Table 3). The results showed that the linear correlation was significant in

TABLE 1. EFFECT OF SUBLETHAL DOSAGES OF MALATHION ON THE BEHAVIORAL RESPONSES OF MALE ASIAN CORN BORER TO SEX PHEROMONE IN A WIND TUNNEL

	Percentage of behavioral response (%)[a]			
Dosage (ng/male)	TF	OR	UF	SC
30	95 ± 2ab	62 ± 3a	45 ± 4ab	13 ± 3bc
60	97 ± 2a	60 ± 5a	47 ± 2ab	20 ± 1b
90	87 ± 1b	41 ± 3b	30 ± 3bc	8 ± 2c
120	75 ± 6b	30 ± 6b	21 ± 2c	8 ± 1c
150	16 ± 3c	7 ± 3c	6 ± 3d	2 ± 1d
Control[b]	98 ± 1a	67 ± 5a	60 ± 7a	51 ± 5a

Data in the same column followed by different letters differ significantly (LSD test, $P < 0.05$). TF: taking flight, OR: orientation towards pheromone source, UF: upwind flight to pheromone source, SC: pheromone source contact. For each treatment, 60 males were tested. All males tested were 3 d old.

[a] Mean ± 95% confidence intervals.

[b] 3-d-old males treated with 1 μl of acetone were used as control.

TABLE 2. EFFECT OF DAYS POST-TREATMENT WITH SUBLETHAL DOSAGES OF MALATHION ON THE BEHAVIORAL RESPONSES OF MALE ASIAN CORN BORER TO SEX PHEROMONE IN A WIND TUNNEL

Time (d)	Percentage of behavioral response (%)[a]			
	TF	OR	UF	SC
1	75 ± 17a	41 ± 9a	31 ± 7b	7 ± 1b
2	74 ± 14a	39 ± 6a	38 ± 7b	11 ± 1b
3	80 ± 5a	38 ± 2a	32 ± 3b	10 ± 1b
4	72 ± 19a	44 ± 12a	32 ± 7b	15 ± 4b
5	68 ± 25a	38 ± 10a	26 ± 6b	8 ± 2b
Control[b]	98 ± 1a	67 ± 5a	60 ± 7a	51 ± 5a

Data in the same column followed by different letters differ significantly (LSD test, $P < 0.05$). TF, OR, UF and SC as described in Table 1. All males tested were treated with 90 ng/male of malathion.
[a] Standard deviation represent 95% confidence intervals.
[b] The 3-d-old males treated with 1 μl of acetone were used as control.

taking flight, orientation, and upwind flight ($P < 0.01$), and in source contact ($P < 0.05$). There was no significant correlation between response rates and the time since treatment (absolute value of r_y lower than $r_{1,23,\ 0.05\ =\ 0.396}$) although the response rates were significantly correlated with the doses ($P < 0.01$ each absolute value of r_x was more than $r_{1,23,0.01\ =\ 0.505}$; see Table 3). The standard regression coefficients (B_x, B_y) further suggested that the decrease in the response to pheromone was mainly caused by the dosages of malathion applied. The regression coefficients for the doses tested, at the stages of taking flight, orientation, upwind flight, and source contact, declined gradually in absolute value. This suggested that the later the stage in the flight sequence, the smaller the response rate with increasing sublethal dose of malathion.

TABLE 3. COEFFICIENTS FOR THE REGRESSION EQUATIONS

Behavior	R	r_x	r_y	b	B_x	B_y
TF	0.72**	0.72**	0.036	15.06** ± 1.93	0.72** ± 0.15	0.25 ± 0.15
OR	0.71**	0.71**	0.128	10.32** ± 1.25	0.70** ± 0.15	0.09 ± 0.15
UF	0.67**	0.66**	0.141	9.18** ± 1.20	0.66** ± 0.16	0.11 ± 0.16
SC	0.56*	0.56**	0.073	6.38** ± 0.91	0.56** ± 0.18	0.06 ± 0.18

Notes: R: Multiple correlation coefficient, r_x: partial correlation coefficient for parameter of dose, r_y; partial correlation coefficient for parameter of duration after treatment, b: intercept, B_x: standard regression coefficient for parameter of dose, B_y: standard regression coefficient for parameter of duration after treatment.
*Significant difference at level $P < 0.05$. TF, OR, UF and SC as defined in Table 1.
**Significant difference at level $P < 0.01$.

Treatment of male Asian corn borer with sublethal doses of malathion also affected physiological responses. EAG responses of malathion-treated male moths to several ratios of pheromone blends (*E*12-14:OAc/*Z*12-14:OAc) were lower than those of the control insects (Figure 2). The average EAG value of malathion-treated males was 0.40 mV, compared to control males with 0.53 mV. The EAG responses of malathion-treated males at all pheromone dosages tested (ranging from 0.1 to 100,000 ng) were lower than those of acetone-treated controls (Figure 3). The saturation dose of perception for males in both the treated and control groups was 10,000 ng.

In wind tunnel tests, control males treated with acetone only showed typical behavioral responses to different pheromone blend ratios (Klun et al., 1980; Cheng et al., 1981; Huang et al., 1998). The ratio of *E*12-14:OAc and *Z*12-14:OAc from the Asian corn borer of Chinese population was about 5:5 (Yang and Du, 2003). Control males showed highest SC percentages at ratios of 4:6, 5:5, and 6:4. At the other ratios, the SC percentages were significantly lower (Figure 4). However, males treated with malathion showed different behavioral response patterns. At most ratios, the SC percentages were not different except at the ratio of 9:1, and SC percentages were low throughout (Figure 4). Furthermore, the dose response of acetone-treated control males peaked at the 10^2 and 10^3 ng levels, and further increment of the dosage caused lower response percentages. In contrast, the dose responses of malathion-treated

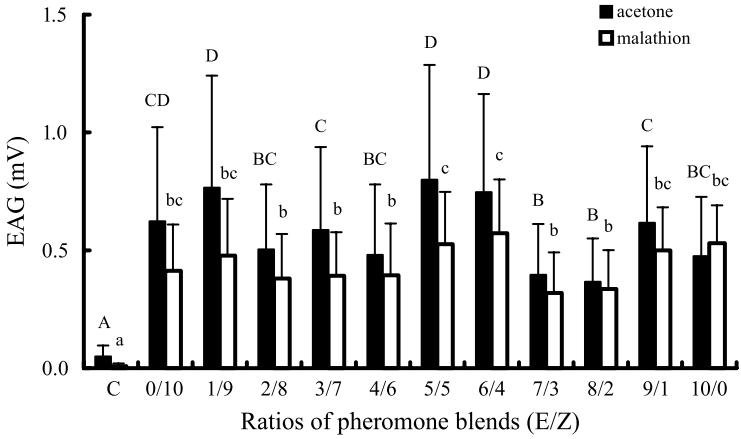

FIG. 2. Electroantennogram responses of male Asian corn borers to binary blends of *E*12-14:OAc and *Z*12-14:OAc (1 μg) after treatment with acetone or malathion (treated with 1 μl of malathion solution at the concentration of 90 ng/μl for each moth). Bars labeled by different letters differ significantly (Duncan's multiple range test, $P < 0.05$). Error bars represent 95% confidence intervals.

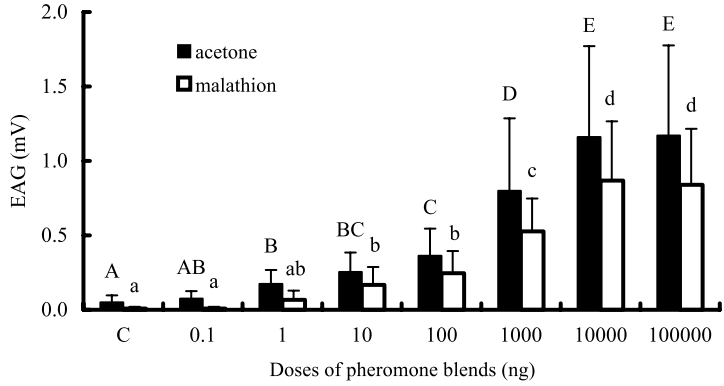

FIG. 3. Electroantennogram responses of male Asian corn borers after treatment with acetone or malathion (treated with 1 μl of malathion solution at the concentration of 90 ng/μl for each moth) to dosages of 1:1 blends of *E*12-14:OAc and *Z*12-14:OAc. Bars labeled by different letters differ significantly (Duncan's multiple range test, $P < 0.05$). Error bars represent 95% confidence intervals.

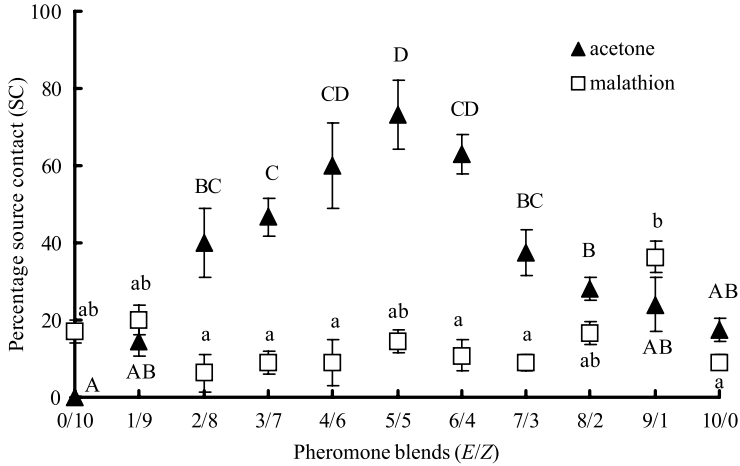

FIG. 4. Source contacts of male Asian corn borers responding to binary blends of pheromone after treatment with acetone or malathion (treated with 1 μl of malathion solution at the concentration of 90 ng/μl for each moth). $N = 60$ moths per experiment. The data series of the same pheromone ratio with the same letter are not significantly different at $P < 0.05$ (*t*-test). Error bars represent 95% confidence intervals.

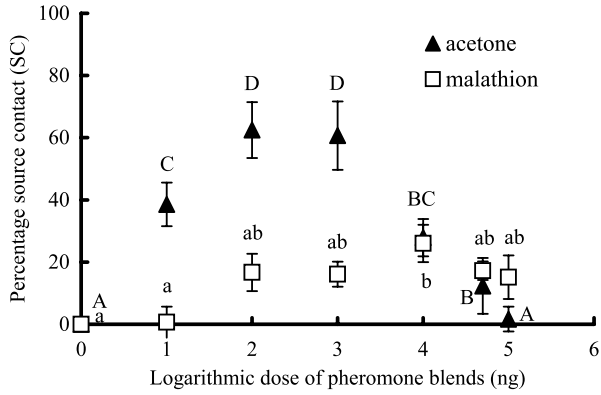

FIG. 5. Source contacts by male Asian corn borers responding to dosages of pheromone (*E*/*Z* = 1:1) after treatment with malathion (treated with 1 μl of malathion solution at the concentration of 90 ng/μl for each moth). The number of insects treated in each experiment was 60. The data series of the same logarithmic dose with the same letter are not significantly different at the level of 5% (*t*-test). Error bars represent 95% confidence intervals.

males were lower than those of acetone-treated males to 10^1, 10^2, and 10^3 ng doses, but not to 10^4 and above (Figure 5). These data suggested that malathion strongly affected behavioral responses of males to normal and abnormal pheromone blends and doses.

DISCUSSION

The present study revealed numerous sublethal effects of malathion treatment on the behavioral responses of male Asian corn borers, *O. furnacalis* (Guenée), to their sex pheromones. Sublethal treatment with malathion not only made males less likely to take flight, but also reduced the number contacting the pheromone source. In addition, the ratios of pheromone components that elicited the largest responses shifted from approximately 5:5 to 1:9 (*E*/*Z*) and 9:1. We conclude that treatment with sublethal doses of malathion significantly affected the reproductive behaviors of male Asian corn borers.

Sublethal effects of insecticides include both behavioral and physiological responses. Sublethal effects may influence the function of sense organs and thus alter pest behaviors by reducing or changing sensory information reaching the brain. For instance, sublethal insecticide treatment significantly affected sex pheromone communication in the parasitoid, *Trichogramma brassicae* (deltamethrin and chlorpyrifos, Delpuech et al., 1999, 2001), cabbage looper

(*Trichoplusia ni,* cypermethrin, Clark and Haynes, 1992), and diamondback moth (*Plutella xylostella,* fenvalerate, Tabashnik and Rethwisch, 1992).

Malathion is one of the organophosphate (OP) insecticides. OPs work by inhibiting enzymes in the nervous system, specifically acetylcholinesterases (AChE). The enzyme is phosphorylated when it becomes attached to the phosphorous moiety of the insecticide, a binding that is irreversible. AChE is involved with the transmission of nerve impulses. When this enzyme is inhibited, the transmission system "jams," resulting in restlessness, hyperexcitability, convulsions, paralysis, and death (Ware, 2000). The reduction of physiological and behavioral responses of the Asian corn borer males treated with sublethal doses of malathion may be connected with disruption of normal nerve cell transmission. As indicated by Haynes (1988), adult behavior is the final outcome of a sequence of neurophysiological events involving sensory neurons, interneurons, motor neurons, and finally muscular contractions. Disruption of any or all of these events will result in incorrect or inappropriate behavior. Because the sensitivity of male moths responding to given doses of pheromone blends and the preference for particular ratios of pheromone components are controlled by the central nervous system (CNS) (Linn and Roelofs, 1986), malathion may reduce neurophysiological responses by acting on the CNS at particular synapses between neurons (Zhang, 1987). The blocking or disruption of neural signals by insecticides will thus affect courtship behavior.

Haynes and Baker (1985) demonstrated that sublethal doses of permethrin effectively blocked key stages in the behavioral responses of pink bollworm male moths, but these effects disappeared 4 d after treatment. In our study, we did not find such a recovery after 5 d (Table 2). Pyrethroid insecticides like permethrin rapidly knock down the insect at sublethal doses, but treated insects may recover from the effects after a certain period. The difference between our data and other studies may be due to the different chemicals and/or insect species used in the experiments.

Efficient mate location in sexually reproducing organisms requires a signaling system in which both signal sender and receiver are tuned to the same signal. Female moths typically release a specific blend of sex pheromone components in a ratio to which the male's receptors are tuned. Our results show that Asian corn borer male moths treated with malathion responded optimally to unnatural ratios of pheromone blends, and that they showed a reduction in sensitivity to normal sex pheromones. Asian corn borer female moths also release a significantly lower amount of sex pheromone when treated with sublethal doses of malathion (unpublished data). These sublethal effects of insecticides may result in a lower percentage of males successfully locating females, and lower reproductive success. A better understanding of the interaction between behavioral responses to sex pheromones and sublethal

exposure to insecticides may help us to understand whether physiological and behavioral changes caused by exposure to sublethal doses of insecticides may contribute to the overall effectiveness of insecticides.

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POTENTIAL ALLELOCHEMICALS FROM AN INVASIVE WEED *Mikania micrantha* H.B.K.

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Abstract—Phytotoxicity-directed extraction and fractionation of the aerial parts of *Mikania micrantha* H.B.K. led to the isolation and identification of three sesquiterpenoids: dihydromikanolide, deoxymikanolide, and 2,3-epoxy-1-hydroxy-4,9-germacradiene-12,8:15,6-diolide. These sesquiterpenoids inhibited both germination and seedling growth of tested species with deoxymikanolide possessing the strongest phytotoxicity. In a bioassay against lettuce (*Lectuca sativa* L.), deoxymikanolide reduced radicle elongation at low concentration (IC_{50} = 47 μg/ml); dihydromikanolide showed a weaker effect (IC_{50} = 96 μg/ml), and 2,3-epoxy-1-hydroxy-4,9-germacradiene-12,8:15,6-diolide exhibited the least effect (IC_{50} = 242 μg/ml). Deoxymikanolide caused yellowish lesions at the root tips of lettuce at a concentration of 50 μg/ml, and a 250 μg/ml solution killed lettuce seedlings. A bioassay against the monocot ryegrass (*Lolium multiforum*) revealed similar results on radicle elongation, which implied that the growth inhibition by these compounds was not selective. To evaluate their phytotoxicity to plants in natural habitats, three common companion tree species in south China, *Acacia mangium*, *Eucalyptus robusta*, and *Pinus massoniana*, were also tested and similar results were obtained. This is the first report on the isolation of 2,3-epoxy-1-hydroxy-4,9-germacradiene-12,8:15,6-diolide as a naturally occurring product.

Key Words—Phytotoxicity, allelochemical, *Mikania micrantha*, exotic species, dihydromikanolide, deoxymikanolide, 2,3-epoxy-1-hydroxy-4, 9-germacradiene-12,8: 15,6-diolide.

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INTRODUCTION

Mikania micrantha H.B.K. is a herbaceous vine that propagates by means of wind-dispersed achenes and stem fragments that root easily at the nodes. It grows and spreads so quickly that it is also called "mile-a-minute weed." This weed smothers trees and crops, suppressing the vigor and eventually killing the affected plants. Although native to tropical South and Central America, *M. micrantha* has become a serious problem in southeast Asia and the Pacific region, particularly in disturbed forests and plantation crops such as tea, teak, rubber, and oil palm. The weed changes the pattern of growth of affected plants, and it has been listed as one of the world's worst weeds (Parker, 1972; Holm et al., 1977). Records show that *M. micrantha* first invaded Hong Kong as an exotic species in 1919 (Kong et al., 2000), and specimens kept at the Herbarium of the South China Institute of Botany indicated that *M. micrantha* was transferred to China no later than 1984. In the past few years, *M. micrantha* has expanded widely in South China. On Neilingding Island, a natural reserve area, *M. micrantha* covers 40–60% of the surface of the island and nearly 33% of the native trees have been seriously affected. Furthermore, the weed creeps up banana trees on the island, thereby destroying a food source of native monkeys (He et al., 2000; Kong et al., 2000; Lan and Wang, 2001).

M. micrantha not only perturbs the growth and development of trees, crops, and ornamental plantings but also reduces the density of wild herbaceous species. Besides competition for nutrients with other plants, *M. micrantha* is believed to have allelopathic effects on neighboring plants (Ismail and Mah, 1993; Cock et al., 2000). Our field surveys suggested that phytotoxins might contribute to the growth suppression of plants in its vicinity. Previous studies demonstrated that leachate and aqueous extracts of *M. micrantha* inhibited germination and growth of some plants, and its debris, either incorporated or remaining on the surface of the soil, also inhibited the growth and germination of neighboring plants (Ismail and Chong, 2002). Ismail and Mah (1993) and Ismail and Chong (2002) identified four phenolic acids from leaf extracts of this weed, but no allelopathic sesquiterpenoids have been reported. In this study, we isolated and characterized three potential allelopathic sesquiterpenoids from *M. micrantha* extracts by activity-directed extraction and fractionation procedures.

METHODS AND MATERIALS

Instrumentation. IR spectra were measured on a Perkin-Elmer 783 spectrometer with KBr disks. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker

DRX-400 instrument with TMS as an internal standard and DMSO-d_6 as solvent. UV spectra were taken on a Perkin Elmer Lambda 25 UV/VIS spectrometer. EIMS were measured on a Micromass Platform EI-200 GC/MS instrument at 70 eV by direct insertion probe.

Preparation of Aqueous Extracts. Aerial parts of *M. micrantha* H.B.K. were collected from the Fairy Lake Botanical Garden of Shenzhen, China in December, 2000. Plant tissues were air dried at room temperature and ground into powder. Five g of the aerial parts were blended in 100 ml distilled water for 24 hr at room temperature, and aqueous extracts (0.05 g/ml) were obtained. pH values of the extracts and the solvent for control (distilled water) were adjusted to 6.0 with 2 M NaOH and HNO_3 (Quayyum et al., 1999).

Preparation of Organic Extracts. Five g of dried aerial parts of the plant were extracted three times with petroleum ether, ethyl acetate, and ethanol sequentially at intervals of 24 hr. Extracts were concentrated under reduced pressure and diluted to 0.05 g/ml.

Phytotoxicity Assays of Extracts. Growth inhibitory effects of the aqueous and organic extracts from the branches and leaves were evaluated through bioassays against three plants: radish (*Raphanus sativus*), ryegrass (*Lolium multiforum*), and white clover (*Trifolium repens* L.). Experimental seeds were surface sterilized with 0.5% $HgCl_2$ and 10 seeds were placed in each sterile Petri dish (9 cm diam) lined with Whatman No. 3 filter paper. Five ml of test extract were added to each Petri dish. For assays of the organic extracts, the method described by Anaya et al. (1999) was used with minor changes. Five ml of distilled water were added after the solvent had completely evaporated, and 5 ml of distilled water (pH 6.5) were added to each control Petri dish. Petri dishes were sealed with parafilm to prevent water loss and stored in the dark at 22 ± 2°. Treatments were allotted in a complete randomized design with three replicates for each treatment. After 5 d of incubation, length of radicles (primary roots) and plumules (primary shoots) were measured as described by Anaya et al. (1999) and Quayyum et al. (1999).

Phytotoxicity Assays of Purified Compounds. Bioassays against lettuce (*Lactuca sativa* L.), a commonly used species for phytotoxicity bioassays, and a monocotyledonous species, ryegrass, were conducted as previously described by Viles and Reese (1995). In these assays, five concentrations of sesquiterpenoids (25, 50, 100, 250, and 500 μg/ml) were used. Because deoxymikanolide showed the strongest inhibitory activity, bioassays against a monocotyledonous (radish) and a dicotyledonous plant (chives) were also performed. In order to confirm the effects, three ecologically relevant tree species, *Acacia mangium*, *Eucalyptus robusta*, and *Pinus massoniana,* which tend to be affected in actual habitats, were included in the assays (Romeo, 2000), and Harness (common name: Acetochlor, Monsanto Co., St. Louis, MO, USA), a commonly used herbicide, was used as a positive control in these assays.

Isolation of Phytotoxins. Dried and ground branches and leaves of *M. micrantha* (4.0 kg) were exhaustively extracted with 95% ethanol at room temperature. After filtration, the filtrate was concentrated under reduced pressure to yield a dark brown residue (300 g, 7.5% yield). The residue was suspended in water, and the suspension was sequentially extracted with petroleum ether, ethyl acetate, and *n*-butanol to yield petroleum ether extract (30 g, 0.75% yield), ethyl acetate extract (4.5 g, 0.11% yield), and *n*-butanol extract (10 g, 0.25% yield), respectively. Results from the bioassay with lettuce indicated that the ethyl acetate extract exhibited the strongest inhibitory effect. Thus 4.5 g of the ethyl acetate extract were fractionated on silica gel eluted with a step gradient elution ($CHCl_3$, $CHCl_3$–MeOH at 98:2, 96:4, 9:1, 8:2, 7:3, 6:4, 1:1, MeOH). Sixteen major fractions (M_1–M_{16}) were collected based on TLC profiles. Fraction M_8, which exhibited the most potent inhibitory effect, was further purified on silica gel eluted with benzene–$CHCl_3$ (1:1, 1:2, 1:3) to afford compounds A (35 mg, 0.00087% yield), B (41 mg, 0.001% yield), and C (22 mg, 0.00055% yield).

Scanning Electron Microscopy (SEM) Study of Root Morphology and Anatomy of Lettuce Seedlings. Specimens were prepared following the procedure outlined by Hedge and Miller (1992), and viewed with a JEOL JSM-T300 scanning electron microscope (data not shown).

Statistical Analyses. The significance of effects of aqueous and organic extracts from *M. micrantha* on seedling growth of tested species was first examined by ANOVA ($P < 0.05$) and then analyzed using Fisher's LSD test at $P < 0.05$ level. For the effects of the sesquiterpenoids on tested species, paired *t* tests at $P < 0.01$ level were conducted to determine the significant differences between treatments and controls.

RESULTS

Phytotoxicity Assays of Aqueous and Organic Extracts. Table 1 shows the effects of the aqueous and organic extracts on the growth of the tested plants. The aqueous extract of the aerial parts of *M. micrantha* showed a significant effect, inhibiting total seedling growth (radicle plus shoot growth) of radish, ryegrass, and white clover by 52%, 70%, and 72%, respectively. The petroleum ether and ethanol extracts caused no significant inhibition of tested plants. The ethyl acetate extract had the most significant effect, inhibiting more than 90% of seedling growth of all tested species. Therefore, the ethyl acetate extract was selected for further investigation.

Identification of Phytotoxins. Compounds A and B: Two compounds from the exotic extract were identified as dihydromikanolide (compound A) and

TABLE 1. EFFECTS OF AQUEOUS AND ORGANIC EXTRACTS OF *M. micrantha* ON SEEDLING GROWTH OF RADISH, RYE GRASS, AND WHITE CLOVER[a]

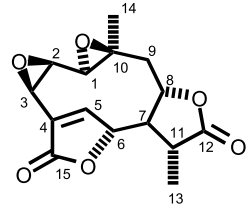
	Radish		Rye grass		White clover	
	Radicle length (cm)	Shoot length (cm)	Radicle length (cm)	Shoot length (cm)	Radicle length (cm)	Shoot length (cm)
Control	6.66 ± 0.78a	3.81 ± 0.25a	2.71 ± 0.20a	1.42 ± 0.10a	1.15 ± 0.18a	2.71 ± 0.15a
Aqueous	3.07 ± 0.52b	1.95 ± 0.18b	0.6 ± 0.06b	0.63 ± 0.09b	0.4 ± 0.05b	0.69 ± 0.06b
Petroleum ether	4.31 ± 0.4ab	2.94 ± 0.46ab	2.31 ± 0.17a	1.52 ± 0.10a	1.02 ± 0.15a	1.67 ± 0.28ab
Ethyl acetate	0.44 ± 0.11c	0.49 ± 0.06c	0.06 ± 0.01c	0.11 ± 0.05c	0.12 ± 0.02c	0.12 ± 0.02c
Ethanol	3.78 ± 0.24ab	3.74 ± 0.18a	1.54 ± 0.38ab	1.69 ± 0.15a	0.55 ± 0.10ab	1.77 ± 0.24ab

Means within a column followed by the same letter are not different at $P = 0.05$ level according to Fisher's LSD test.
[a] Each value is the mean of three replicates ± SE.

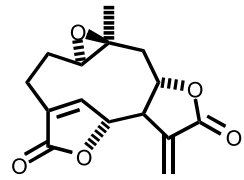
deoxymikanolide (compound B) by comparison of their spectral data with the values reported by Herz et al. (1970) and Cuenca et al. (1988).

Compound C: Colorless prisms, mp 281–282° (acetone); $[\alpha]_D^{24}$ + 59.1° (ca. 0.132, acetone); UV (MeOH) λ_{max} nm (log ε): 215 (3.59); EIMS *m/z* (%): 293 $[M + H]^+$ (1), 275 (0.5), 263 (5), 95 (100). Its IR and ^1H NMR data were in agreement with those previously reported by Herz et al. (1970) and indicated that compound C was 2,3-epoxy-1-hydroxy-4,9-germacradiene-12,8:15,6-diolide. The previously unreported ^{13}C NMR data are presented as follows: δ 68.3 (d, C-1), 60.5 (d, C-2), 52.1 (d, C-3), 129.1 (s, C-4), 144.9 (d, C-5), 78.6 (d, C-6), 48.7 (d, C-7), 74.6 (d, C-8), 120.6 (d, C-9), 142.1 (s, C-10), 37.4 (d, C-11), 176.7 (s, C-12), 12.1 (q, C-13), 17.8 (q, C-14), 170.7 (s, C-15) ppm. This compound, previously obtained by Herz et al. (1970) from the acid-catalyzed rearrangement of dihydromikanolide, was isolated as a naturally occurring compound in the present study.

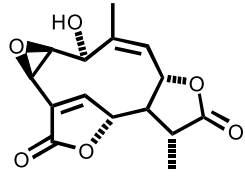
Bioassay of Purified Phytotoxins. The sesquiterpenoids isolated from the ethyl acetate extract (Figure 1) were tested for their growth inhibitory effects, and all of them exhibited biological activity. Their phytotoxicity varied with concentration as well as individual structures of the compounds. Figure 2 shows their inhibitory effects on seed germination of lettuce. Germination rates of all tested species were inversely proportional to concentration. Within the concentration range of 10–200 μg/ml, seeds germinated



A) Dihydromikanolide



B) Deoxymikanolide



C) 2,3-Epoxy-1-hydroxy-4,9-germacradiene-12,8:15,6-diolide

FIG. 1. Chemical structures of dihydromikanolide, deoxymikanolide, and 2,3-epoxy-1-hydroxy-4,9-germacradiene-12,8:15,6-diolide isolated from *Mikania micrantha*.

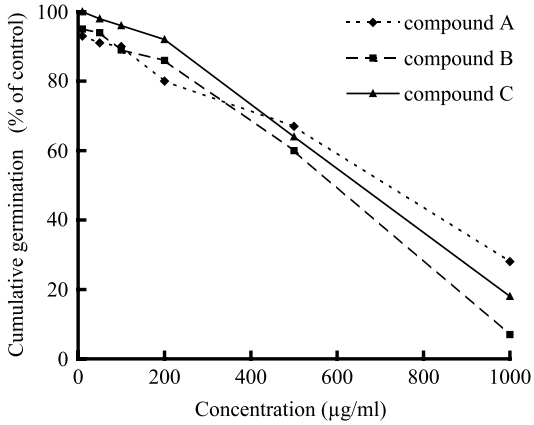


FIG. 2. Impact of three sesquiterpenoids on seed germination of lettuce. A = Dihydromikanolide, B = Deoxymikanolide, C = 2,3-Epoxy-1-hydroxy-4,9-germacradiene-12,8:15,6-diolide.

normally. However, all sesquiterpenoids induced a significant inhibition of germination at a concentration of 500 μg/ml. At 1000 μg/ml, seed germination rates were 28% (dihydromikanolide), 7% (deoxymikanolide), and 18% (2,3-epoxy-1-hydroxy-4,9-germacradiene-12,8:15,6-diolide) of those of controls.

A seed germination test with ryegrass also revealed that all sesquiterpenoids started to show significant inhibitory effects at 500 μg/ml. The 1000 μg/ml solution resulted in germination rates of 10% (dihydromikanolide), 4% (deoxymikanolide), and 29% (2,3-epoxy-1-hydroxy-4,9-germacradiene-12,8:15,6-diolide), compared to controls.

Figure 3 shows the effects of six different concentrations of the sesquiterpenoids on radicle elongation of lettuce seedlings. Deoxymikanolide had the strongest effect, and linear regression analysis indicated an IC_{50} value of 47 μg/ml. It reduced radicle elongation to 8% of the control at a concentration of 500 μg/ml. At the 50 μg/ml concentration, deoxymikanolide started to cause yellowish lesions at the root tip. A 250 μg/ml solution caused the formation of brown lesions throughout the entire radicle surface and deformation of both hypocotyls and radicles. At a concentration of 500 μg/ml, radicles turned brown, resulting in gradual dehydration and arrested growth of the roots. To exclude any possible effects caused by low pH values of the culture solutions, pH values were measured but no significant differences were detected between treatments and controls. Dihydromikanolide had less effect on radicles (IC_{50} = 96 μg/ml), and 2,3-epoxy-1-hydroxy-4, 9-germacradiene-12,8:15,6-

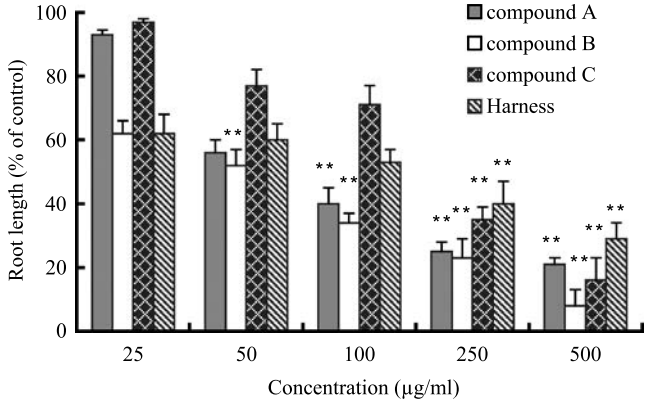


FIG. 3. Impact of three sesquiterpenoids on radicle growth of lettuce. **Significant difference between treatment and control as determined by the paired t test at $P < 0.01$ level. Compounds A, B, C as in Figure 2. D = Harness (commercial herbicide).

diolide showed the least effect (IC_{50} = 242 μg/ml). All sesquiterpenoids caused yellowish to brown lesions on radicles at high concentrations of 500 μg/ml.

Figure 4 shows the shoot lengths of lettuce seedlings treated with the three sesquiterpenoids. All three compounds had less effect on shoot growth compared to that on radicle growth.

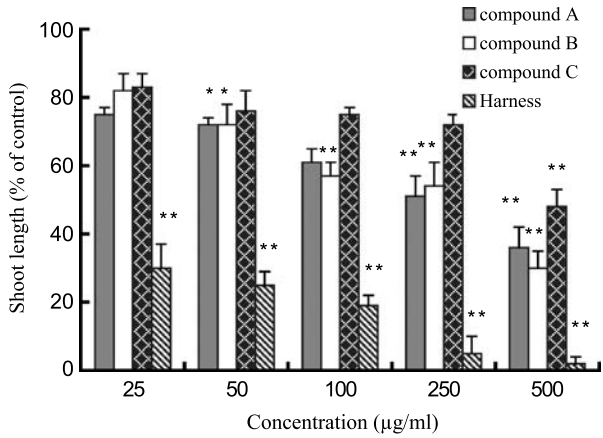


FIG. 4. Impact of three sesquiterpenoids on shoot growth of lettuce. **Significant difference between treatment and control as determined by the paired t test at $P < 0.01$ level. Compounds A, B, C as in Figure 2.

Bioassay against ryegrass showed similar results. For root growth, IC_{50} values for treatments were 230 μg/ml (dihydromikanolide), 67 μg/ml (deoxymikanolide), and 290 μg/ml (2,3-epoxy-1-hydroxy-4,9-germacradiene-12,8:15,6-diolide). Compounds did not significantly reduce the shoot length of ryegrass until the concentration increased to as high as 500 μg/ml.

Because deoxymikanolide had the strongest inhibitory activity, it was selected for further investigations. Bioassays against radish, a dicotyledonous plant, and chives, a monocot, showed similar results to those of lettuce and ryegrass (figures not shown), further confirming the inhibitory activity. The IC_{50} values on root length were 74 μg/ml for radish and 88 μg/ml for chives.

These sesquiterpenoids were also toxic to three selected ecologically relevant tree species. Figure 5 shows the effects of these compounds on the root growth of *Acacia mangium*. They also affected the seedling growth of *Eucalyptus robusta* and *Pinus massoniana* (data not shown). The IC_{50} values on root length of *Acacia mangium* were 159 μg/ml (dihydromikanolide), 95 μg/ml (deoxymikanolide), and 201 μg/ml (2,3-epoxy-1-hydroxy-4, 9-germacradiene-12,8:15,6-diolide). For *Eucalyptus robusta*, the IC_{50} values were 149, 59, and 155 μg/ml, whereas for *Pinus massoniana*, the values were 120, 66, and 128 μg/ml.

Results of Scanning Electron Microscopy. Scanning electron microscopy (SEM) studies were conducted in plants treated with deoxymikanolide, the most toxic of the three compounds. Pregerminated lettuce seeds were treated with 50 and 100 μg/ml of deoxymikanolide solutions at 25°C for 26 hr. A 50 μg/ml deoxymikanolide solution reduced root hair length and density of lettuce seed-

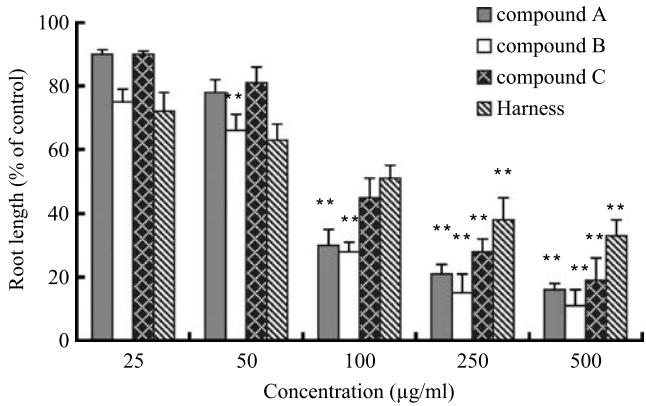


FIG. 5. Impact of three sesquiterpenoids on radicle growth of *Acacia mangium*. **Significant difference between treatment and control as determined by the paired *t* test at $P < 0.01$ level. Compounds A, B, C as in Figure 2.

lings significantly. There was no visible root hair development on radicles of seedlings treated with 100 μg/ml deoxymikanolide solution. After 5 d of continuous incubation, however, several root hairs with abnormalities developed. The root tip swelled into a small ball-like structure, which indicated its physiological response to the effects of the compound. SEM of the internal root anatomy was also characterized, but no significant difference was revealed (data not shown).

DISCUSSION

The widespread distribution of *M. micrantha* has received much attention worldwide. Despite its fast-growing characteristics, the growth of *M. micrantha* in its native habitat is restrained, probably due to control by its natural enemies, such as phytophagous insects and fungi. In a new ecosystem with a lack of natural enemies, this weed can spread quickly (Cock, 1982; Barreto and Evans, 1995). It has been speculated that allelopathy plays an important role on *M. micrantha*'s dominance beyond its competition for light and nutrition (Ismail and Mah, 1993; Cock et al., 2000). Several different sesquiterpenoids have been reported as allelochemicals (Macias et al., 1996, 2000; Bagchi et al., 1997), but the phytotoxic effects of these three sesquiterpenoids from *M. micrantha* are reported here for the first time.

Herz et al. (1970) noticed that extracts of many members of the large genus *Mikania* were used as folk medicine and suspected the presence of sesquiterpene lactones on phylogenetic grounds. They isolated and identified six new sesquiterpene dilactones from *Mikania scandens* (L.) Willd including compound A (dihydromikanolide) and compound B (deoxymikanolide). They also obtained compound C (2,3-epoxy-1-hydroxy-4,9-germacradiene-12,8:15,6-diolide) from the acid-catalyzed rearrangement of dihydromikanolide. Cuenca et al. (1988) identified dihydromikanolide and deoxymikanolide from *M. micrantha* during a study of Argentine Compositae. Although all three sesquiterpenoids were phytotoxic, the potency varied with structures. For example, the double bond between C-11 and C-13 may be important for potency, whereas the 2,3-epoxy group possibly decreases its activity.

Our bioassays demonstrated that these compounds are a group of important phytotoxins in this plant and may play a role in allelopathy. These sesquiterpenoids may be leached down to the soil by rainfall or gradually released with the decomposition of leaf litter, affecting the growth of the surrounding plants, and assisting *M. micrantha* to become a dominant species in new ecosystems. However, to demonstrate the possible allelopathic role of these compounds, it is necessary to verify that these sesquiterpinoids can accumulate in the soil in

concentrations sufficient to affect neighboring plants. (Kamo et al., 2003; Inderjit and Weston, 2000).

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EXTENDING THE ELEMENTAL DEFENSE HYPOTHESIS: DIETARY METAL CONCENTRATIONS BELOW HYPERACCUMULATOR LEVELS COULD HARM HERBIVORES

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Abstract—Previous work has shown that hyperaccumulator levels of some metals can defend plants against herbivores, but the possibility of defense by metal concentrations at accumulator or normal levels is unexplored. This study tested the hypothesis that metals can defend plants at low concentrations. We determined the relative toxicities of eight metals commonly acquired by plants: Cd, Co, Cr, Cu, Mn, Ni, Pb, and Zn. Larvae of the diamondback moth (*Plutella xylostella*), a representative crucifer specialist, were fed with artificial diet amended with concentrations of metal varying from 2 to 3,000 μg/g. Different concentration ranges were used for each of the eight metals, and larval survival at 10–14 days was calculated for each concentration. All metals were toxic to diamondback moth larvae at hyperaccumulator levels. All metals, however, were also toxic to larvae at accumulator concentrations, far below those found in hyperaccumulating plants. Five metals (Cd, Mn, Ni, Pb, and Zn) were toxic below accumulator levels, Cd and Pb were toxic near the concentration ranges of normal plants, and Zn was toxic at a concentration within the normal range. Our results indicate that uptake of certain metals may provide a defensive benefit for plants, and that elemental defenses may be effective at concentrations far lower than previously hypothesized. This study implies that elemental defenses are more widespread in plants than previously believed, and that the ecological consequences of even low levels of metal accumulation need to be explored.

Key Words—Accumulator, diamondback moth, elemental defense, hyperaccumulator, metal, metal toxicity, *Plutella xylostella.*

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INTRODUCTION

The concentrations of metals in the aboveground tissues of plants can be used to divide them into three somewhat arbitrary categories: normal plants, accumulators, and hyperaccumulators. These categories are defined in a relative fashion for each metal (Reeves and Baker, 2000), with normal plants having relatively low metal concentrations and accumulator and hyperaccumulator plants having increasingly extreme metal concentrations. According to Reeves and Baker (2000), normal plants typically contain low concentrations (generally <5 μg/g on a dry mass basis in aboveground parts) of several metals, including Cd (0.1–3 μg/g), Co (0.03–2 μg/g), Cr (0.2–5 μg/g), and Pb (0.1–5 μg/g). Normal concentrations of Ni and Cu can be somewhat greater: 5–25 μg Cu/g and 1–10 μg Ni/g. Still greater concentrations of Mn or Zn are typical, with normal concentrations of both metals ranging from 20 to 400 μg/g. Accumulator plants typically grow on mineralized soils and contain greater concentrations of metals (Baker, 1981; Macnair, 2003). Minimum accumulator concentrations, signifying unusually elevated metal concentrations, are 20 μg/g for Cd and Co, 50 μg for Cr/g, 100 μg/g for Ni, Cu, or Pb, and 2000 μg/g for Mn and Zn (Reeves and Baker, 2000).

Hyperaccumulators have exceptionally high concentrations of metals in their tissues (Brooks et al., 1977). These plants are almost exclusively found on mineralized or ultramafic soils (Brooks, 1987, 1998; Reeves and Baker, 2000; Boyd et al., 2004) that, in part because of their high metal concentrations, are unfavorable environments for most plants (Sieghardt, 1990; Reeves and Baker, 2000; Prasad and Strzalka, 2002). However, a number of species (about 400) actively take up and concentrate (hyperaccumulate) one or more of these metals (Baker et al., 2000; Reeves and Baker, 2000). Minimum tissue concentrations used to define hyperaccumulators of the above metals are as follows: 100 μg/g for Cd; 1000 μg/g for Co, Cu, Cr, Ni, and Pb; and 10,000 μg/g for Mn and Zn (Brooks, 1987; Pollard and Baker, 1997).

We know of no comprehensive study that has compiled the numbers of species in hyperaccumulator and accumulator categories for each metal. Pollard et al. (2002) showed that Ni levels among *Alyssum* species form a bimodal distribution with relatively few accumulator species, but noted also that the data were incomplete. Most surveys of plant metal concentrations are designed to discover hyperaccumulator species and, therefore, target taxa and geographic areas likely to yield them (e.g., Reeves et al., 1999). Researchers also may not proceed with detailed analyses of species that do not seem promising as hyperaccumulator candidates in initial sample screenings (e.g., Reeves et al., 1999). Thus, it seems likely that the number of accumulator species in the literature is underreported. For example, a recent survey of Cuban ultramafic species by Reeves et al. (1999) examined specimens from 277 species and documented 50 taxa of Ni hyperaccumulators and 25 of Ni accumulators.

The toxicity of hyperaccumulators to herbivores has been addressed by an increasing (but still relatively small) number of experiments. Much of this research has focused on determining the advantages a plant may receive from hyperaccumulation of an element (Boyd, 2004). Martens and Boyd (1994) suggested that hyperaccumulation may serve as an "elemental defense" against herbivory. Experiments with Ni (Boyd and Martens, 1994; Martens and Boyd, 1994; Boyd and Moar, 1999; Davis and Boyd, 2000; Boyd et al., 2002), Se (Hanson et al., 2003, 2004), Cu (Ernst, 1987), and, in some cases, Zn (Pollard and Baker, 1997; Jhee et al., 1999; Behmer et al., 2005) have supported this defense hypothesis. Other experiments have shown that not all herbivores are negatively affected by hyperaccumulator metal concentrations (e.g., Boyd and Martens, 1999), especially for Zn (Huitson and Macnair, 2003; Noret et al., 2005).

The above experiments deal with only a few of the elements hyperaccumulated by plants, targeting Ni, Zn, Cu, or Se hyperaccumulators. While Reeves and Baker (2000) reported that Ni is the most commonly hyperaccumulated metal, with 318 taxa (78% of known hyperaccumulators), a number of plants hyperaccumulate other metals: to date, there are 26 Co hyperaccumulators, 35 Cu hyperaccumulators, 14 Pb hyperaccumulators, and 16 Zn hyperaccumulators (Brooks et al., 1998; Reeves and Baker, 2000). There are also at least 11 species of Mn hyperaccumulators and at least one Cd hyperaccumulator (Brooks et al., 1998; Reeves and Baker, 2000). The protective function of these elements in plants has yet to be explored.

Although herbivore feeding experiments can demonstrate the toxicity of metals at hyperaccumulator concentrations, they do not show the minimum plant tissue metal concentration that can produce a negative or toxic effect (Boyd and Martens, 1998). Our experiment was, therefore, designed to investigate the toxic thresholds of Ni and Zn, as well as six other hyperaccumulated metals (Cd, Co, Cr, Cu, Mn, and Pb). Our goals were twofold: (1) to determine whether these metals are toxic to herbivores at hyperaccumulator levels, thus extending the defense hypothesis to other hyperaccumulated metals; and (2) to determine whether any of these metals could serve as elemental defenses at concentrations within the ranges of accumulator or normal plants, thus extending the defense hypothesis to new categories of plant species.

METHODS AND MATERIALS

Experiment Rationale. Our experiment used a representative herbivore raised on artificial diet amended with a range of metal concentrations. We used larvae of the diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), a folivore that attacks plants of the Brassicaceae (Talekar and Shelton, 1993), a family that contains about 25% of all hyperaccumulator taxa

(Reeves and Baker, 2000). Lepidopteran larvae have been shown to be important herbivores of wild-growing species of *Streptanthus* (Brassicaceae) plants and can easily be responsible for the death of a plant. For example, Shapiro (1981a) reported that *Pieris sisymbrii* (Lepidoptera: Pieridae) is a specialist herbivore of several *Streptanthus* species, including *S. glandulosus* and *S. breweri*, both of which are found on ultramafic soils in California. He also noted that some populations of *S. polygaloides* may be attacked by *P. sisymbrii* (Shapiro, 1981b), and that a single *P. sisymbrii* oviposition on a plant of *S. tortuosus* could be effectively lethal if the resulting larva destroys the plant's leaves and fruits (Shapiro, 1981c).

Advantages of using DBM also include its short generation time, small size, ease of culture on artificial diet, and the commercial availability of a standardized artificial diet. Its short generation time (about 25 d from egg to adult for our colony) allowed us to rapidly assess survival at the completion of the larval stage (about 11–12 d). Use of artificial diet provided us with direct control over the concentrations of metal that the insects consumed. Experiments that have varied soil metal concentrations to obtain different plant metal concentrations have reported difficulties obtaining consistency within a treatment (Boyd and Moar, 1999), have had problems generating subhyperaccumulator concentrations of a metal within a plant species (Boyd and Moar, 1999), or have found it difficult to use a single plant species to accumulate the range of metals that we desired to study (e.g., Boyd and Davis, 2001).

Experimental Colony. The DBM colony used was established using eggs obtained from a colony at Cornell University (Harvey, 2002). Founder moths from the Cornell colony were collected from a field in Geneva, NY, USA. After the colony was established in Auburn, it was supplemented with wild individuals collected from Auburn, AL, USA. Artificial diet for the colony was obtained from Bio-Serv® (Frenchtown, NJ, USA). The diet's exact ingredients are proprietary information, but wheat germ and cabbage leaf powder are two of the main ingredients (Carpenter and Bloem, 2002). The diet was used to maintain the colony following a procedure similar to that of Shelton and Collins (2000).

Our colony maintenance protocol was as follows. Sheets of DBM eggs obtained from the established colony were sterilized in 10% bleach solution for 20 sec, rinsed in water for 1 min, and allowed to dry. Dried egg sheets were cut into strips, containing about 300–400 eggs per strip, and each strip was placed in a 250-ml paperboard cup with about 1 cm of congealed artificial diet covering its bottom. The cups were placed in an incubator (37°C, ~30–50% relative humidity) until they hatched and the first instar larvae began to feed (~60 hr). Empty egg sheets were then removed, and larvae were allowed to feed until pupation (~11–12 d after hatching). Pupae were then placed in screen cages where adults could emerge to mate and lay eggs (~4 d from pupation to

emergence). Eggs were collected on scored aluminum foil sheets that had been dipped in sterilized collard juice (an oviposition stimulant) (Shelton and Collins, 2000). Peak oviposition occurred between 2 and 6 d after adult emergence. Eggs collected from this time period had the greatest vitality; eggs collected after this time period produced larvae with 25–50% lower survival (from egg to adult) than average for the colony. Consistent with the Cornell colony, mean survival of larvae from egg to pupa was at least 50% (Shelton and Collins, 2000).

Artificial Diets Containing Metal. Artificial diet was amended with varying concentrations of the eight metals studied by using stock solutions of metal chloride salts (obtained from Sigma-Aldrich, St. Louis, MO, USA). These amended diets were used to determine metal toxicity by feeding different sets of DBM larvae different concentrations of a given metal. Experimental concentrations selected were based on preliminary results, and we attempted to include at least one nontoxic dose of each metal chloride salt. Five to seven concentrations were tested for each of the eight metals. For each concentration, 100 ml of diet were made to produce the concentrations listed in Table 1. Diet was distributed into 30-ml plastic cups to give 14 replicates of each

TABLE 1. METAL CONCENTRATIONS IN ARTIFICIAL DIET[a]

Metal	Units	Concentration						
Cd	mM $CdCl_2$	0	0.018	0.044	0.070	0.088	0.132	
	μg Cd/g	0	7.50	19.0	31.5	38.5	56.5	
Co	mM $CoCl_2$	0	0.022	0.066	0.088	0.176	0.264	
	μg Co/g	0	2.8	9.0	12.3	25.0	40.0	
Cr	mM $CrCl_2$	0	0.22	0.44	0.88	1.76	3.52	
	μg Cr/g	0	24.0	53.0	106	210	418	
Cu	mM $CuCl_2$	0	0.88	1.32	1.76	2.64	3.52	4.4
	μg Cu/g	7[b]	130	195	280	400	500	660
Mn	mM $MnCl_2$	0	4.4	8.8	17.6	26.4	35.2	
	μg Mn/g	15[b]	350	680	1370	2000	2750	
Ni	mM $NiCl_2$	0	0.22	0.44	0.88	1.76	3.08	
	μg Ni/g	0	20.0	35.0	70.0	136	245	
Pb	mM $PbCl_2$	0	0.044	0.088	0.220	0.308	0.440	
	μg Pb/g	2[b]	15.0	30.0	100	165	250	
Zn	mM $ZnCl_2$	0	0.22	0.44	0.55	1.10		
	μg Zn/g	30[b]	140	275	350	700		
Ca/Cl	mM $CaCl_2$	0	1	20	30	40		
	μg Ca/g	5900[b]	6900	7840	8540	9500		
	μg Cl/g	0	1660	3330	4570	6250		

[a] mM data, as millimoles of metal chloride per liter, calculated from dilutions of stock solutions; μg/g data from elemental analysis of diet samples. Fourteen replicates were used for each concentration of each metal.

[b] Amounts of these elements were present in the original diet mix (Mn, Zn, Cu, and Ca) or result from contamination during sample preparation for ICAP analysis (Pb).

concentration, with each cup receiving about 2–3 ml. A 30-ml diet sample of each metal concentration was reserved for elemental analysis.

Use of chloride salts introduced the potential for chloride toxicity to affect the results. Thus, we created an experiment using diet amended with $CaCl_2$ to serve as a partial control. For this test, a range of $CaCl_2$ concentrations was used that included the highest molar concentration of chloride from all metals tested (Table 1). If the $CaCl_2$ amended diet did not cause significant larval mortality, whereas diets amended with other metals did, then we could conclude that chloride toxicity did not significantly influence our results.

Elemental Analysis. Elemental analysis of dried diet samples was used to determine metal concentrations expressed as parts per million (μg/g) of metal in each batch of diet. Diet was analyzed using inductively coupled argon plasma spectroscopy (ICAP) (Jarrell-Ash ICAP 9000: Genesis Laboratory Systems, Grand Junction, CO, USA) for Cd, Co, Cr, Cu, Mn, and Pb and atomic absorption spectroscopy (AA) (Instrumental Laboratory, IL 215) for Ni and Zn. For both processes, the 30-ml preserved sample of diet were dried at ~60°C for 5 d and ground to a fine powder. The AA samples were wet-ashed with nitric and perchloric acids, dissolved in HCl, diluted with deionized water, and analyzed. The ICAP samples were dry-ashed in a muffle furnace, oxidized with boiling nitric acid, dissolved in HCl, diluted with deionized water, and analyzed.

Metal Toxicity Tests. Each set of concentrations for a given metal was tested for toxicity to DBM larvae using eggs collected from a single cage containing adults that were 2–4 d posteclosion. Eggs were collected at the completion of a 24-hr period and sterilized as described above. After egg sheets dried, they were cut into strips that were divided among the 70–100 cups (of five to seven metal concentrations) that composed a single metal toxicity test. Between 60 and 100 eggs were placed in each cup of diet, yielding 30–50 first instars per cup.

Cups were placed into an incubator with the same temperature and humidity conditions used for the colony. Egg sheets were removed from cups after larvae hatched and had begun to feed (60–72 hr after the initial egg sheet was collected and sterilized). When egg sheets were removed, an initial count was made of first instars present in each cup. The number of larvae alive was then counted at 2- or 3-d intervals. When larvae began to pupate (generally 10–12 d after eggs were collected), the number of pupae plus larvae alive at that time was used to represent survival in each cup, and survival was expressed as the percentage of initial first instar larvae. Counting for all cups within a metal set was terminated when adults started to emerge in the control cups, usually 14–17 d after the eggs were collected.

Statistical Analysis. Survival data for each metal were analyzed to determine each metal's minimum toxic concentration. Survival values were arcsine square root transformed to satisfy the normality assumption (Zar, 1996) underlying one-way analysis of variance (ANOVA), and ANOVA (StatView:

Abacus Concepts, 1998) was used to determine if metal concentration significantly affected survival at $\alpha \leq 0.05$. For each metal, Fisher's protected least significant difference (PLSD) test was used to determine which concentrations significantly decreased survival relative to the control treatment at $\alpha \leq 0.05$ (Abacus Concepts, 1998).

RESULTS

Toxic effects of metal treatments on DBM larvae were due to metals and not to chloride toxicity. The test for chloride toxicity showed that concentrations of

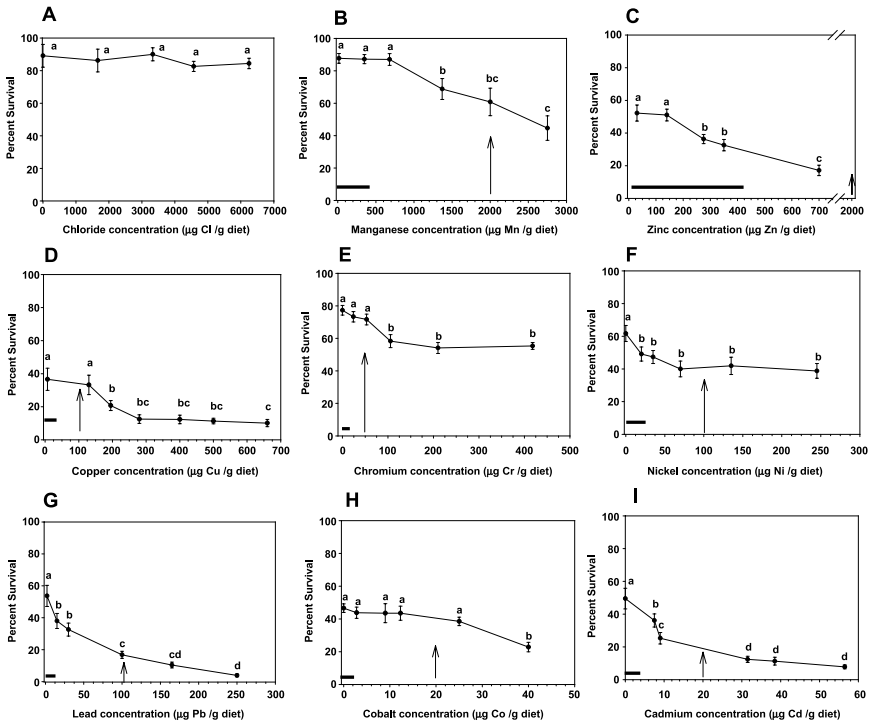

FIG. 1. Mean survival of DBM on $CaCl_2$ (A), $MnCl_2$ (B), $ZnCl_2$ (C), $CuCl_2$ (D), $CrCl_2$ (E), $NiCl_2$ (F), $PbCl_2$ (G), $CoCl_2$ (H), and $CdCl_2$ (I) amended diets. Bars represent the standard error of each mean. Means with the same letter are not significantly different (Fisher's PLSD test at $\alpha \leq 0.05$). A horizontal line denotes the normal concentration range for each metal in plant tissues (dry mass basis), and a vertical arrow shows the minimum concentration for accumulator plant status (Reeves and Baker, 2000).

TABLE 2. ANOVA RESULTS OF LARVAL MORTALITY DATA FROM THE EXPERIMENT FOR EACH METAL

Metal	*F*	*df*	*P* value
Cd	21	5, 78	<0.001
Co	5	6, 90	<0.001
Cr	10	5, 78	<0.001
Cu	7.7	6, 75	<0.001
Mn	4.7	5, 84	<0.001
Ni	3.4	5, 71	0.008
Pb	27	5, 70	<0.001
Zn	14	4, 76	<0.001

$CaCl_2$, which at their greatest exceeded the highest concentration of chloride of any other metal used (Mn: Table 1), produced no significant toxic effects (ANOVA: $F_{8,102} = 0.794$, $P = 0.609$) (Figure 1a). The mean survival for all experimental controls (no metal added to diet) was about 60%, a value above the minimum average survival of insects within the colony. These two control systems (chloride test and no-metal diet) showed that the main variable affecting larval survival was the addition of metals.

All metals affected larval survival at some concentration (Table 2). A concentration of 1,370 μg Mn/g produced the first significant decrease in survival for that metal (Figure 1b). For Zn, survival decreased at 275 μg Zn/g (Figure 1c). The lowest dose of Cu that produced significant toxicity was 195 μg Cu/g (Figure 1d), with that for Cr at 106 μg Cr/g (Figure 1e).

TABLE 3. NORMAL RANGE, MINIMUM ACCUMULATOR LEVEL, MINIMUM HYPERACCUMULATOR LEVEL, AND LEVEL TOXIC TO DBM LARVAE FOR THE METALS USED IN THIS EXPERIMENT[a]

Metal	Normal range	Minimum accumulator level	Minimum hyperaccumulator level	Minimum toxic level to DBM
Cd	0.1–3	20	100	7.5 (0.018 mM)
Co	0.03–2	20	1,000	40 (0.264 mM)
Cr	0.2–5	50	1,000	106 (0.88 mM)
Cu	5–25	100	1,000	195 (1.3 mM)
Mn	20–400	2,000	10,000	1,370 (17.6 mM)
Ni	1–10	100	1,000	20 (0.22 mM)
Pb	0.1–5	100	1,000	15 (0.044 mM)
Zn	20–400	2,000	10,000	275 (0.44 mM)

[a] Normal range, minimum accumulator level, and minimum hyperaccumulator level refer to tissue concentrations in field-collected plants and follow Reeves and Baker (2000). All values are expressed in μg/g (dry mass basis) with toxic levels also expressed parenthetically in mM wet diet so that metal toxicities can be compared.

We did not determine the toxic thresholds for Ni and Pb, as the lowest dose tested for each, 20 μg Ni/g and 15 μg Pb/g, showed significantly toxic effects (Figure 1f and g). For Co, 40 μg/g significantly decreased DBM survival (Figure 1h). The lowest dose of Cd tested, 7.5 μg/g, significantly decreased DBM survival (Figure 1i). Minimum toxic doses for each metal are summarized in Table 3, along with the normal range, minimum accumulator threshold, and minimum hyperaccumulator threshold for these metals.

DISCUSSION

Minimum toxic concentrations (in wet diet) of metals to DBM larvae varied greatly. Most toxic were Cd and Pb (<0.05 mM; Table 3) followed by Ni and Co at <0.3 mM. Zinc and Cr were toxic at <1 mM, with Cu toxic at 1.3 mM and Mn as least toxic at 17.6 mM (Table 3). The ranking of these metals in terms of their toxicity to DBM was not surprising, as essential metals such as Mn and Cu are generally less toxic than nonessential metals such as Cd and Pb (Yasutake and Hirayama, 2002). We note that we did not differentiate between toxic (where metal in diet caused a toxic effect on larvae) and antifeedant (where metal in diet caused larvae to stop feeding and starve) effects of dietary treatments and, thus, cannot address the mechanisms whereby these metals affected DBM larvae. Studies that address these questions (e.g., Behmer et al., 2005) are valuable extensions of the work reported here. However, regardless of the mechanism(s) involved, our experiments still allow us to make conclusions about the effectiveness of these metals as elemental defenses.

We conclude that hyperaccumulator concentrations of all metals tested are toxic to DBM. Table 3 shows that toxic levels were far below hyperaccumulator thresholds, in every case <20% of the minimum value used to define hyperaccumulation. Thus, for the representative crucifer herbivore used in our study (DBM), hyperaccumulation of any of the metals we examined would cause plant tissues to be toxic. Because these results show that metal hyperaccumulation could protect plants via toxic effects on a folivore, they support the defense hypothesis of metal hyperaccumulation. These results also support other studies that have shown toxic effects of hyperaccumulator species of Ni (e.g., Boyd and Martens, 1994; Martens and Boyd, 1994; Boyd and Moar, 1999; Boyd et al., 2002) and Zn (Pollard and Baker, 1997; Jhee et al., 1999; Behmer et al., 2005) to most folivores tested. Furthermore, our results extend the defense hypothesis to hyperaccumulated metals that have not yet been experimentally tested *in planta* (Cd, Co, Cr, Cu, Mn, and Pb) and suggest that, at least for DBM, metal hyperaccumulation would be a potent plant chemical defense. We should note that metal toxicity may depend on other constituents of

the diet, and further experimentation *in planta* is needed to confirm our results. In regard to this, recent research on the defensive effects of Zn hyperaccumulation (Behmer et al., 2005), using both Zn-amended artificial diet and plants containing varying levels of Zn, has shown that defensive effects occurred at similar Zn concentrations in both experimental situations.

All of the metals we studied were also toxic to DBM at concentrations in the accumulator range. Toxic levels extended into the accumulator range for Co, Cr, and Cu and below that range for Cd, Mn, Ni, Pb, and Zn (Table 3). Thus, for these metals, accumulator concentrations in plant tissues could also constitute elemental defenses. Five metals (Cd, Mn, Ni, Pb, and Zn) could have defensive effects at concentrations below accumulator levels. One of these metals (Zn) was toxic at a concentration within the normal range of plant tissues (Table 3). The other four were toxic at concentrations between the upper limit of the normal range and the start of accumulator levels. We, therefore, conclude that, in the case of Cd, Zn, Ni, and Pb, concentrations below the accumulator level may be toxic to some folivores and act as elemental defenses.

While the matrix within which a herbivore ingests a metal may serve to decrease that metal's toxicity, the reverse is also possible. Metals in plants may act in combination with other defense compounds in ways that may contribute to their effectiveness at low concentrations. This would result in the possibility that even lower concentrations of metals than shown by our study may contribute to plant defense. Our experiments determined the toxicity of single metals added to artificial diet. Under natural conditions, a single metal is only one chemical component of a particular plant's tissues. Some plants hyperaccumulate or accumulate more than one metal (Reeves and Baker, 2000). Furthermore, plants usually contain one or more of a wide variety of organic (or secondary) chemicals that also may be toxic to some herbivores. For example, members of the Brassicaceae, a family which contains many Ni hyperaccumulator species (Reeves and Baker, 2000), commonly produce glucosinolates that have defensive effects against some herbivores (Bodnaryk, 1992). Combinations of chemicals may act together, either additively or synergistically (Dyer et al., 2003), to be more toxic to a herbivore than each is alone. If this is the case with elemental defenses, as suggested by Boyd (2004), then concentrations less than those used in our single-metal studies could contribute to defensive effects against herbivores when combined with other plant chemicals. By this reasoning, the minimum toxic concentrations we detected may not be the least quantity of each metal that can have defensive value to a plant. Combination effects may decrease the effective defensive threshold of metals to levels even less than those shown in our study. A recent study by Jhee (2004) has shown just such combination effects for Zn paired with Cd, Ni, and Pb, and for Ni paired with some organic defense chemicals (tannic acid and

alkaloids). These considerations imply that elemental defenses are more important than heretofore recognized.

A defensive effect of metals at levels below hyperaccumulator concentrations is also notable because it suggests a mechanism whereby hyperaccumulation may have evolved. Boyd (2004) proposed that natural selection, driven by a defensive effect of metals against herbivores and/or pathogens, could have caused a stepwise magnification of metal levels in plants culminating in hyperaccumulation. Our study supports this hypothesis by showing that accumulation can itself have selective value against herbivory, leading to the survival of more toxic variants. Recent genetic studies of hyperaccumulation, summarized by Pollard et al. (2002), have demonstrated the existence of quantitative genetic variation in hyperaccumulation ability. Action of natural selection on similar variation in levels of accumulation could have resulted in the evolution of hyperaccumulation.

In summary, we suggest that elemental defenses may be more widespread among plants than previously suspected and may be more ecologically important than previously thought (Boyd, 2004). Our results show that plant species that accumulate or hyperaccumulate the metals that we studied will be toxic to DBM. These results also suggest that elemental defenses may be more widespread geographically than previously supposed. Most hyperaccumulator and many accumulator plant species are found on mineralized soils (Reeves and Baker, 2000), but the low concentration at which some metals (e.g., Zn; Figure 1c and Table 3) can be an effective defense suggests that some species growing on less mineralized soils may also be defended by metals. Our study extends the elemental defense hypothesis to plants other than hyperaccumulators, and suggests that hundreds or even thousands of additional plant species may be protected by elemental defenses.

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FOLIVORY IN FRUIT BATS: LEAVES PROVIDE A NATURAL SOURCE OF CALICUM

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Abstract—Leaves are an important dietary source of carbohydrates and protein, and an especially rich source of calcium for bats. Most studies of leaf eating by fruit bats have suggested that only male bats feed on leaves. In this study, 23 wild-caught Tongan fruit bats (*Pteropus tonganus*) were used in feeding trials conducted in an outdoor enclosure. The number of leaves and percentage of each leaf eaten were recorded for each bat on a daily basis, and these data were then multiplied by a calcium constant that was derived from a chemical analysis of leaves from *Callophylum neo-ebudicum*. Leaves of *C. neo-ebudicum* that were available in the enclosure were consumed by 82.7% of the bats. Overall, males consumed leaves in greater quantities and with higher frequency than females. Bats that consumed leaves on a regular basis consumed up to 46% more calcium to their diet compared with bats that did not regularly consume leaves. Leaves may represent a readily available, widely used, concentrated source of minerals for foraging bats, and have the potential to contribute significantly to the total amount of ingested calcium.

Key Words—*Pteropus tonganus*, *Callophylum neo-ebudicum*, calcium, folivory.

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INTRODUCTION

Folivory, or leaf eating by bats, is a well-documented phenomenon (Marshall, 1985; Lowry, 1989; Funakoshi et al., 1993; Kunz and Ingalls, 1994; Kunz and Diaz, 1995; Banack, 1996; Tan et al., 1998; Ruby et al., 2000). Leaves are an important dietary source of minerals, carbohydrates, and protein for bats, and they are especially rich in calcium (Tan et al., 1998; Nelson et al., 2000; Ruby et al., 2000). Leaves also provide a consistent food source for fruit bats as they are available year-round and are predictable in time and space (Kunz and Ingalls, 1994; Rajan et al., 1999). Thus, leaves may provide a greater net return per foraging bout than ingestion of large amounts of more widely dispersed and low-protein fruit or the active pursuit of insects (Kunz and Ingalls, 1994; Tan et al., 1998). In addition, steroid hormones found in leaves of some species may have an influence on the timing of bat reproduction (Wickler and Seibt, 1964; Kunz and Diaz, 1995).

Bats consume leaves by leaf fractionation. This process includes masticating the leaves into a bolus, swallowing the liquid portion, and ejecting the flattened fibrous pellet (Lowry, 1989; Kunz and Ingalls, 1994; Kunz and Diaz, 1995). By rejecting the fibrous portion, bats are able to consume leaf nutrients without altering their digestive tract or increasing wing loading (Kunz and Ingalls, 1994). Frugivorous bats appear to be preadapted for folivory by leaf fractionation; their dentition and gut morphology are specialized for extracting and digesting a largely liquid diet (Tedman and Hall, 1985; Kunz and Ingalls, 1994). To shift their diet alternately between one of fruits to leaves would involve little or no change in function of the gut or dentition (Kunz and Diaz, 1995).

Folivory was once thought to be rare among fruit bats, with leaves taken only when other food sources were scarce (Marshall, 1985; Funakoshi et al., 1993; Pierson et al., 1996). However, recent studies have shown that leaf eating is both common and widespread among Old World flying foxes (Banack, 1996; Tan et al., 1998; Ruby et al., 2000). Folivory has been reported for at least 17 species of Old World Megachiroptera, and leaves eaten by bats include 44 species of plants (Kunz and Diaz, 1995). For example, *Cynopterus brachyotis* fed regularly on the leaves of 14 plant species (Tan et al., 1998), and *Pteropus dasymallus* on nine species (Funakoshi et al., 1993). The incidence of leaf pellets under feeding roosts was 37–50% and occurred almost throughout the year (Funakoshi et al., 1993). However, this estimate may be low because leaf pellets produced by foliage roosting bats often go unnoticed because the pellets are inconspicuous among other plant material on the forest floor (Kunz and Ingalls, 1994).

Calcium is of particular interest in bat biology (Barclay, 1994, 1995; Kunz et al., 1995; Bernard and Davison, 1996). It has been postulated that females

may be stressed for calcium because of the mineral demands of both pregnancy and lactation (Barclay, 1994, 1995). To compensate for the relatively large size of their offspring, bats donate their own skeletal calcium to build the bones of their young (Barclay, 1995). Calcium concentrations are often much higher in leaves than in fruit (Nelson et al., 2000; Ruby et al., 2000). While some fruits may be high in calcium, the calcium is not readily available if the Ca/P ratio is less than the optimum ratio of 2 to 1 (McDowell, 1992; Robbins, 1993). The Ca/P ratio is three times higher in leaves than in fruits, which suggests that leaves may be sought by bats for their high calcium content (Kunz and Diaz, 1995; Ruby et al., 2000).

This study examined folivory among captive, wild-caught Tongan fruit bats (*Pteropus tonganus*). It is the first study to examine the amount of leaves that are consumed by individual fruit bats in a single night and to estimate how much calcium that folivory contributes to total daily calcium intake. We also describe sex and age differences in leaf consumption and suggest why this pattern may exist.

METHODS AND MATERIALS

Research was conducted from December 2000 to August 2001 on the island of Tutuila, American Samoa (14°S, 170°W) in the South Pacific Ocean. All 23 (13 males, 10 females) Tongan fruit bats (*P. tonganus*) were captured using large mist nets and transported to the "bat house." Upon being removed from nets, bats were placed into cloth holding bags and transported to a field laboratory or "bat house." Four adult males, 9 juvenile males, 4 adult females (of which two were lactating), and 6 juvenile females were used for experimentation. The bat house consisted of a 4 × 3 m outdoor wooden structure with an adjoining 4 × 3 m screened outdoor pen constructed specifically to house bats. The outdoor pen was built around a single *Callophylum neo-ebudicum* (Clusiaceae) tree for roosting and was the only source of leaves in the present study (Trail, 1994; Whistler, 1994). Bats were able to fly and move easily within the outdoor enclosure. Each night, bats were offered twice their body mass in bat foods known from the island (Banack, 1996). Fruits were suspended individually from the ceiling of the pen on a dowel rod, and fruit remains were analyzed separately (see Nelson, 2003). Fruit types supplied to the bats varied each day depending on their availability on the island. Salt rings composed of salt (96–99%) and mineral oil (Pet Products, Inc., Hauppauge, NY, USA) as well as collected rainwater were available to the bats *ad libitum*. Feeding trials were conducted on individual bats and lasted 3–5 d preceded by a 2 d acclimation period, for a total of 7 d in captivity. Bats were tested one at a time in the outdoor enclosure. Raised screen platforms were placed on the floor of the

enclosure to catch food and leaves dropped by the bats while they were feeding. We assumed that the amount of time that bats spent in captivity neither altered nor adversely affected their mineral status. If this assumption is correct, any possible deficiencies or excesses from their native diet should not influence their consumption patterns while in captivity.

The number of leaves and percentage of a leaf eaten were recorded for each bat on a daily basis. Samples of *C. neo-ebudicum* leaves were collected and dried at 105°C for 24 hr. Leaf samples included both whole leaves and leaves partially eaten by the bats. To assess the calcium content of leaf tissue actually consumed by bats, we only analyzed the portion of leaves that did not contain the midrib. These samples were then compared with whole leaves that contained the midrib. Leaf samples were dried to a constant mass and digested according to Miles et al. (2001). Calcium concentrations (ppm) were assessed by atomic absorption spectrophotometry (Perkin-Elmer AAS 5000, Norwalk, CT, USA) in the Nutrition Laboratory at the University of Florida and were calculated on a dry matter basis.

Males and females, juveniles, and adults were compared to identify possible differences in leaf-eating behavior observed during the experiments. Bats that consumed leaves more than 50% of the days during the 5 d experimental period were classified as habitual leaf eaters. Bats that only occasionally consumed leaves for fewer than half of the days they were housed for the experiments were classified as occasional leaf eaters. Some bats never consumed leaves and were classified as nonleaf eaters. These three groups were compared for total calcium consumption.

Values of supplementary calcium were calculated for each group as the total amount of leaf matter eaten by a bat multiplied by the calcium (Ca) concentration of the leaves (8,861.47 mg/g Ca). To estimate how much calcium that folivory contributed to total daily calcium intake, we calculated the average amount of supplemental calcium consumed for each of the three groups and compared this value to the average total calcium ingested by that group (Nelson, 2003). Differences in consumption of leaves between sex and age were compared using two-tailed *t*-tests. The Kolmogorov–Smirnov test was used to evaluate assumptions of normality for each variable, and Levene's test was used to evaluate the assumption of normality between groups (Sokal and Rohlf, 1995). Total calcium consumption for the three leaf-consumption groups was compared using a one-way ANOVA.

RESULTS

Ninety-four feeding trials were performed on 23 Tongan fruit bats. Leaves were consumed by 82.7% of the bats in this study. More males (92%) consumed

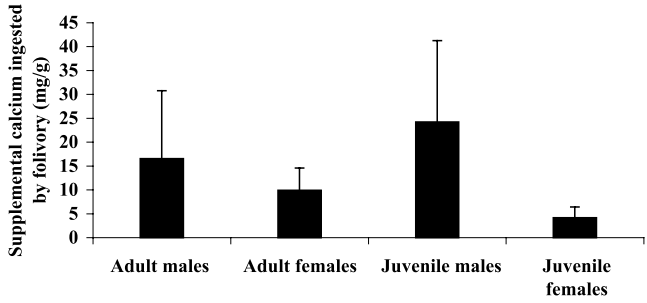

FIG. 1. Supplemental calcium ingested (mg/g) by folivory for adult males, juvenile males, adult females, and juvenile females.

leaves than females (70%). The total leaf mass eaten (g) differed ($P = 0.02$) between the sexes; males consumed an average of 9.55 ± 6.99 SD g, and females consumed an average of 2.97 ± 2.04 g over the period of the feeding trial. This resulted in 22.14 ± 16.00 mg/g of additional calcium for males and 7.06 ± 4.56 mg/g for females (Figure 1). The amount of additional ingested calcium was different ($P = 0.02$) between males and females. Juvenile male and juvenile female bats differed ($P = 0.04$) in their consumption of leaves (24.22 ± 17.03 and 4.19 ± 2.24 g, respectively), but adult male and female bats did not ($P = 0.40$). Twice as many juvenile males ate leaves than juvenile females

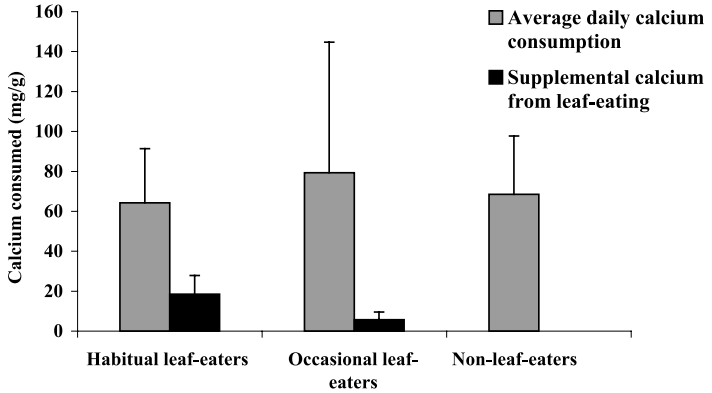

FIG. 2. A comparison of total calcium ingested among habitual, occasional, and nonleaf eaters. Total calcium is average daily calcium and supplemental calcium from leaf eating combined. Total average daily calcium values for each group are calculated from values found in Nelson, 2003.

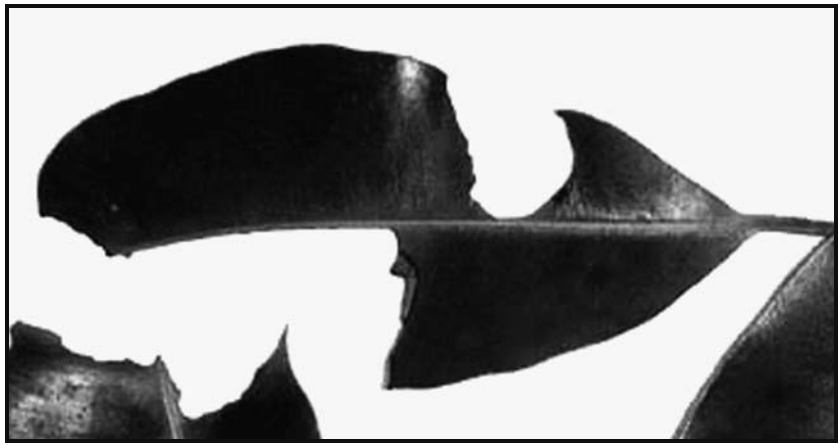

FIG. 3. Typical pattern of leaf consumption by *Pteropus tonganus* in American Samoa.

(eight males/four females). The number of leaves eaten by individuals over the 5 d experimental period ranged from 0.75 to 26 leaves. One young male ate 26 leaves in a single night.

Overall, folivory provided 2.3–32.26 mg/g of additional calcium to the diet of individual fruit bats. The numbers of habitual and occasional leaf eaters were similar (11 and eight, respectively); however, habitual leaf eaters consumed significantly more calcium than occasional leaf eaters ($P = 0.04$). Habitual leaf eaters consumed an average of 10 ± 6.56 leaves, which contributed an additional 23.89 ± 15.05 mg/g of dietary calcium (Figure 2). This represented an average dietary increase in calcium of 11–46% when compared to the daily calcium consumption for each bat in that group. Occasional leaf eaters consumed an average of 2.34 ± 1.54 leaves, which added an additional 5.75 ± 3.82 mg/g of calcium to their diet. Occasional leaf eating represented an average dietary increase in calcium of 3–22% when compared to the daily calcium consumption for each bat. Nonleaf eaters ($N = 4$) added no additional calcium to their diet.

When eating leaves, *P. tonganus* often avoided the fibrous midrib and instead ate around it (Figure 3). The portions of leaves with the midrib contained 15.08 mg/g of calcium, whereas leaves without midribs contained 8.86 mg/g of calcium. The manner in which Tongan fruit bats consumed leaves suggests that they are actively avoiding the fibrous midrib, possibly because of high levels of tannins or other secondary compounds. Unfortunately, this analysis was largely exploratory, and several leaves resulted in only one analyzed sample leaves containing the midrib and those without. Thus, a statistical analysis of these samples was not possible.

DISCUSSION

Previous studies reporting evidence of folivory (Marshall, 1985; Lowry, 1989; Funakoshi et al., 1993; Kunz and Ingalls, 1994; Kunz and Diaz, 1995; Banack, 1996; Tan et al., 1998; Ruby et al., 2000) were based on indirect and anecdotal means of quantifying leaf eating by bats. Thus, they were unable to determine either the amount of leaves eaten per bat or the sex and age of the consumer. The present study quantified both the number and quantity of leaves consumed by individual bats and how much this contributed to total dietary calcium intake. The majority (83%) of wild-caught Tongan fruit bats engaged in leaf eating during a 5-d experimental period. Both sexes consumed leaves, but male bats consumed more leaves than females, and juvenile males consumed both the greatest number and quantity of leaves for all groups. Habitual leaf-eating bats increased their dietary calcium consumption by 46%. Our results indicate that folivory is both commonly and frequently practiced by *P. tonganus* and thus has the potential to contribute significantly to the total amount of ingested calcium. In the present study, leaf consumption by the Tongan fruit bat involved only one plant species (*C. neo-ebudicum*) known from American Samoa, but levels of folivory could be even higher if leaves of other species had been available.

Kunz and Diaz (1995) observed only mature males carrying leaves, and they hypothesized that folivory may be limited to adult male bats. Leaf eating was practiced by both adult males and females in the present study, but it was observed most often in juvenile males. Other research has suggested that compounds extracted from leaves by fruit bats may influence their reproductive activity (Kunz and Ingalls, 1994; Kunz and Diaz, 1995). Leaves of *Erythrina*, consumed by *P. tonganus* on the island of Tonga (Harris and Baker, 1959), may contain one or more metabolites (alkaloids) important for reproduction. In addition to being a rich calcium source that supports rapid growth, leaves may also influence reproductive activity in young male bats. It is currently unknown if leaf consumption by female *P. tonganus* is influenced by hormonal compounds present in leaves.

Female bats may consume leaves to gain access to nutrients (e.g., protein) and minerals not available in some fruits (Kunz and Diaz, 1995). In general, leaves tend to have higher levels of calcium, sodium, manganese, and magnesium than ripe native or agricultural fruits (Nelson et al., 2000). Leaves are also widely available in both the wet and dry season (Whistler, 1994). Leaves analyzed from American Samoa provide rich sources of calcium and other macrominerals, and they may be particularly important for reproduction among Tongan fruit bats living there. Banack (1996) found that female Tongan fruit bats in American Samoa gave birth year-round, and young were observed on mothers during all months of the year. She also observed copulations with

pregnant females, suggesting that the female was nursing while allocating her own calcium for the skeletal formation of a new offspring (Barclay, 1995; Banack, 1996). Both gestation and lactation are nutritionally demanding, and their combined effect may promote leaf eating in females as a way to obtain additional minerals. Feeding trials on *P. tonganus* in American Samoa demonstrate high levels of calcium retention that suggest calcium stress (Bronner, 2003; Nelson, 2003). Together, the cumulative demands of gestation and lactation, with overlapping generations, and a diet low in calcium, may promote leaf eating by female bats in an attempt to gain supplemental calcium and other minerals.

Leaves also are widely available in both the wet and dry season (Whistler, 1994) in American Samoa, and leaf consumption is possible for *P. tonganus* throughout the year. Reports of year-around leaf consumption in other species are based on either analysis of fecal remains, leaf parts discarded beneath roosts (Lowry, 1989; Parry-Jones and Augee, 1991), or direct observation (Zortea and Mendes, 1993). Banack (1996) described year-round leaf consumption by both *Pteropus samoensis* and *P. tonganus* in American Samoa. Although folivory can contribute significantly to the total dietary calcium of leaf-eating bats, the motivation for folivory still cannot be attributed to a single factor. Our research has demonstrated that leaf eating is common among the Tongan fruit bat, and the leaves of *C. neo-ebudicum* provide a rich and concentrated calcium supplement to the potentially calcium-poor diet of this plant-visiting species in American Samoa.

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GEOGRAPHIC VARIATION IN ALKALOID PRODUCTION IN *Conium maculatum* POPULATIONS EXPERIENCING DIFFERENTIAL HERBIVORY BY *Agonopterix alstroemeriana*

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Abstract—*Conium maculatum*, a Eurasian weed naturalized in North America, contains high concentrations of piperidine alkaloids that act as chemical defenses against herbivores. *C. maculatum* was largely free from herbivory in the United States, until approximately 30 yr ago, when it was reassociated via accidental introduction with a monophagous European herbivore, the oecophorid caterpillar *Agonopterix alstroemeriana*. At present, *A. alstroemeriana* is found in a continuum of reassociation time and intensities with *C. maculatum* across the continent; in the Pacific Northwest, *A. alstroemeriana* can cause severe damage, resulting in some cases in complete defoliation. Studies in biological control and invasion biology have yet to determine whether plants reassociated with a significant herbivore from the area of indigeneity increase their chemical defense investment in areas of introduction. In this study, we compared three locations in the United States (New York, Washington, and Illinois) where *C. maculatum* experiences different levels of herbivory by *A. alstroemeriana* to determine the association between the intensity of the interaction, as measured by damage, and chemical defense production. Total alkaloid production in *C. maculatum* was positively correlated with *A. alstroemeriana* herbivory levels: plants from New York and Washington, with higher herbivory levels, invested two and four times more N to alkaloid synthesis than did plants from Illinois. Individual plants with lower concentrations of alkaloids from a single location in Illinois

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experienced more damage by *A. alstroemeriana*, indicative of a preference on the part of the insect for plants with less chemical defense. These results suggest that *A. alstroemeriana* may act either as a selective agent or inducing agent for *C. maculatum* and increase its toxicity in its introduced range.

Key Words—Insect–plant interactions, *Conium maculatum*, *Agonopterix alstroemeriana*, chemical defenses, alkaloids, γ-coniceine, coniine, conhydrinone, evolution, herbivory.

INTRODUCTION

Flowering plants and the insects that eat them collectively constitute almost half of all terrestrial species (Berenbaum, 1995). Interactions between plants and herbivorous insects are of central importance in determining community structure in a wide range of terrestrial systems. Thus, perturbations that affect interactions can have profound impacts within terrestrial systems. Among the global perturbations of increasing concern is the introduction and incorporation of nonnative species into communities within which they have not evolved. Once established, invasive plants can cause direct economic damage by reducing crop yields and livestock growth, and indirect damage by altering community composition via displacement of native species (Vitousek et al., 1996; Pimentel et al., 2000). Invasive species can influence the evolution of native species via niche displacement, competitive exclusion, introgression, hybridization, and even extinction (Mooney and Cleland, 2001).

Because the rate at which invasive species enter and become established in the United States has been increasing (Mooney and Cleland, 2001), there is considerable interest within the scientific community in understanding the dynamics of invasion. Characterizing "invasibility," however, has proven difficult (Crawley, 1987; Rejmanek and Richardson, 1996; Reichard and Hamilton, 1997). Transport of a species out of its native habitat generally results in a reduction in herbivory by coevolved specialist insects. Indeed, the idea that plant populations in areas of indigeneity are regulated by herbivores underlies the practice of classical biological control of weeds (Huffaker, 1959; Willis et al., 2000). The fact that plants tend to grow taller and produce more seeds in areas of introduction than in areas of indigeneity has been attributed in part to release from herbivorous natural enemies (Crawley, 1987). Blossey and Nötzold (1995), in proposing what they call the evolution of increased competitive ability (EICA) hypothesis based on optimal defense theory (Zangerl and Bazzaz, 1992), argue that invasiveness results from changes in biomass allocation patterns; in areas of introduction, where herbivores are absent, genotypes with reduced resource allocation to herbivore defense and increased resource allocation to competitive abilities are favored. Although

preliminary tests of this hypothesis with purple loosestrife (*Lythrum salicaria*) were suggestive (Blossey and Nötzold, 1995) and have been confirmed in other systems (Siemann and Rogers, 2001; Blair and Wolfe, 2004), additional tests failed to document intraspecific variation in herbivore resistance according to plant origin (Willis et al., 1999; Stastny et al., 2005), and a broader survey suggests that differences in sizes of plants in indigenous vs. nonindigenous habitats may represent plastic phenotypic variation rather than evolutionary change (Willis et al., 2000). More recently, a comprehensive comparison of size, fecundity, and leaf areas of nonindigenous and indigenous populations of *Hypericum perforatum* provided compelling evidence of the capacity for rapid contemporary evolution of these traits in invasive species (Maron et al., 2004a).

In general, little quantitative and ecologically relevant information is available on phytochemical changes in plants that occur after introduction into a nonindigenous area and release from interactions with longtime insect associates (Daehler and Strong, 1997; Willis et al., 1999; Siemann and Rogers, 2003; Maron et al., 2004b). Perhaps equally important, little information is available on phytochemical changes that ensue when coevolved herbivore associates resume interacting with a host plant in a nonindigenous area. This scenario is of no small consequence in that classical biological control involves reconstructing such plant–herbivore associations in the area of introduction. The possibility exists that, in the area of introduction, a newly resumed interaction will differ dramatically in its dynamics from such interactions in the area of indigeneity, given the differences in the structure of the surrounding community. Understanding the selective impact of reassociated herbivores on the chemistry of their host plants in areas of introduction is of interest not only in the context of understanding the basic dynamics of plant–insect interactions, but also in predicting potential trajectories of classical weed biological control programs.

A system in which the chemical consequences of reassociation with a coevolved herbivore may be thoroughly examined involves the interaction between *Conium maculatum* (L.) (Apiaceae) (poison hemlock), a Eurasian weed, and its monophagous associate *Agonopterix alstroemeriana* (Clerck) (Lepidoptera: Oecophoridae), a leaf-rolling European caterpillar known only to feed on *C. maculatum*. *C. maculatum* is an herbaceous Eurasian biennial that is extensively naturalized in temperate North America, as well as in other parts of the world, including Australia, New Zealand, and South America (Parsons, 1976; Holm et al., 1979). The weed is generally regarded as noxious; all aerial parts are poisonous to livestock and to humans (Sperry et al., 1964; Widner, 1984; Markham, 1985; Hannam, 1985; Jessup et al., 1986; Panter et al., 1988; Panter and Keeler, 1989). The toxicity of *C. maculatum* to vertebrates is attributable primarily to its production of relatively high concentrations of coniine

and related piperidine alkaloids, including methylconiine, coniceine, and conhydrine (Fairbairn, 1971). Its tendency to invade fields of alfalfa and other forage crops has led to livestock death through contamination of green-chopped hay (Kubik et al., 1980; Panter et al., 1988). *C. maculatum* is frequently a target of eradication programs in populated areas because of its toxicity, as well as to its rank odor and profuse growth.

Relative to other introduced weed species, *C. maculatum* is attacked by few insect herbivores. In an extensive survey of poison hemlock in southern California, Goeden and Ricker (1982) reported "amazingly few insect species or individuals thereof. A clear majority, 16 (70%) of the 20 [*sic*] phytophagous insect species found on this weed were rare and were only encountered as a few individuals at one or two sites." Of the relatively few native insect species that have colonized the plant extensively throughout its range, the majority are species that feed generally on native and introduced plants in the Apiaceae; these species include *Papilio zelicaon* Lucas (Goeden and Ricker, 1982), *P. polyxenes asterius* Stoll (Feeny et al., 1985; personal observations) (Lepidoptera: Papilionidae), and *Euleia fratria* (Loew) (Diptera: Tephritidae) (Berenbaum, 1981; personal observations). The most abundant insect associate of the plant in California until recently has been an aphid, *Hyadaphis foeniculi* (Passerini), accidentally introduced from Europe, where it also feeds on poison hemlock (Goeden and Ricker, 1982).

A. alstroemeriana, a leaf-rolling oecophorid caterpillar monophagous on *C. maculatum* throughout its native range in Europe, was first reported on *C. maculatum* populations in the United States in Tompkins County, NY, in 1973 (Berenbaum and Passoa, 1983). *A. alstroemeriana* was subsequently reported in 1983 in northern California, Oregon, and Utah and by 1987 was established in mesic areas of Washington, Idaho, and Colorado (Powell and Passoa, 1991), where it remains reliably "collectible in large numbers" (Anonymous, 1995). An adult *A. alstroemeriana* collected in 1990 near Columbus, OH, marked the first appearance of this species in the Midwest (Powell and Passoa, 1991). In 1993, the existence of established populations of *A. alstroemeriana* in central Illinois was confirmed (Berenbaum and Harrison, 1994); McKenna et al. (2001) reported substantial populations of this insect at several sites throughout Champaign County, IL.

Thus, throughout its range in North America, populations of *C. maculatum* exist with varying histories of reassociation with a specialist herbivore from its area of indigeneity. This continuum of association allows us to test whether a specialist insect (and potential biological control agent) may act as a selective agent on the defense chemistry of its host plant. In this study, we set out to determine whether chemical defense production by *C. maculatum* changes in response to reassociation with a principal herbivore from its area of indigeneity.

METHODS AND MATERIALS

Sampling. Study sites were located in Champaign County, IL, USA (40.109°N, 88.203°W), Tompkins County, NY, USA (42.443°N, 76.503°W), and Whitman County, WA, USA (46.733°N, 117.161°W). These locations were selected because they represent a continuum in time of association with and intensity of herbivory by *A. alstroemeriana* on *C. maculatum*, with longer association and higher levels of herbivory in Washington and New York and shorter association and lower levels of herbivory in Illinois. In June 2003, we selected four sites located more than 2 km apart within each region. *A. alstroemeriana* larvae can be found on *C. maculatum* for a short period of time, between 30 and 45 days, with some differences in life cycle timing among the regions studied. Thus, *A. alstroemeriana* larvae can be found from late April to early June in Illinois (Berenbaum and Harrison, 1994), from early May to mid-June in New York (Berenbaum and Passoa, 1983), and from early June to mid-July in Washington (Piper, personal observation). *C. maculatum* sampling was conducted in early June in Illinois and New York and in late June in Washington to coincide with the presence of *A. alstroemeriana* larvae. Plants are in approximately the same developmental stage across regions when *A. alstroemeriana* is abundant (early to mid-flowering). At each site within a region, we estimated *A. alstroemeriana* damage levels. Five levels of intensity were identified: level 0, *A. alstroemeriana* leaf rolls absent; level 1, leaf rolls present, but no visible leaf damage; level 2, minor defoliation, with up to one quarter of leaf area damaged (as estimated by eye); level 3, mild defoliation, between one quarter and one half of leaf area damaged; level 4, major defoliation, with more than one half of leaf area damaged; and level 5, complete defoliation, with most of leaf area damaged.

In each region, we randomly sampled between 12 and 20 *C. maculatum* plants per site. Two subsamples of green foliage per plant, one for alkaloid analysis and one for nitrogen and leaf water content analysis, were placed separately into Eppendorf tubes and frozen *in situ* on dry ice. Samples were shipped on dry ice to our UIUC laboratory and stored at −80°C until analyzed.

Plant Extraction and Chemical Analyses. Leaf material (ca. 200 mg FW) was ground using a ball mill and extracted on a shaker for 1 hr with 1.5 ml of acidified methanol (70% MeOH, 30% 0.1 N HCl). After centrifuging, 1 ml of the resulting extract was concentrated to approximately 0.2 ml on a centrifugal evaporator (Jouan RC 10.10), extracted with hexane to remove nonpolar compounds, and placed back into the centrifugal evaporator to remove the residual hexane. The remaining solution was then basified with 10 M NaOH; these were extracted in 200 μl hexane with 0.01% hexadecane. Alkaloids were analyzed by flame ionization detection on a gas chromatograph equipped with capillary column (Alltech EC-1, 30 m, 0.23 mm) coupled with an autosampler

(HP 5890). Hexadecane was used as internal standard. The samples were run with the following temperature program: initial temperature 50°C, ramp 5°C min^{-1} up to 105°C, ramp 35°C min^{-1} up to 290°C, 5 min at 290°C. (±)-Coniine (Sigma) was used as a standard. Alkaloid concentrations were expressed as coniine equivalents on a dry weight basis and per milligram of nitrogen. Total alkaloid concentrations were calculated by adding the concentrations of each individual alkaloid.

Total nitrogen was measured to estimate the relative resource investment in defense compounds among the three regions studied. A subsample of fresh leaf material was weighed, oven-dried at 60°C overnight, and weighed again to obtain the FW/DW ratio. Samples (10 plants per site) were then ground and analyzed for total nitrogen in an elemental combustion analyzer (Costech Instruments ECS 4010).

Isolation and Identification of Alkaloids. Leaves of *C. maculatum* were collected during March 2004 at Phillips Tract Experimental Station located at 5 km of Urbana, IL, USA. Fresh leaves (200 g FW) were extracted in a blender with 70% methanol, 30% 0.1 N HCl and filtered through Whatman 1 filter paper. The residue was re-extracted two more times; fractions were bulked together (1 l in total) and filtered through reversed-phase C18 (Baker; 40 μm) previously rinsed with methanol to remove nonpolar compounds. The eluate containing the alkaloids was concentrated by rotary evaporator at low temperature (max 45°C) until the volume was reduced by half; the eluate was then partitioned with chloroform (3×'s) to further remove nonpolar compounds. In a separation funnel, the extract was basified with 10 M NaOH and liquid–liquid partitioning was conducted with chloroform (5×'s) to extract the alkaloids. The chloroform fractions were combined, mixed with 20% HCl in MeOH, and evaporated to obtain a mixture of alkaloids in hydrochloride form. Bulk alkaloids were resuspended in ethanol/0.1 N HCl (1:1), basified with 10 M NaOH, and extracted (3×'s) with a small volume of chloroform. Individual alkaloids were isolated using a 25 × 2.5 cm silica gel (Merck; 32–63 μm) gravity column eluted with 150 ml of chloroform/ethanol/NH_3OH (70:30:1) (Leete and Olson, 1972) at ca. 1 ml min^{-1}. Fractions (2 ml each) were monitored by spotting 5 μl on a TLC silica gel plate (Baker; 250 μm) and sprayed with Dragendorff (Jungreis, 1985) or 0.2% ninhydrin reagents (Sigma). Although individual alkaloids did not completely separate, we could obtain fractions with more than 95% purity for the two major alkaloids to be identified by nuclear magnetic resonance (NMR) (RT 6.5 and RT 10) as explained below. The low concentration of alkaloid RT 12.0 did not allow us to obtain enough pure material for structure elucidation. Coniine identity (RT 5.6) (Figure 1) was established by comparison with authentic material (Sigma).

Liquid Chromatography-ElectroSpray Ionization-Mass Spectrometry (LC-ESI-MS) Analysis for Hemlock Alkaloids. Samples were run on a Finnigan-

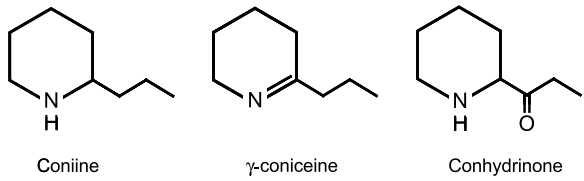

FIG. 1. Major piperidine alkaloids of *Conium maculatum* from Illinois, New York, and Washington.

Thermoquest LCQ LC-MS system (AS3000 autoinjector, P4000 HPLC pump, UV6000 PDA detector, LCQ mass spectrometer) (San Jose, CA) all running under the Xcaliber 1.2 software system. The MS was run with the ESI probe in the positive mode. The column was a 3 × 150 mm Inertsil reverse-phase C-18, ODS 3, 3-μm column (Metachem, Torrance, CA) with a Metaguard guard column. The source inlet temperature was set at 220°C, and the sheath gas rate was set at 90 arbitrary units. The MS was optimized for detection of hemlock alkaloids by using the autotune feature of the software while infusing a solution of coniine with the column effluent and tuning on an atomic mass unit of 128 $[M+H]^+$. The solvent systems were as follows: (A) water with 0.1 M ammonium acetate and 0.25% acetic acid; and (B) methanol with 0.1 M ammonium acetate and 0.25% acetic acid. The column was equilibrated with 2% B at a flow rate of 0.3 ml min^{-1}. After injection, the column was held at the initial conditions for 2 min, then developed with a linear gradient to 40% B over 23 min and then to 50% B over the next 10 min. The column effluent was monitored at 210 nm in the PDA detector. Mass data between 150 and 1000 AMUs was collected. Generally, the most significant sample ion generated under these conditions was $[M+1]^+$.

Gas Chromatography–Mass Spectrometry Analysis for Hemlock Alkaloids. Gas chromatography–mass spectrometry (GC-MS) was performed using a Hewlett-Packard (HP) 6890 GC system attached to an HP 5972A Mass Selective Detector. The column was a fused silica HP-5MS capillary (0.25-μm film thickness, 30 m × 0.25 mm ID). The GC operating parameters were as follows: splitless injection mode; temperature programmed from 50 to 315°C at 5°C min^{-1} with a 10-min initial temperature hold; He carrier gas flow rate at 1.1 ml min^{-1}, with the injector temperature set at 250°C. Spectra were compared to known standards or by computer with the Wiley/NBS Mass Spectral Registry (McLafferty and Stauffer, 1989).

Nuclear Magnetic Resonance Analysis of Hemlock Alkaloids. ^1H, Correlation Spectroscopy (COSY), Distortion less Enhancement by Polarization Transfer (DEPT), Heteronuclear Single Quantum Coherence (HSQC),

and ^{13}C-NMR spectra were obtained with a Bruker (Billerica, MA, USA) Avance 500 spectrometer equipped with a 5-mm inverse broadband Z-gradient probe (^{13}C NMR, 125 MHz; ^{1}H, 500 MHz). Nuclear magnetic resonance spectra were recorded in methanol-d4, which served as the internal reference (^{13}C, 49.0 ppm; ^{1}H, 3.30 ppm). The data were analyzed using the Advanced Chemistry Development, Inc., SpecManager 1D Processor and the HNMR and CNMR Predictor software suite (Toronto, ON).

Structure Confirmation of γ-Coniceine (RT 6.5). Positive ion LC-ESI-MS showed the presence of one major compound with a large mass ion $[M+H]^+$ of *m*/*z* 126. Prominent diagnostic GC-mass spectral ions and their relative intensities are as follows: EI-MS [*m*/*z* (%)]: 125 (M^+, 13), 124 (10), 110 (32), 97 (100), 96 (43), 82 (10), 70 (19), 55 (10), 54 (10); ^{1}H-NMR δ (CD_3OD): 3.65 (2H, bs), 2.82 (2H, m), 2.62 (2H, m), 1.90 (2H, m), 1.86 (2H, m), 1.71 (2H, m), 1.02 (3H, t, J = 7.4 Hz); ^{13}C-NMR δ (CD_3OD): 45.9, 41.0, 30.6, 20.3, 20.3, 17.9, 7.4. The ^{1}H-NMR and ^{13}C-NMR spectra identify a compound with a composition of $C_8H_{15}N$ consistent with the structure of γ-coniceine (Asensio et al., 1993; Fukuda et al., 1991) (Figure 1).

Structure Confirmation of Conhydrinone (RT 10). Positive ion LC-ESI-MS showed the presence of one major peak with a large mass ion $[M+H]^+$ of *m*/*z* 142. Prominent diagnostic GC-mass spectral ions and their relative intensities are as follows: EI-MS [*m*/*z* (%)]: 141 (M^+, 2), 98 (100), 84 (4), 70 (8), 55 (65); ^{1}H-NMR δ (CD_3OD): 4.04 (1H, dd, J = 12.3 Hz, J = 3.3 Hz), 3.39 (1H, m), 2.99 (1H, m), 2.63 (1H, m), 2.38 (1H, m), 1.91 (2H, m), 1.67 (2H, m), 1.54 (1H, m), 1.09 (3H, t, J = 7.2 Hz); ^{13}C-NMR δ (CD_3OD): 207.4, 64.4, 44.9, 32.6, 27.1, 23.2, 23.0, 7.4. The ^{1}H-NMR showed signals for an isolated ethyl group adjacent to a carbonyl carbon. The downfield proton is consistent with a proton attached to the carbon in the ring that is both adjacent to the nitrogen and attached to the side chain bearing the ketone. All assignments for conhydrinone could be obtained from the COSY spectrum. The ^{13}C-NMR revealed the presence of a keto carbonyl at 207.4 ppm. All the assignments were obtained from DEPT and HSQC experiments. The spectra match that of a compound with a composition of $C_8H_{15}NO$ corresponding to conhydrinone (Leete and Olson, 1972) (Figure 1).

Information on an Unknown Alkaloid (RT 12). Positive ion LC-ESI-MS showed the presence of one major compound with a large mass ion $[M+H]^+$ of *m*/*z* 126 and several minor contaminating TIC peaks. The retention time of this compound in the GC is significantly longer than of γ-coniceine; on this basis, we conclude that the compound has a different chemical structure. Prominent diagnostic GC-mass spectral ions and their relative intensities are as follows: EI-MS [*m*/*z* (%)]: 125 (M^+, 1), 124 (6), 110 (18), 97 (100), 96 (31), 82 (9), 69 (7), 55 (15). Examination of purified fractions containing this peak by NMR and IR yielded conflicting data as to the exact chemical structure of this compound.

At this time, we can say it appears to be a coniceine isomer with the exact location of the double bond undetermined.

Herbivory and Alkaloids. To determine whether *C. maculatum* chemistry is associated with differences in *A. alstroemeriana* abundance, we randomly selected 29 plants from one site in Champaign County, IL ("Yard waste site") in June 2003. For each plant, we counted the number of leaf rolls; two leaf samples per plant were then taken and alkaloids were analyzed as described. The number of leaf rolls was used as an estimate of herbivory intensity assuming a proportional relationship between the number of larvae and the number of leaf rolls made during larval development.

Statistical Analyses. All statistical analyses were performed using Statistica 6.0 (Statsoft, Tulsa, OK, USA). A one-way analysis of variance with "site" nested in "region" (Illinois, New York, and Washington) was performed to examine differences among populations in total alkaloid content, individual alkaloid content, and nitrogen content. A *t*-test was conducted to analyze the relationship between herbivory level and total alkaloid and N concentrations. *Post hoc* comparisons for "region" or "herbivory level" were conducted using Tukey's HSD test. The association between alkaloids and intensity of *A. alstroemeriana* herbivory was tested by conducting a simple regression analysis between total alkaloid concentrations and number of leaf rolls and a multiple regression analysis with coniine, γ-coniceine, conhydrinone, and RT 12 as independent variables and leaf roll number as the dependent variable. Data were log transformed to fit normality when necessary.

RESULTS

Herbivory. A. alstroemeriana herbivory levels on *C. maculatum* were lower in Illinois compared with New York and Washington, ranging from absence of leaf rolls (level 0) to minor defoliation (level 2) (Figure 2). In New York, herbivory damage ranged from minor defoliation (level 2) to total defoliation (level 5). However, in New York, especially at the Etna site (ET), the sampling was conducted earlier in the season compared with the other regions, as shown by the number of early instars found, and thus the actual damage to plants may be higher over the course of the season than that estimated here. In Washington, the damage inflicted by herbivores was severe, ranging from major (level 4) to complete defoliation (level 5) (Figure 2), and plants in many stands were found to be totally desiccated as a result of the damage by *A. alstroemeriana*.

Plant Chemistry. We found a total of four alkaloids in *C. maculatum* foliage: coniine, γ-coniceine, conhydrinone, and an unknown alkaloid (RT 12) (Table 1). Not all compounds were present in every plant, and when present,

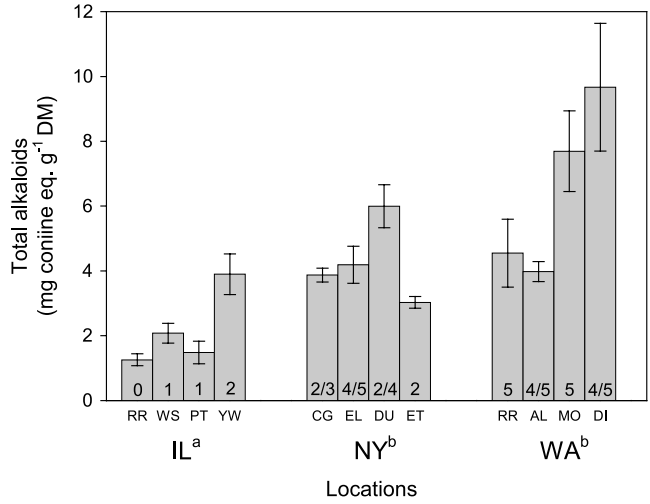

FIG. 2. Total alkaloid concentrations (mean ± SE) of *C. maculatum* plants by site in each region. In Illinois (IL): Railroad (RR), Windsor Rd (WS), Phillips Tract (PT), and Yard Waste (YW). In New York (NY): Coy Glenn (CG), Elm St (EL), Dump (DU), and Etna (ET). In Washington (WA): Railroad (RR), Albion (AL), Moscow (MO), and Ditch (DI). Averaged *Agonopterix alstroemeriana* herbivory levels for each site are indicated inside the graph bars (see a description of each level in Methods and Materials). Different letters indicate significant differences among regions at $P < 0.05$ by Tukey's *post hoc* comparisons test.

their concentrations were highly variable among individuals. Total alkaloid concentrations in plants expressed on a dry weight basis varied among the three regions (ANOVA, $P < 0.05$) and were lower in Illinois than in New York and Washington (Figure 2, Table 1). These lower levels reflected primarily lower concentrations of γ-coniceine, the major alkaloid in all three regions, constituting 80, 91, and 89% of the total alkaloids in Illinois, New York, and Washington, respectively. Plants in New York and Washington, although not different in total alkaloid content, differed in the concentrations of conhydrinone and RT 12, with lower values of both compounds in New York (Table 1). Coniine was present in about 16% of the plants growing in Illinois; however, in these plants, coniine reached such high concentrations that it constituted the second most abundant alkaloid after γ-coniceine. Coniine was not present either in New York or Washington (Table 1). Alkaloid concentrations expressed by unit N were significantly lower in Illinois and higher in Washington, partially because of differences in leaf N concentrations among regions (Figure 3). Total alkaloid concentrations were significantly higher in plants under increasing

TABLE 1. TOTAL ALKALOID CONCENTRATIONS (mg g^{-1} DM), INDIVIDUAL ALKALOID CONCENTRATIONS (mg g^{-1} DM), AND ALKALOID RELATIVE ABUNDANCE (%) IN *Conium maculatum* FROM ILLINOIS (IL), NEW YORK (NY), AND WASHINGTON (WA)

	Retention time (min)	IL	NY	WA
Total alkaloids		2.49[a]*	4.07[b]	6.48[b]
Coniine	5.7	[1.1][a] [(50.9%)]	0[b]	0[b]
γ-Coniceine	6.5	2.0[a] (80.8%)	3.96[b] (97.3%)	5.94[b] (91.7%)
Conhydrinone	10.0	0.22[a] (9.02%)	0.08[a] (1.96%)	0.37[b] (5.72%)
RT 12	11.9	0.07[a] (2.73%)	0.03[b] (0.68%)	0.16[c] (2.51%)

*Different letters indicate significant differences among regions at $P < 0.05$ by Tukey's *post hoc* comparisons test. Values of coniine shown in brackets indicate the average concentrations for the 16% of plants from the Illinois population that contain those alkaloids.

levels of *A. alstroemeriana* herbivory (Figure 4). No significant trends were found for herbivory levels relative to N concentrations (Figure 4).

Herbivory and Alkaloids. The number of leaf rolls in plants from Illinois, used as an estimate of *A. alstroemeriana* herbivory, was marginally negatively

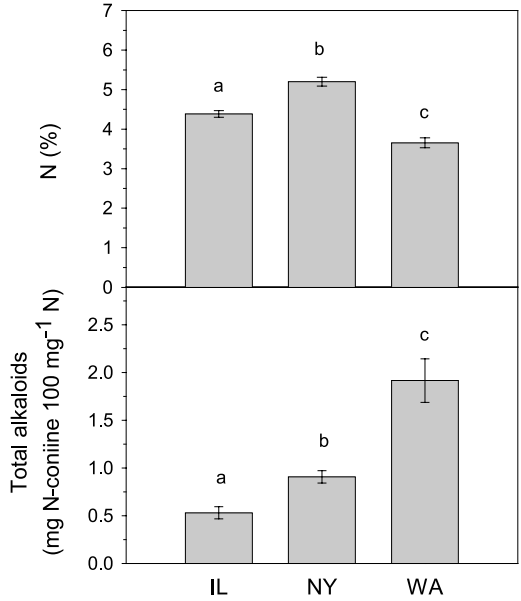

FIG. 3. Total N and total alkaloid concentrations (mean ± SE) expressed on N basis of *C. maculatum* growing in Illinois (IL), New York (NY), and Washington (WA). Different letters indicate significant differences at $P < 0.05$ by Tukey's *post hoc* comparisons.

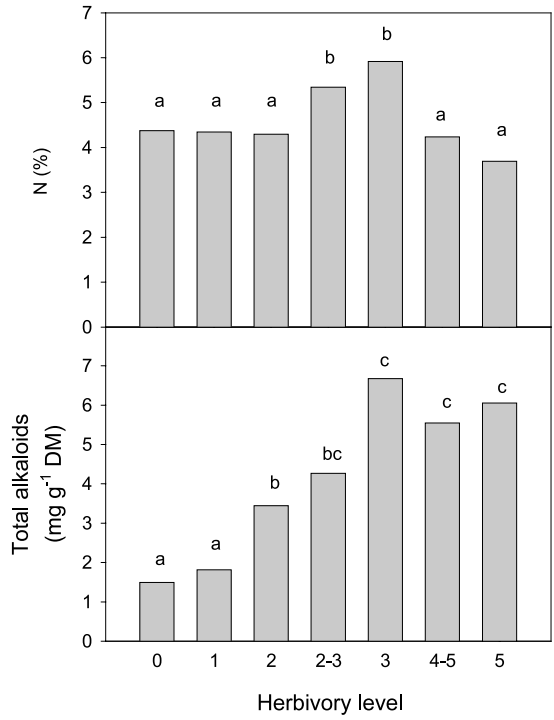

FIG. 4. Mean of N and total alkaloid concentrations at different *A. alstroemeriana* herbivory levels, from 0 to 5 with increasing levels of herbivory, as described in Methods and Materials. For total alkaloids, the back-transformed mean is shown. Different letters indicate significant differences at $P < 0.05$ by Tukey's *post hoc* comparisons.

correlated with total alkaloids (Figure 5a). In a multiple regression analysis, the number of leaf rolls was significantly correlated with γ-coniceine (Figure 5b), but no significant relationships were found for coniine, conhydrinone, or RT 12 (data not shown).

DISCUSSION

Poison hemlock has been established in Illinois for over 100 yr (Vasey, 1861; Patterson, 1876; Jones and Fuller, 1955). Despite the fact that the plant is extremely abundant locally, intermittent inspection over the last decade has consistently revealed few insect associates and little leaf damage by herbivores (personal observation). This same pattern has been documented in other parts

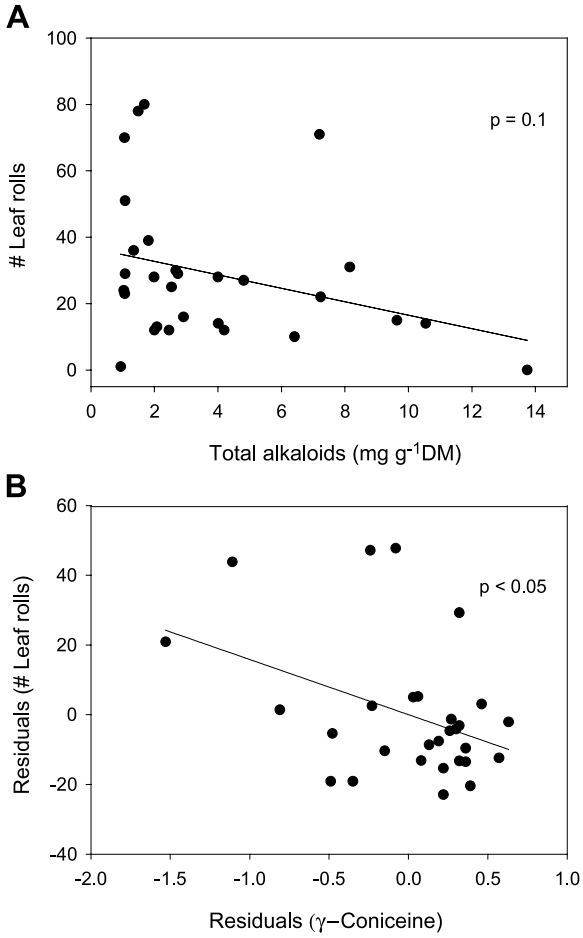

FIG. 5. (A) Simple regression between total alkaloid concentration and number of leaf rolls of 29 *C. maculatum* plants from Illinois. (B) Correlation between the residuals of γ-coniceine and the residuals of leaf roll number after performing multiple regression analyses with the independent variables coniine, γ-coniceine, conhydrinone, and RT 12.

of the United States—by Berenbaum (1981) in central New York and by Goeden and Ricker (1982) in southern California. Failure of insects to colonize this plant in large numbers over historical time may be attributable to its formidable array of chemical defenses. However, populations of poison hemlock in several localities in the United States are now experiencing unprecedented levels of herbivory because of colonization by and population

growth of *A. alstroemeriana*. It is of ecological and evolutionary interest to monitor changes in plant secondary compounds that may accompany this colonization. In this study, the chemistry of poison hemlock populations largely free from *A. alstroemeriana* (Illinois populations) was compared to populations in which *A. alstroemeriana* has become successfully established (New York and Washington populations) to determine under field conditions whether these herbivores, touted as potential biocontrol agents (http://www.bio-control.com, http://www.integratedweedcontrol.com), could reduce plant fitness and at the same time select for chemically based resistance factors (Berenbaum et al., 1986).

Changes in plant resource allocation between growth and chemical defenses driven by herbivore selective pressures have been discussed by the "optimal defense" hypothesis (Zangerl and Bazzaz, 1992) and the derivative "evolution of increased competitive ability" hypothesis, formulated in the context of invasion biology (Blossey and Nörtzold, 1995). According to these theoretical frameworks, when a plant species invades a new habitat where its native herbivores are absent, those genotypes with higher investments in growth and/or reproduction and reduced investments in defense are expected to have higher fitness and to increase their frequencies in the population. A logical corollary to this hypothesis is that defense investments should increase in the area of introduction commensurate with increases in herbivory, either by newly colonizing native species or by reassociation with introduced enemies from the area of indigeneity (as in the case of biological control). With the reassociation between the plant and the herbivore, such is the case of *C. maculatum* and *A. alstroemeriana*, the genotypes with increased levels of chemical defense should be favored by selection, particularly if, as is suggested in our study, high levels of alkaloid defense are deterrent to *A. alstroemeriana*. We found that C. maculatum plants from populations experiencing high levels of *A. alstroemeriana* herbivory (New York and Washington) had higher alkaloid concentrations in foliage than plants under lower levels of herbivory (Illinois). Because alkaloid concentrations in other species tend to increase with higher plant N availability (Gershenzon, 1984), concentrations in *C. maculatum* foliage were also analyzed to determine whether variation in the geographic pattern in *C. maculatum* alkaloid production could be related to differences in soils among regions. Nitrogen concentrations in the three populations varied significantly, with higher concentrations in New York and lower concentrations in Washington, but these differences were not consistent with the observed differences in alkaloid concentrations. Plants from New York and Washington invested two and four times more N to alkaloid synthesis, respectively, compared with plants from Illinois. In Washington populations, higher allocation of N to alkaloids was accompanied by lower total N concentration in foliage, which is suggestive of selection for higher constitutive or inducible alkaloid levels under more intense herbivory. Mechanistically, this pattern may

result from greater mortality on or avoidance of high levels of alkaloids by *A. alstroemeriana*; the negative relationship between the number of leaf rolls, and thus herbivory intensity, and the total alkaloid concentrations or γ-coniceine concentrations in Illinois plants is consistent with an increase in larval mortality with higher alkaloid concentrations.

Other environmental factors, such as water availability, could contribute to variation in the concentrations of alkaloids in *C. maculatum* across the United States. The different levels of alkaloid concentrations observed may reflect differential induced responses to herbivory (Castells et al., unpublished). Future studies, including a common garden experiment, will be necessary to determine definitively whether the geographic differences in alkaloids are genetically based and the result of a selection response to herbivory.

Although it was not deliberately introduced into the United States for biological control, *A. alstroemeriana* has demonstrated potential for systematic use as a biocontrol agent for poison hemlock. In the western United States, this insect has quickly become established naturally in infested locations and has established itself when it has been intentionally released (Piper, personal communication). Where it is established, it causes severe injury, including complete defoliation and destruction of inflorescences (Anonymous, 1995). However, the value of a biological control agent must be assessed not only by its ecological effect on population sizes, but also by its evolutionary impact on its target host plants. This study suggests that successful biological control agents may have the potential to alter the chemistry of their host plant, leading to increased allelochemical content, and potentially greater toxicity to livestock and humans who mistakenly ingest it.

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WITHIN-PLANT VARIATION IN GLUCOSINOLATE CONCENTRATIONS OF *Raphanus sativus* ACROSS MULTIPLE SCALES

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Abstract—Variation in chemical defenses remains underexplored. In particular, little is known about patterns of variation at small scales within leaves and spatial variation of induction. I examined variation in glucosinolate concentrations in the leaves of *Raphanus sativus* at several different spatial scales in two related experiments. I used samples equivalent in area to the amount an intermediate-sized caterpillar might eat in 1 d, a smaller scale than used in most previous studies. I examined variation due to induction and leaf age and small-scale spatial variation within leaves. The mean and variance of glucosinolate concentrations were higher in induced plants, young leaves, and the proximal half of leaves. Higher glucosinolate concentrations in the proximal half of leaves are previously unreported. Small-scale variation was extreme, accounting for 57% of the total random variation, and spatially random. There was no spatial autocorrelation found at scales as small as 1–2 cm. The high degree of small-scale, spatially random variation in glucosinolate concentrations in leaves is previously unreported. This small-scale variation and the variation caused by induction may have significant effects on herbivores and could be an important component of plant defense.

Key Words—Chemical defenses, induction, spatial patterns, small-scale variation, *Raphanus sativus*, glucosinolates.

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INTRODUCTION

Many plants have high degrees of variation in chemical defenses, and numerous authors have proposed hypotheses about why this variation has been maintained and how it might improve the effectiveness of a plant's defense (e.g., Denno and McClure, 1983; Berenbaum, 1995; Hartmann, 1996). Spatial variation in defenses within or among individuals could slow the evolution of resistance by herbivores by creating uneven selective pressure on herbivores (Dolinger et al., 1973; Whitham, 1981, 1983; Schultz, 1983b). Chemical variation might also improve the effectiveness of a defense in ecological time. A plant that can change its defensive phenotype in response to damage may become a "moving target" for herbivores (Adler and Karban, 1994). If defenses have nonlinear effects on herbivores, such that high doses have much stronger effects than low or moderate doses, a plant may have a stronger defense if it distributes its defenses unevenly (Karban et al., 1997; Shelton, 2000).

Induction of defenses is currently the most thoroughly examined source of variation in chemical defenses. When a plant systemically increases its levels of defense in response to herbivores, this increases the spatial variation within a population of plants and increases temporal variation within a plant. Only a few studies, however, have examined how induction affects spatial variation within an individual. Orians et al. (2000) examined the induction of proteinase inhibitors (PIs) in tomato plants in each leaf in response to mechanical damage of the second leaf. They found that vascular connections among leaves had strong effects on induction: some leaves had large increases in PIs in response to the damage, others had slight increases, and still others had no increase at all, depending on the strength of vascular connections to the damaged leaf.

There also has been little effort to document patterns of chemical variation within leaves at the small scales relevant to herbivorous insects. Individual leaves or leaflets are usually the smallest scale examined. For many small herbivores, however, smaller scales may have important effects. Only a handful of studies have looked at chemical variation within individual leaves (Whitham, 1983; Niemalä et al., 1984; Stout et al., 1996; Orians, 2002), and these have shown variation within leaves. For many small herbivores with limited mobility, small-scale variation may be important (Shelton, 2004), and patterns at larger scales may not accurately represent the variation that exists at smaller scales.

Another aspect of chemical variation that has not been sufficiently examined is variation that cannot be explained by predictable patterns. Most studies have examined chemical variation due to environmental variation caused by light, moisture, or nutrient levels (e.g., Louda and Rodman, 1983a; Mattson and Haack, 1987) or in reference to optimal defense theory (McKey, 1974; Rhoades and Cates, 1976), which states that valuable or threatened parts of

plants should be protected more than other parts. Even after predictable patterns are accounted for, a great deal of variation remains unexplained (see Denno and McClure, 1983), and the potential consequences of this unpredictable variation on herbivore behavior and fitness have not been explored.

In this paper, I examine chemical variation at multiple spatial scales within *Raphanus sativus* plants, including small-scale variation within individual leaves, and at three different levels of damage. I used leaf samples of a size equivalent to that eaten by an intermediate-sized caterpillar in one feeding bout (Shelton, data not shown), so the smallest scale of variation corresponds to a biologically relevant size for these herbivores. *Raphanus* is an excellent study system for the study of chemical variation because its chemistry, particularly of predictable patterns of variation of glucosinolates, is well documented (e.g., Larsen, 1981; Feeny and Rosenberry, 1982; Louda and Rodman, 1983b; Chew, 1988; Rosa and Rodrigues, 1998) as is its induced response to damage by herbivores (e.g., Bodnaryk, 1992; Agrawal et al., 1999).

Here, I present the results of two observational experiments that build on the known patterns of glucosinolate distribution. I examined two levels of damage, two leaf ages, and four regions of individual leaves, subsequently three levels of damage, and focused on the spatial arrangement of small-scale variation of glucosinolates within leaves. These experiments reveal new information about the effect of induction on variation of glucosinolates, the magnitude of variation at different scales, and the patterns and variation in glucosinolates at small scales within individual leaves.

METHODS AND MATERIALS

Study System. R. sativus (Brassicaceae) is a cosmopolitan, herbaceous annual plant. It is common in disturbed areas, roadsides, and agricultural fields in northern California. Like all members of the Brassicaceae family, its dominant defenses are glucosinolates, which are effective against an array of pathogens and nonspecialized herbivores (Ettlinger and Kjaer, 1968; Chew, 1988). Glucosinolates are attractants for a number of specialist herbivores (e.g., Chew, 1975; Siemens and Mitchell-Olds, 1996) but may be deleterious even to some of these specialists at high doses (Agrawal and Kurashige, 2003). At least 15 different glucosinolates have been isolated from *R. sativus* (Fahey et al., 2001), many of which, primarily indole glucosinolates, are inducible, increasing in response to damage by herbivores (Koritsas et al., 1991; Bodnaryk, 1992; Doughty et al., 1995; Agrawal et al., 1999). These compounds differ in their inducibility (Fahey et al., 2001) and effects on herbivores (Louda and Mole, 1991; Renwick, 1996; Lambrix et al., 2001). I measured only total glucosinolate

concentration. Total glucosinolate concentration is a conservative measure of chemical variation because analysis of specific glucosinolates would magnify variation.

I collected *R. sativus* seeds from wild populations growing in Santa Cruz, CA, USA, planted seeds from the current year in 15.25-cm pots with ProMix HP soil mix topped with 1 cm of vermiculite, and weeded the pots to a single seedling after germination. Plants were kept in the Thimann Greenhouse at UC Santa Cruz at ambient light and temperature. All plants were pest-free throughout the experiment (with the exception of the herbivores used to impose the damage treatments). Plants were kept far enough apart to prevent any shading, and because all experiments were completed while plants were still rosettes, there was no self-shading. The plants were watered as needed, with plain water during the first 2 wk, and with 100-ppm solution of Peter's 20:20:20 N/P/K fertilizer thereafter.

Plants for Exp. A were grown from August to September of 1999. Plants for Exp. B were grown from May to August 2001. Because of logistical constraints, each of the three damage treatments in Exp. B was applied to a separate group of plants grown at different times in summer 2001. This confounds the treatment effect with any potential time effect, although such effects are likely to be minimal because of the controlled conditions in the greenhouse. In addition, I found similar effects of damage in Exp. A, in which all plants were grown at the same time, and in other experiments (Shelton, 2002, and unpublished data).

Experimental Design. I used one level of damage in Exp. A, one leaf eaten by caterpillars (low damage), and compared it with undamaged controls. In Exp. B, I added an additional damage level of two leaves eaten by caterpillars (high damage; Table 1). Damage treatments and sampling were performed according to the developmental age of the plant. One week after the seeds germinated,

TABLE 1. SUMMARY OF THE LEVELS OF EACH TREATMENT USED IN EACH EXPERIMENT

	Experiment A	Experiment B
Damage levels (no. of leaves eaten)	No damage (0) Low damage (1)	No damage (0) Low damage (1) High damage (2)
No. of plants/treatment	10 (no damage) 10 (low damage)	6 (no damage) 5 (low damage) 4 (high damage)
Leaf ages	1 young 1 old	1 young
Leaf parts (replicates per part)	Main leaflet (12), Proxima leaflets (4)	Main leaflet (7–25), Proxima leaflets (0–25)

I placed a *Pieris rapae* (Lepidoptera: Pieridae) caterpillar on each leaf to be damaged. At this time, most plants had two true leaves (range two to four leaves). For the low damage treatment, I placed a caterpillar on the first true leaf, and for the high damage treatment, I placed caterpillars on both the first and second true leaves. I restricted caterpillars to a single leaf with clip cages (constructed from 4-cm plastic petri dishes with the edges padded to protect the leaf, drilled with ventilation holes, and attached to an aluminum hair clip). At the time of the damage, leaves were small enough to fit entirely within the cages, so there was minimal damage from the cage. I removed the cages and caterpillars once they had entirely consumed the leaf (2–3 d).

I sampled plants in the undamaged and low damage treatments 2 wk after germination, when plants had produced four to six leaves (counting the consumed leaves). Because essentially all the true leaves were consumed in the high damage treatment, there were no leaves to sample at the 2 wk point. Therefore, I sampled the high damage plants 3 wk after germination, when they had regrown one to two leaves that were at the same developmental stage as the leaves sampled in the other treatments.

Developmental age of leaves has strong effects on glucosinolate concentrations, particularly the degree of leaf expansion (Porter et al., 1991; Fahey et al., 2001), so I sampled leaves at the same developmental stage, rather than the same absolute age or leaf number. In Exp. A, I sampled one young leaf, and one mature leaf, which I defined as the youngest fully expanded leaf, from each of 10 plants. Young leaves varied in leaf number (first true leaf = 1) from 3 to 5, and old leaves were leaves 1–3. In Exp. B, I sampled one young leaf only from

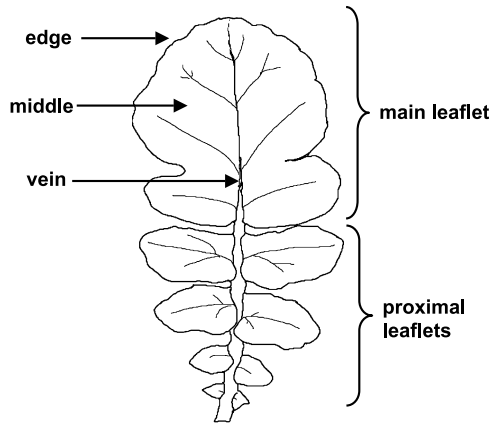

FIG. 1. Diagram of a typical leaf of *Raphanus sativus* indicating the regions of the leaf defined in Exp. A.

four to six plants in each treatment. I defined young leaves as the leaf closest to 50% of full expansion.

In Exp. A, I defined four regions of each leaf (Figure 1): three parts of the main terminal leaflet—the area including and within 1 cm of the midvein (vein), the area within 1 cm of the edge of the leaf (edge), and the rest of the main leaflet (middle)—and the proximal leaflets (leaflets). I cut four samples from each of the four leaf regions (for a total of 16 samples per leaf). The precise location within a defined region of the leaf varied among leaves.

In Exp. B, I sampled the entire leaf by dividing it into a grid of samples. I cut each leaf from the plant and scanned the leaves to a digital image. I then cut each leaf into separate samples with a razor blade and marked the spatial arrangement of the samples on the digital image. The area of samples was chosen to correspond to the amount eaten in 1 d by a third to fourth instar *Trichoplusia ni* caterpillar (Shelton, unpublished data). Glucosinolates tend to be present at high concentrations in young leaves and decrease as the leaf expands (Porter et al., 1991). As a result, leaf area may not be an ideal measure for glucosinolate concentration in rapidly expanding leaves. Therefore, I divided leaves according to the size and shape of the leaf rather than strictly controlling for area. The area of samples varied among the damage treatments (mean area = 2.7 cm^2 in no damage, 3.4 cm^2 in low damage, and 1.3 cm^2 in high damage). Smaller area of samples could overestimate variation within a leaf. To test whether this influenced the results, I summed the glucosinolate concentration of adjacent pairs of samples in the high damage treatment and statistically analyzed the data with these pairs treated as a single unit to increase the area of each sample. This had no qualitative difference on any of the results.

Chemical Analyses. Glucosinolates are stable and inactive in their intact form, but when cells are damaged, glucosinolates are exposed to thioglucosidase enzymes that break them into glucose and their composite mustard oils. I avoided degradation of glucosinolates by cutting samples from the leaves with a razor blade to minimize cell damage and the release of the enzymes. Within 15 min of removal from the plant, I cooked the samples in a microwave oven at full power for 2 min to denature the enzymes (Chew, personal communication). This method of heating has been shown to result in almost no residual myrosinase activity (Verkerk and Dekker, 2004). There was no detectable change in glucosinolate concentration between removal from the plant and completion of cooking of all samples (Shelton, unpublished data). I dried the samples in a 40°C oven overnight and stored them until analysis. Immediately before chemical analysis, each dried sample was weighed to the nearest 0.1 mg to standardize the chemical results to glucosinolate concentration per gram of dry leaf.

I quantified total glucosinolate concentrations following the protocol originally developed by Heaney and Fenwick (1981), modified by Siemens

and Mitchell-Olds (1996), and further modified by Stowe (personal communication). This method uses ion-exchange columns to isolate the glucosinolates, and then thioglucosidase enzyme is added to split off the glucose. Each molecule of glucosinolate releases one molecule of glucose, so glucose concentration is a direct proxy for the glucosinolate concentration. I ran two replicates for each sample with a glucose-reacting color reagent and measured the absorbance of the samples on a Labsystems Multiskan MCC/340 plate-reading spectrophotometer at 492 nm. I used DeltaSoft 3 spectrophotometer software (version 2.243 for the Macintosh, BioMetallics, Inc., 1999) to calculate the glucosinolate concentrations from glucose standards on each plate and divided these results by the sample weight to obtain glucosinolate concentration in μmol glucose/g dry leaf.

Spatial Analyses. I plotted the results of Exp. B using ArcView GIS software (v. 3.1) to visualize spatial patterns and to measure the distance between pairs of samples. I tested for spatial autocorrelation by calculating a variogram (Cressie, 1991) for each leaf with 1-cm increments. I determined confidence intervals for each variogram with randomization tests (Crowley, 1992; Hilborn and Mangel, 1997). To do this, I shuffled the measured glucosinolate concentrations within each leaf while retaining the spatial arrangement of samples, and then calculated a variogram for the randomized data. I used 10,000 randomizations per leaf and computed the 95% confidence intervals from the frequency distribution of the randomizations.

Statistical Analyses. I compared the differences in mean glucosinolate concentrations among treatments, leaves, and leaf regions with an ANOVA for each experiment. Even after $\log_{10}$ transformation, some heteroscedasticity remained, but because sample sizes are large, the data are reasonably balanced, and there was not a single group with particularly high variance, it should not significantly affect the results of the ANOVA (Underwood, 1997). The nested design of these experiments combined with the fixed effects of damage, leaf age, and leaf region includes both fixed and random effects.

The model for Exp. A includes the leaf and leaf region terms twice, once as a fixed effect and again as a nested random effect. This was necessary to separate the fixed effect of leaf age and leaf region across leaves, as well as to account for the random variation due to the specific leaf analyzed and the specific parts of the leaf that were sampled from a specified leaf region. The model for Exp. A was $G_{ijklmn} = \mu + T_i + A_k + B_l + P_{j(i)} + L_{k(ij)} + R_{l(ijk)} + S_{m(ijkl)} + \varepsilon_{n(ijklm)}$, where G is the log-transformed glucosinolate concentration of chemical replicate n of leaf sample m from region l on a leaf of age k on plant j with treatment i. T_i is the fixed effect of damage treatment, A_k is the fixed effect of leaf age, and B_l is the fixed effect of leaf region. The random effect of individual plant j in treatment i is represented by $P_{j(i)}$, $L_{k(ij)}$ is the random effect of a leaf, $R_{l(ijk)}$ is the random effect of the specific samples within a leaf region, and

TABLE 2. TOTAL GLUCOSINOLATE CONCENTRATIONS[a] FOR EACH LEVEL OF DAMAGE

	Treatment		
	No damage	Low damage	High damage
Exp. A			
Young leaves			
Main leaflet	7.27 ± 4.22 (116)	9.94 ± 6.16 (114)	
Edge	7.02 ± 4.50 (39)	9.82 ± 6.51 (40)	
Vein	7.14 ± 4.12 (38)	9.42 ± 6.28 (36)	
Middle	7.66 ± 4.11 (39)	10.55 ± 5.76 (38)	
Proximal leaflets	9.00 ± 5.85 (38)	13.82 ± 9.37 (39)	
Old Leaves			
Main leaflet	4.04 ± 2.88 (120)	7.11 ± 4.33 (124)	
Edge	4.35 ± 3.97 (40)	6.20 ± 3.70 (42)	
Vein	4.06 ± 2.05 (40)	7.23 ± 3.87 (40)	
Middle	3.74 ± 2.26 (40)	7.93 ± 5.12 (42)	
Proximal leaflets	5.47 ± 3.54 (36)	12.76 ± 10.47 (32)	
Exp. B			
Young leaves			
Main leaflet	3.46 ± 1.61 (64)	5.23 ± 2.66 (55)	9.85 ± 8.34 (90)
Proximal leaflets	3.40 ± 2.20 (37)	5.36 ± 3.52 (30)	10.51 ± 10.30 (60)

[a] Glucosinolate concentrations are means ± standard deviation in μmol glucosinolate/g dry leaf weight. Sample sizes are in parentheses.

$S_{m(ijkl)}$ is the random effect of individual leaf samples within a specific leaf region.

Exp. B did not include a fixed effect for leaf age because only young leaves were examined, and because the entire leaf was sampled, there is no random effect of samples within a leaf region. Thus, the statistical model was $G_{ijklm} = \mu + T_i + P_{j(i)} + B_k + S_{l(ijk)} + \varepsilon_{m(ijkl)}$ where G is the log-transformed glucosinolate concentration of chemical replicate m of plant sample l within region k of plant j in treatment i. T_i is the fixed effect of damage treatment, $P_{j(i)}$ is the random effect of plants, B_k is the fixed effect of leaf regions, $S_{l(ijk)}$ is the random effect of samples within a leaf region, and $\varepsilon_{m(ijkl)}$ is the experimental error. I determined the appropriate tests for each factor from the expected mean squares (Tables 2 and 3).

Because I was also interested in differences in variation in addition to mean differences, I used Levene's test for homogeneity of variances (Snedecor and Cochran, 1989) to test for differences in variation among damage treatments and among leaves within damage treatments. I performed these tests on the untransformed data because it better represents the variation experienced by caterpillars. I did all of the statistical analyses in PROC GLM of SAS version 6.12 for the Macintosh.

TABLE 3. ANOVA RESULTS FOR EXP. A

Source	*df*	Type III SS	MS	Denominator MS[a]	*F* value	*P* value	Variance components[b] (%)
Tmt	1	12.692	12.692	Plant (tmt)	6.98	0.017	–
Leaf age	1	8.551	8.551	Leaf (tmt, plant)	8.04	0.011	–
Leaf part	3	3.079	1.026	Part (tmt, plant, leaf)	7.09	<0.001	–
Plant (tmt)	18	32.748	1.819	Leaf (tmt, plant)	1.71	0.127	11.2
Leaf (tmt, plant)	19	20.203	1.063	Part (tmt, plant, leaf)	7.34	<0.001	27.3
Part (tmt, plant, leaf)	115	16.654	0.145	Sample (tmt, plant, leaf, part)	1.19	0.111	2.7
Sample (tmt, plant, leaf, part)	461	56.153	0.122	Error	64.71	<0.001	57.0
Error	618	1.163	0.002				1.8

[a] Used to construct *F*-tests for each factor as determined from expected mean squares.
[b] Variance components were calculated only for random effects.

RESULTS

Differences Among Damage Treatments. Damage by herbivores increased both the mean and variation of glucosinolate concentrations in both experiments (Table 2). Consumption of one leaf increased glucosinolate concentrations approximately 1.5 times compared to the undamaged treatment in both experiments, and consumption of two leaves increased concentrations by 2.9 times. The increase in glucosinolates due to damage treatments was statistically significant in Exp. A (Table 3) but not in Exp. B (Table 4). This may be due to reduced power in Exp. B because fewer plants were examined. Variation of glucosinolates was significantly higher in the damaged treatments for both experiments (Levene's test: Exp. A, $F = 7.95$, $P = 0.011$; Exp. B, $F = 32.95$, $P < 0.001$). Among the three damage treatments in Exp. B, the high damage treatment had significantly higher variation than the undamaged treatment (Tukey's test, $P < 0.05$).

Differences Among Leaves. Young leaves had significantly higher glucosinolate concentrations than old leaves (Table 3). According to Levene's test, young leaves were also more variable than older leaves ($F = 15.15$, $P < 0.001$). There were differences among leaves within a damage treatment (random effects) in both Exps. A and B. Exp. A includes separate effects for plants and

TABLE 4. ANOVA RESULTS FOR EXP. B

Source	*df*	Type III SS	MS	Denominator MS[a]	*F* value	*P* value	Variance components[b] (%)
Tmt	2	7.245	3.623	Part	1.60	0.243	
Part	1	0.001	0.001	Sample (tmt leaf part)	0.00	0.948	
Leaf (tmt)	12	27.242	2.270	Sample (tmt leaf part)	7.20	<0.001	34.5
Sample (tmt leaf part)	318	100.230	0.315	Error	15.12	<0.001	57.4
Error	329	6.859	0.021				8.1

[a] Used to construct *F*-tests for each factor as determined from expected mean squares.
[b] Variance components were calculated only for random effects.

leaves because both old and young leaves were sampled from each plant. The leaf effect was significant, but the plant effect was not (Table 3). Only one leaf per plant was sampled in Exp. B, so differences among leaves and plants are confounded. This plant/leaf effect was significant and contributed about 34.5% of the random variation in the data (Table 4). This is comparable to the sum of the random variation explained by the separate plant and leaf effects in Exp. A (38.5%).

Glucosinolate concentrations in Exp. B, which included only young leaves, were lower than concentrations for young leaves in Exp. A (Table 2). The reasons for this are unclear because the plants were of the same age, and the leaves were of the same relative age. It may be a result of differences in seeds or greenhouse conditions between years. The range of glucosinolate concentrations varies widely in other studies because of environmental or genetic effects. The values in both experiments of this study correspond to values found for similar species in other studies (Fahey et al., 2001; Agrawal and Kurashige, 2003; Brown et al., 2003).

Within-Leaf Spatial Patterns. Among the four leaf regions examined in Exp. A, the proximal leaflets had significantly more glucosinolates than all three regions of the main leaflet (Table 3). The three regions of the main leaflet (edges, veins, and middle) were not statistically different from each other. Proximal leaflets did not differ from the main leaflet in Exp. B (Table 4). However, there was a tendency for the proximal half of a leaf to have higher glucosinolate concentrations than the distal half. Of the highest 10% of observed glucosinolate concentrations in Exp. B, 82% (28 of 34) were in the proximal half of leaves ($X^2 = 11.155$, $P < 0.001$). The random variation among samples within a single leaf region was highly significant and accounted for approximately 57% of the total random variation in both experiments (Tables 3 and 4). This means that the

differences in glucosinolate concentrations among the replicate samples from a single leaf region (e.g., proximal leaflets) of one leaf were extremely variable.

This variation can be visualized in Figure 2, which shows the glucosinolate concentration for each section of the leaves in Exp. B. The glucosinolate

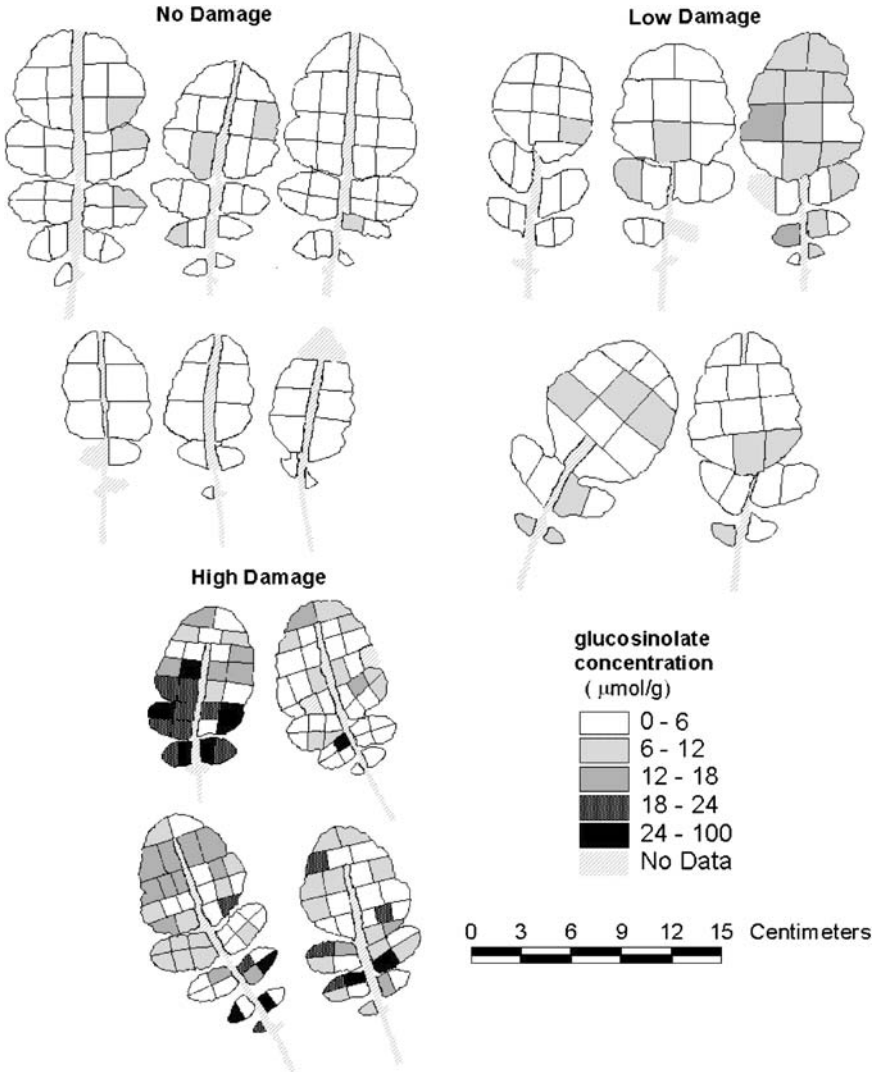

FIG. 2. Spatial distribution and glucosinolate concentrations of samples in Exp. B. Glucosinolate concentrations are measured as μmol/g dry leaf.

concentrations are patchily distributed within leaves, particularly in the high damage treatment. Leaves in the undamaged treatment had little variation among samples in a leaf, and leaves in the high damage treatment had a great deal of variation within a leaf. In addition, the frequency of high glucosinolate concentrations increased with the amount of damage.

Variograms for each leaf in Exp. B confirm the patchy distribution of glucosinolates. A variogram that increases with distance indicates spatial auto-

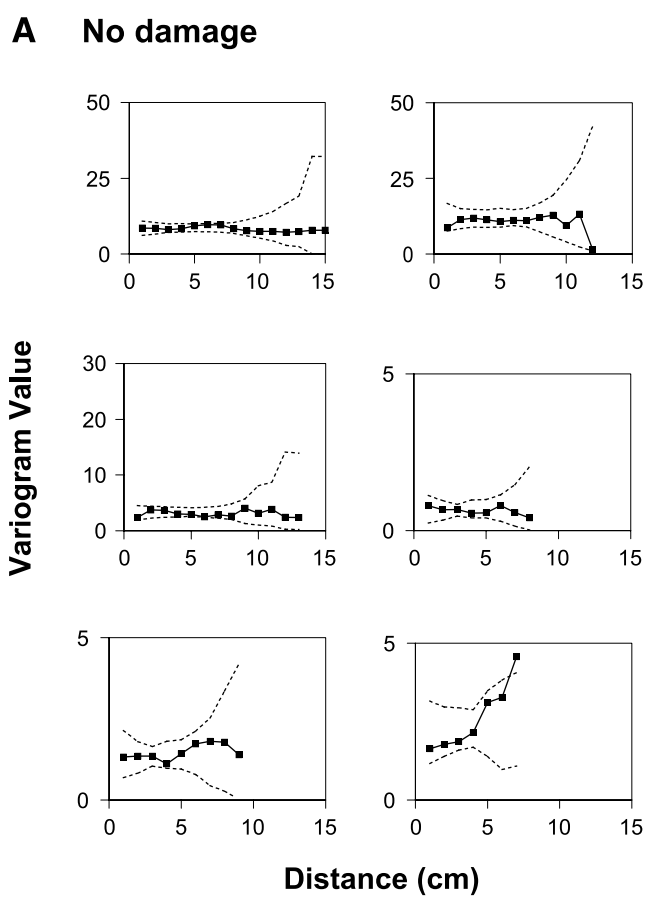

FIG. 3. Variograms for leaves from the no damage (A), low damage (B), and high damage (C) treatments in Exp. B. Solid lines are the variograms. Dotted lines are the 95% confidence intervals as determined by randomization tests. The differences in scales for the variograms are due to differences in the relative magnitude of variation within leaves.

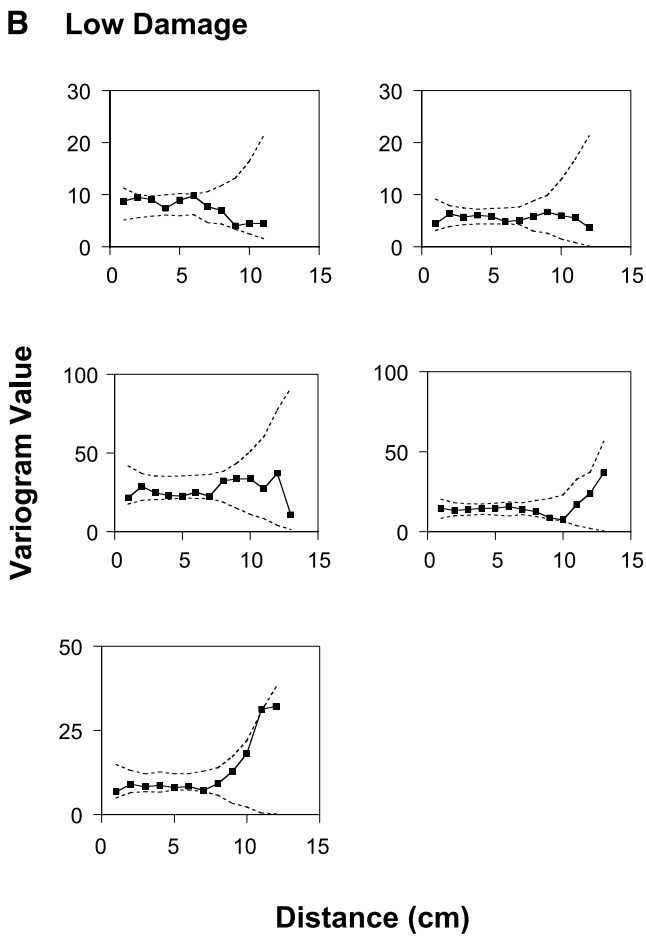

FIG. 3. Continued.

correlation, i.e., the closer the two samples are on the leaf, the more similar they are. A variogram that is level with respect to distance indicates that sample values are randomly distributed with respect to their distance from one another. The glucosinolates appear to be spatially random, indicated by the flat relationship of the variograms to distance (Figure 3). In the variograms for each leaf, only 7 of the 157 total points (15 leaves × 8–15 distances/leaf) fell outside the confidence intervals, matching the expected number according to a binomial probability at the 5% level (binomial mean, $N \times P = 157 \times 0.05 = 7.85$). This indicates that there is no spatial autocorrelation in the data at this scale. The

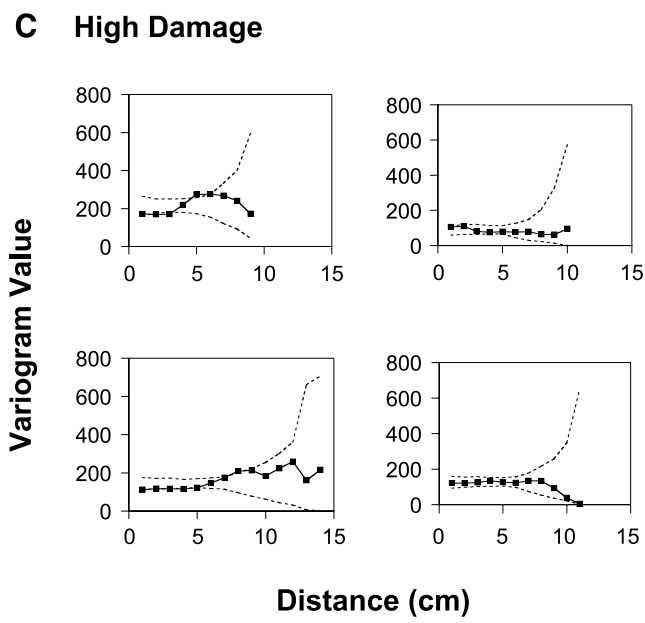

FIG. 3. Continued.

lack of spatial autocorrelation in these data may seem to contradict the differences in the frequency of high glucosinolate concentrations between the proximal and distal halves of leaves. However, because variograms examine samples in all directions from a focal point, they are not likely to detect a difference along a single gradient. The differences in the scales of the variograms among the three damage treatments are a result of the differences in variation among these treatments, but some of the increased variation in the high damage treatment is a statistical artifact due to the smaller size of samples.

The different factors that resulted in higher glucosinolate concentrations—herbivore damage, young leaf age, and proximal part of the leaf—all contributed to increased variation within leaves and to an increased frequency of high glucosinolate concentrations. Few samples from the least defended leaf type (undamaged, old leaves, main leaflets) had glucosinolate concentrations greater than 10 μmol/g, but over half the samples from the most defended leaf type (damaged plant, young leaf, leaflet) had concentrations this great (Figure 4). However, in leaf types with intermediate mean concentrations, the majority of samples had relatively low concentrations. Even in the most defended leaf types, low concentrations of glucosinolates are common (Figure 4).

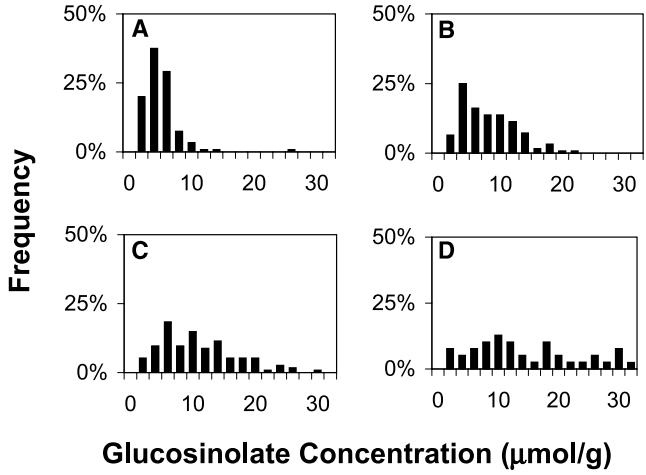

FIG. 4. Frequency of samples in Exp. A with different glucosinolate concentrations for four representative leaf types. Both the mean and variance of glucosinolates increased with the inclusion of each factor that led to higher defenses (damaged plant, young leaf, and proximal leaflets). The individual leaf types differed significantly in variation (Levene's test, $F = 8.71$, $P < 0.001$). Leaf type with the lowest mean and variation of glucosinolates: main leaflets of old leaves on undamaged plants (A). Intermediate glucosinolates: main leaflets of old leaves on damaged plants (B). Intermediate glucosinolates: main leaflets of young leaves on damaged plants (C). Leaf type with the highest mean and variation of glucosinolates: proximal leaflets of young leaves on damaged plants (D). Glucosinolates are μmol/g dry leaf weight.

DISCUSSION

There are three key results from this study. First, damage by herbivores not only increased mean concentrations of glucosinolates but also significantly increased the variation of glucosinolates. Second, the smallest scale examined—variation among samples within a specific region of a leaf—had the greatest variation of any of the scales examined, and this variation was spatially random within a leaf. Third, the proximal half of leaves had higher mean and variation of glucosinolates than the distal half of leaves.

Damage by herbivores increased the degree of small-scale stochastic variation. Damage and other factors, such as young leaf age or the proximal part of a leaf, that caused an increase in the mean concentration of glucosinolates did not cause higher glucosinolate concentrations throughout the leaf, but rather caused an increase in the frequency of high glucosinolate concentrations. Low concentrations were still common even in the leaves with the highest mean

concentrations (Figure 4). These results indicate that induction did not uniformly increase glucosinolate concentrations in a leaf. Concentrations increased dramatically in a few small patches of the leaf, and other parts were relatively unchanged.

Variation among samples within a leaf part accounted for 57% of the random variation in both experiments. Variation among leaves and plants explained only 34–38% of the random variation. Such high variation at a small scale is unusual and indicates that a great deal, perhaps the majority, of the variation in glucosinolate concentrations occurred at this small scale. The spatial results from Exp. B indicated that glucosinolate concentrations in adjacent samples were uncorrelated. This small-scale variation was the result of extreme patchiness of glucosinolates within leaves, which was spatially random.

The proximal part of leaves, defined as the distinct proximal leaflets in Exp. A and as the proximal half of the leaf in Exp. B, had higher means and variances than the distal part of leaves. To my knowledge, differences in glucosinolate concentration among different parts of leaves have not been previously reported in the Brassicaceae.

The differences due to damage and leaf age followed the predictions of optimal defense theory: previous damage presumably indicates an increased risk of future damage, and young leaves are more valuable because they have a higher future photosynthetic potential (Harper, 1989). Glucosinolate concentrations have also been shown to be higher in young leaves due to dilution of the chemicals through leaf expansion (Porter et al., 1991; Brown et al., 2003). It is less clear why the proximal part of leaves had higher levels of glucosinolates than the distal part. This pattern may be adaptive if herbivores often crawl onto a leaf from the base of the petiole and use the quality of the proximal part of the leaf as an indication of the quality of the leaf as a whole. In this case, plants may gain protection for the whole leaf by allocating more defenses to the proximal part of the leaf, causing it to act as a barricade for the rest of the leaf. This pattern might also be nonadaptive and purely physiological, resulting from vascular architecture, differences in cell size, or other factors. Behavioral experiments with herbivores are necessary to determine whether there is an adaptive purpose for higher glucosinolates in the proximal half of leaves.

Only a handful of other studies have directly examined patterns of chemical variation within plants. Suomela and Ayres (1994) and Suomela et al. (1995) compared several different traits, including concentrations of phenols, carbohydrates, and nitrogen across multiple different scales in the leaves of mountain birch. For most of these traits, the majority of variation was explained by the highest level differences among trees. This is the reverse of the pattern seen in glucosinolate concentrations in *R. sativus*. A gradient of defensive chemicals along the length of leaves has previously been reported for *Populus*

angustifolia, but the pattern is the opposite of that found in *R. sativus* leaves. Phenolics are higher at the tips of *P. angustifolia* leaves than at the base, and this difference causes aphids to preferentially place galls at the base of leaves (Whitham, 1981; Zucker, 1982).

Stout et al. (1996) examined within-plant spatial variation in induction by comparing the levels of several chemical defenses at different leaf and leaflet positions in relation to the site of the experimental damage. They found that damaged plants were more variable than undamaged plants: they found different patterns of induction at different leaf positions for each of the chemicals examined. In addition, the standard error of chemical concentrations was higher in damaged plants compared with undamaged plants. Vascular connections can explain some of the variation in induction. Orians et al. (2000) showed that in tomato plants, the near side of leaves adjacent and orthostichous to a damaged leaf had high levels of induction of proteinase inhibitors. The far side of these leaves and both sides of leaves on the opposite side of the plant showed little induction. The leaflets that had strong induction had direct vascular connections to the damaged leaves. Leaves with weaker vascular connection showed weaker induction, and leaves with no direct connections showed no detectable increase in proteinase inhibitors in response to the damage imposed.

Recent work has revealed that there are special glucosinolate-rich cells, dubbed "S-cells" for their high sulfur content (Koroleva et al., 2000), located adjacent to myrosinase-rich cells (Husebye et al., 2002). Both types of cells are adjacent to the phloem, and glucosinolates have been shown in other studies to be transported through the phloem and have higher concentrations in the veins of leaves (Mikkelsen et al., 2000; Reintanz et al., 2001). My research did not detect a difference in the veins of leaves compared to other parts, but this may be because my samples were too large to detect localization specifically in the veins.

Several recent papers have used genetic techniques of binding reporter genes to promoter sequences involved in the biosynthesis of glucosinolates and myrosinases in *Arabidopsis thaliana* to visualize where genes are active within plants. These studies have shown that, in addition to the cells adjacent to the phloem, myrosinases are active in the guard cells. The pictures of the leaves shown in these studies (Husebye et al., 2002; Thangstad et al., 2004) show spatial patchiness with pinpoints of high glucosinolate/myrosinase activity throughout the leaves and high concentrations in the veins. Some of the leaves shown in Thangstad et al. (2004) show a tendency for higher activity in the lower half of the leaf, but this is not discussed by the authors and may be only an artifact. The result of this localization of glucosinolates and myrosinases in the phloem and guard cells is a scattered accumulation of glucosinolates throughout leaves. This suggests a plausible physiological explanation for the patterns of glucosinolate distribution observed in this experiment.

Over half of the random variation in the data occurred at a small scale, and this variation was spatially random. This variation may have important effects on herbivore behavior and fitness because small herbivores are likely to experience frequent, unpredictable changes in glucosinolate concentrations. Small-scale stochastic variation in defenses may be adaptive in at least four ways. First, a random distribution of defenses provides the least information possible to herbivores about the location of low and high doses of defenses within a plant. A herbivore cannot discern anything about the quality of a leaf or a plant from feeding on one part of it; a high-quality bite is as likely to be followed by a low-quality bite as a high-quality one. Second, many herbivores have inducible detoxification systems to deal with plant toxins (Snyder et al., 1993; Glendinning and Slansky, 1995; Feyereisen, 1999); the rate of production of detoxification enzymes is determined by the amount of toxins consumed. If toxin levels continually change, and if there is any time lag in production of gut enzymes, herbivore detoxification systems are likely to be frequently out of phase with their food. Third, if the effects of toxins on herbivores increase nonlinearly such that high doses have much stronger effects as predicted by the "nonlinear averaging" theory (Karban et al., 1997; Shelton, 2000), then increasing the proportion of high toxin patches that a herbivore encounters should negatively affect the herbivore. Finally, herbivores exposed to varying levels of defense will experience reduced selection for resistance to the defenses. Thus, variation may enable plants to evolutionarily compete with the short generations and high recombination potential of insect herbivores (Dolinger et al., 1973; Whitham, 1981; Schultz, 1983a; Gardner et al., 1999).

Plants exhibited both predictable and unpredictable variations in glucosinolates at different scales. Herbivores experience both types of variation simultaneously. Plants appeared to distribute glucosinolates according to optimal defense theory at larger scales, but may use stochastic, spatial variation at smaller scales to increase uncertainty to herbivores. Predictable variation should optimize the distribution of a plant's defense, but herbivores can evolve strategies to avoid predictably highly defended plant parts. In contrast, it should be more difficult to evolve strategies to efficiently cope with stochastic variation. In addition, stochastic variation may mask the predictable patterns, slowing the herbivores' ability to evolve responses to them. The defensive strategy of *R. sativus* may be like a minefield. Most of a leaf has relatively low levels of defense, but small, randomly located patches have high doses of glucosinolates, and herbivores never know when they will encounter these toxic patches. When a plant increases its defenses, it increases the number of these high-dose patches in more valuable or more threatened parts, effectively putting more mines into the minefield.

This is one of the few studies to examine the patterns and magnitude of chemical variation at a scale important to herbivorous insects. Most previous

studies have looked at larger scales, such as among leaves or organs, or among plants. Sampling at larger scales will reduce the measured variation by averaging the variation at smaller scales, which would result in a systematic underestimation of the variation experienced by small herbivores. If variation affects herbivore fitness or behavior in any of the ways suggested above, simply examining the mean level of defense in a plant will not accurately represent the effect of the defenses on actual herbivores. To understand how variation affects herbivores, it is essential to examine variation at the scale at which herbivores experience it.

A model (Shelton, 2004) based on the results of this study suggests that larger-scaled predictable patterns of variation affect herbivore behavior and small-scale stochastic variation that may be deleterious to small, low-mobility herbivores such as caterpillars. The high degree of stochastic small-scale variation found in this study complicates the experimental approaches to this question because it suggests that it may not be possible to know precisely what concentrations of chemicals a herbivore consumes when it feeds on a plant. We need cleverly designed experiments with herbivores to determine if chemical variation is an important aspect of plant defense. If it is, studies that examine only mean levels of defenses and average over large areas of a plant may be missing information needed to understand the ecological interactions between herbivores and plant defenses.

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METHYL SALICYLATE, A SOYBEAN APHID-INDUCED PLANT VOLATILE ATTRACTIVE TO THE PREDATOR *Coccinella septempunctata*

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Abstract—Induced volatiles provide a signal to foraging predatory insects about the location of their prey. In Iowa, early in the growing season of soybean, *Glycine max*, many predacious seven-spotted lady beetles, *Coccinella septempunctata*, were observed on plants with heavy infestations of soybean aphid, *Aphis glycines*. We studied whether the attraction of this beetle is caused by the release of specific volatile compounds of soybean plants infested by aphids. Volatile compounds emitted by soybean plants infested by aphids were compared with those of undamaged, uninfested, and artificially damaged plants. Gas chromatography–mass spectrometry analyses revealed consistent differences in the profiles of volatile compounds between aphid-infested soybean plants and undamaged ones. Significantly more methyl salicylate was released from infested plants at both the V1 and V2 plant growth stages. However, release patterns of two other induced plant volatiles, (D)-limonene and (*E*,*E*)-α-farnesene, differed between the two plant growth stages. Gas chromatographic–electroantennographic detection of volatile extracts from infested soybean plants showed that methyl salicylate elicited significant electrophysiological responses in *C. septempunctata*. In field tests, traps baited with methyl salicylate were highly attractive to adult *C. septempunctata*, whereas 2-phenylethanol was most attractive to the lacewing *Chrysoperla carnea* and syrphid flies. Another common lady beetle, the multicolored Asian lady beetle, *Harmonia axyridis*, showed no preference for the compounds. These results indicate that *C. septempunctata* may use

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methyl salicylate as the olfactory cue for prey location. We also tested the attractiveness of some selected soybean volatiles to alate soybean aphids in the field, and results showed that traps baited with benzaldehyde caught significantly higher numbers of aphids.

Key Words—*Glycine max*, soybean volatile, induced plant volatile, methyl salicylate, attraction, enhanced biological control, seven-spotted lady beetle, *Aphis glycines.*

INTRODUCTION

The soybean aphid, *Aphis glycines*, is a new, invasive insect species that threatens U.S. soybean, *Glycine max*, production. It is the only aphid species to develop large colonies on soybean in North America. Infestation stunts the plants so that fewer pods and seeds are developed, thereby lowering yield. Soybean aphids also transmit several plant viruses, including alfalfa mosaic, soybean mosaic, soybean dwarf, soybean stunt, and bean yellow mosaic, that distort soybean's growth and further reduce yield.

In soybean fields, soybean aphids are attacked by several predatory insects, including lady beetles (*Harmonia axyridis* and *Coccinella septempunctata*), lacewings (*Chrysoperla carnea*), true bugs (*Orius insidiosus*), and syrphid flies (Zhu, unpublished data). The complex of these natural enemies seems to play a key role in regulating aphid populations (Han, 1997; Fox et al., 2004). Among these predatory insects, *C. septempunctata* dominates early in the growing season, with an increasing abundance of *C. carnea* and *H. axyridis* as the season progresses.

Studies have shown that predators, parasitoids, and predatory mites locate their hosts by using volatile semiochemicals emitted from their hosts or from food plants infested by their hosts (Noldus, 1989; Vet and Dicke, 1992; Bernasconi et al., 1998; Dicke et al., 1998; De Moraes et al., 1998; Du et al., 1998; Ninkovic et al., 2001; Van den Boom et al., 2004; De Boer and Dicke, 2004). During our soybean aphid field experiments, we observed increased abundance of *C. septempunctata* in soybean fields where higher soybean aphid infestation occurred during the early growing season, compared with fields with lower soybean aphid infestation. We report here the identification of volatile compounds released from soybean plants at vegetative growth stages 1 and 2 (V1 and V2, defined when leaflets on the first through the third node leaf are unrolled). A soybean aphid-induced volatile compound that is attractive to *C. septempunctata* is discussed. We also report a volatile compound emitted from the undamaged soybean plant that is attractive to alate soybean aphids.

METHODS AND MATERIALS

Plants and Insects. V1- and V2-stage soybean plants (Garst Roundup Ready variety [80411203] grown in a greenhouse in small pots were used for infestation and entrainment. These two stages of plants were reported to be colonized by winged aphids emigrating from their overwintering host plant, buckthorn, *Rhamnus cathartica*, into soybean. Soybean aphids were collected from soybean fields at the University Farms in Ames, IA. They were maintained in a cabinet kept at 25 ± 2°C, with photoperiod of 14:10 (L:D) hr as a stock colony with only parthenogenetically produced females. Adults of *C. septempunctata* were collected from soybean fields during early summer and maintained under the same conditions as the soybean aphid colony, with 5% sugar water provided.

Volatile Collection. The collection apparatus for volatile compound collection consisted of two glass cells (9.5 cm ID × 12 cm in depth and 10 cm ID × 15 cm in depth), which when put together formed a chamber around the plant with the stem passing through a small slit. The remaining space around the slit was packed with glass wool held in place with Teflon tape. Moisturized and charcoal-filtered air was pumped into the chamber through the inlet, and the outlet of the chamber was connected with a prebaked (200°C overnight) glass tube (5 cm × 0.3 cm ID) containing 100 mg of Super Q (80/100 mesh, Alltech Associate, Deerfield, IL, USA) sandwiched with glass wool plugs. Airflow rates were measured at 2 l/min from the filtered house air pumped into the entrainment chamber (regulated by a flow meter, Barnant Company/Gilmont Instrument, Barrington, IL, USA) and 400 ml/min from the Super Q collector by using a digital flow meter (Hewlett Packard, Palo Alto, CA, USA). The entrainment was carried under the same condition as described for soybean growth.

Volatile entrainment was conducted from V1- and V2-stage soybean plants. Thirty second instars of soybean aphid were transferred onto leaves of one soybean plant, and collection was started 6 hr later. A control plant of the same stage was set up identically but without aphids (uninfested, undamaged). We also collected volatile emissions from a soybean plant of the respective stage by artificially damaging it with thin tungsten wires (5 μm ID). Leaves were punctured with 30 tiny holes. Volatiles were collected from d 1 to d 12, and the collection was extracted every other day. Volatile collection (24 hr) from only soybean aphids also was conducted in a 355-ml wide-mouth glass bottle with ~200 soybean aphids (second and third instars) under the same conditions described above but without soybean leaves. The trapped volatiles were eluted with 2 ml of HPLC-grade hexane (Burdick & Jackson Brand, High Purity, Des Plaines, IL, USA) containing 250 ng of pentadecane as an internal standard and then concentrated to 200 μl under a gentle nitrogen stream. Two microliters of extract were injected into either combined gas chromatography

and electroantennographic detection (GC-EAD) or gas chromatography–mass spectrometry (GC-MS) for quantitative and qualitative analyses.

Chemical Analyses. For GC-EAD analysis, a Hewlett Packard 5890 Series II gas chromatography equipped with a DB-wax column (30 m × 0.25 mm i.d., J & W Scientific, Folsom, CA, USA) and a 50:50 effluent split allowed simultaneous flame ionization detection (FID) and EAD of the collected volatiles. Helium was used as the carrier gas with a flow rate of approximately 30 ml/min for both FID and EAD. Extracts were injected in splitless mode. The injector temperature was 250°C, and the split valve was opened 1 min after injection. The oven initial temperature was set at 50°C for 3 min and then increased to 250°C at a programmed rate of 15°C/min. The outlet for the EAD was continuously supplied with a purified, moisturized airstream flowing over the antennal preparation at 0.5 l/min. An adult of *C. septempunctata* was restrained on a block of dental wax with thin copper wires for the EAD recording. A glass capillary recording Ag–AgCl electrode filled with 0.5 M KCl solution was brought into contact with the distal segment of the antenna. A ground electrode, filled with the same saline solution, was introduced into the intersegmental membrane of the beetle body. A high-input impedance EAG amplifier (Syntech, Hilversum, the Netherlands) with automatic baseline drift compensation was used for the GC-EAD analysis. GC-EAD software (version 2.3, Syntech) was used to process, record, and analyze the EAD and FID signals on a PC (Micron Inc., Minneapolis, MN, USA).

GC-MS analyses of volatiles collected from soybean plants were performed with a Hewlett Packard 5890 Series II gas chromatography interfaced to a Hewlett Packard 5972 mass selective detector (MSD). The GC-MS was equipped with the same columns as those used in the GC-EAD system described above. The temperature program was the same as that described for the GC-EAD analyses. Mass spectra were recorded from 30 to 550 amu with electronic impact ionization at 70 eV. The assignments of chemical identities to the soybean volatile compounds were confirmed by comparison of the retention indices and mass spectra with those of authentic chemical standards and reference spectra in a mass spectral library (Wiley 138K, John Wiley & Sons, Inc., New York, NY, USA).

Chemicals. All synthetic standards of soybean volatile compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA), and the purity of each compound was examined by GC-MS, ranging from 98 to 99.5% (from solutions containing 200 ng of each standard compound in 2 μl of hexane).

Field Tests. Field trapping tests were conducted in a 10,000-m^2 soybean field in 2003 in Ames, IA, USA. The first test was designed to test the attractivity of selected volatile compounds emitted from undamaged soybean plants to alate soybean aphids. Synthetic compounds at a dose of 100 mg were prepared in either hexane or methylene chloride. A second test examined the

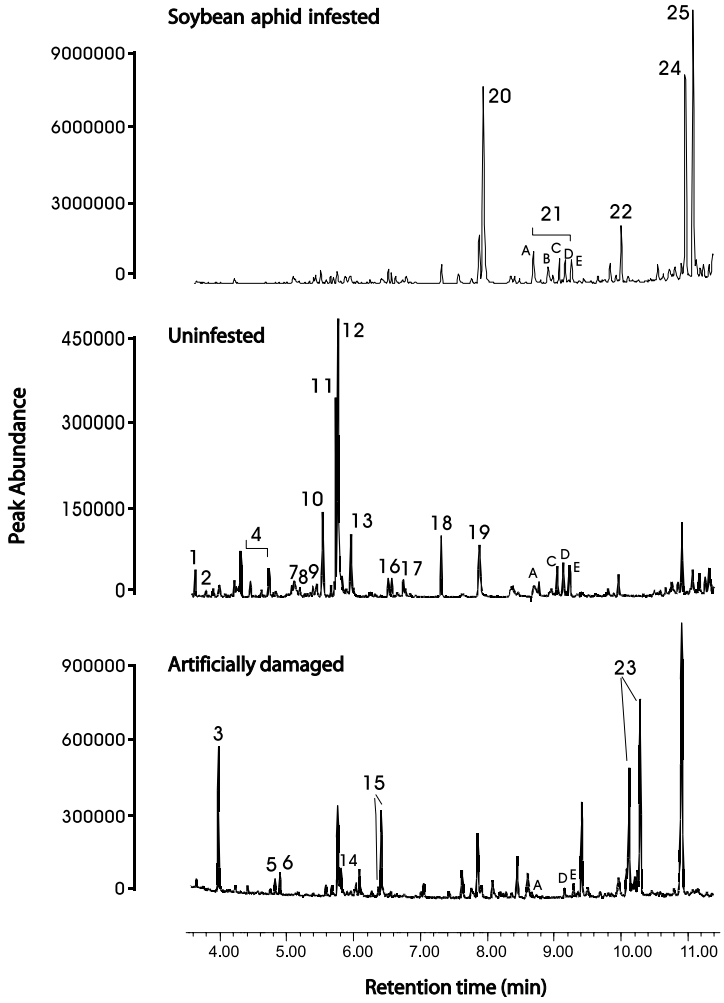

FIG. 1. Typical gas chromatograms (on a DB-5 column) of volatile compounds emitted from soybean aphid-infested, artificially damaged, and undamaged plants of *G. max* (V2 stage). Represented are (*E*)-2-hexenal (1), (*E*)-2-hexenol (2), unknowns (3 and 4), ethyl benzene (5), 1,3-dimethyl benzene (6), benzaldehyde (7), 6-methyl-5 heptanone (8), (*Z*)-3-hexenyl acetate (9), decane (10), 1,2,3-trimethyl benzene (11), dichlorobenzene (12), D-limonene (13), β-ocimene (14), unknown (15), linalool (16), 2-phenylethanol (17), unknown (18), naphthalene (19), methyl salicylate (20), unknown (21), tetradecane (22), unknown (23), α-humulene (24), and (*E*,*E*)-α-farnesene (25) (the labeled numbers on each peaks do not refer to those compounds that are only detected uniquely in each treatment, and the internal standard, *n*-pentadecane, is not shown due to its elution time is after 11.00 min).

trapping efficacy of the induced methyl salicylate and a previously identified lady beetle and lacewing attractant compound, 2-phenylethanol (also at 100-mg dose). In a third dose–response test, serial dilutions of synthetic methyl salicylate were made at doses ranging from 10 to 1000 mg. Medical peerless cotton wicks (5 cm in length, 100% cotton) were used as dispensers, and the trap used was constructed by stapling four yellow sticky traps together, which is similar to the Rebell trap described by Zhu et al. (1999), where the attractant lure was suspended in the center of this trap. The trap was hung from bamboo stakes, 1.2 m above the ground. Within a replicate (N = 10), the distance between traps was 15 m. The traps were checked daily and captured ladybugs were removed from sticky traps after checking. Trap position within a series

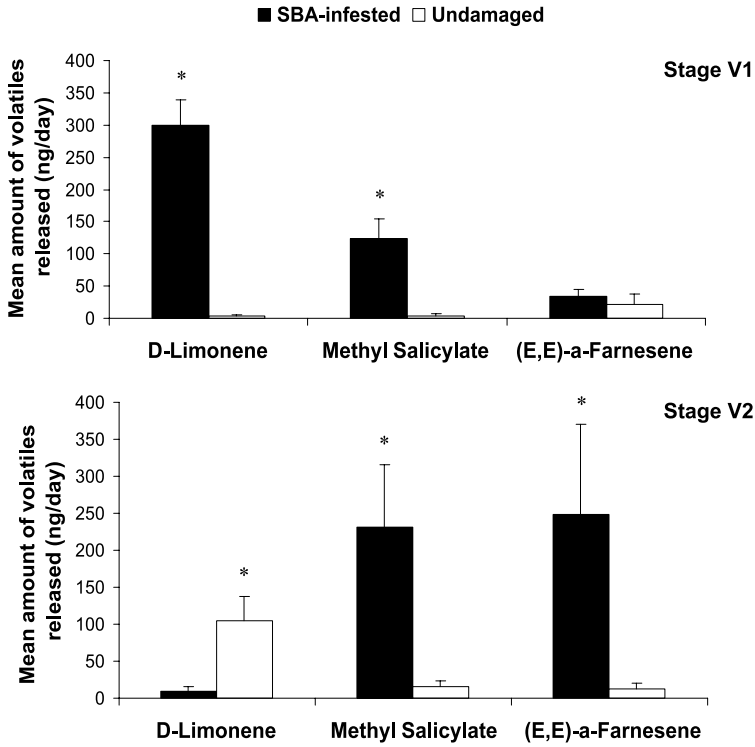

FIG. 2. Mean emission rates of three volatile compounds from plants at V1 (top) and V2 (bottom) stages of *G. max*. Asterisks indicate statistically significant differences in volatile release rates between soybean aphid-infested and undamaged plants (Student's *t*-test, $N = 6$, $P < 0.05$).

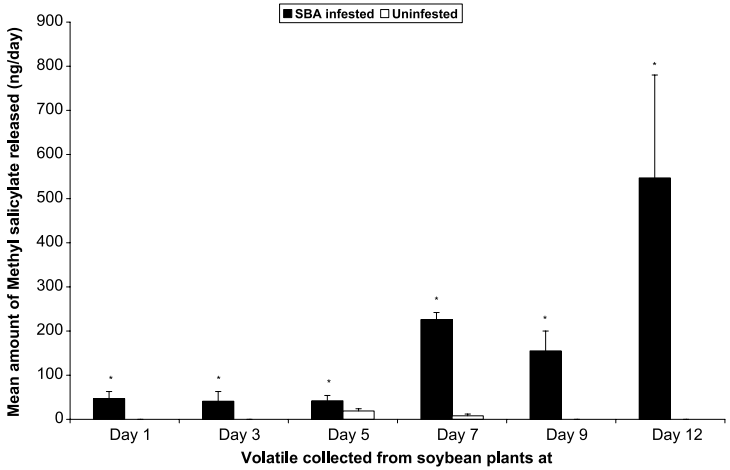

FIG. 3. Average released amounts of methyl salicylate at every other day from soybean aphid-infested and undamaged *G. max* plants during a 12-d collecting period. Asterisks indicate statistically significant differences in amounts of methyl salicylate released between soybean aphid-infested and undamaged *G. max* plants (Student's *t*-test, $N = 6$, $P < 0.05$).

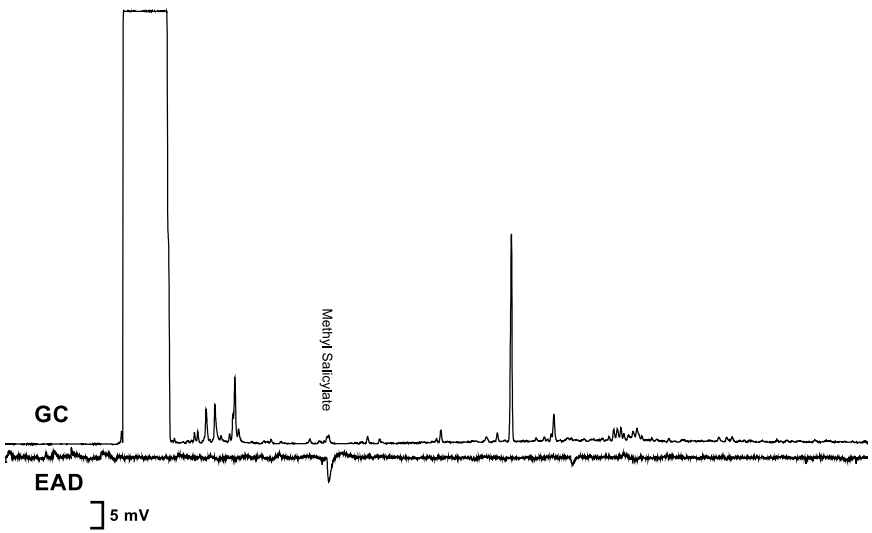

FIG. 4. Simultaneously recorded FID and EAD responses of antennae of a female adult *C. septempunctata* to extracts of soybean aphid-infested *G. max* (analyzed on a DB-Wax column).

was randomized after checking to minimize the effects of habitat heterogeneity. The trapping test lasted for 2 wk.

Statistical Analyses. Differences in volatile emission among treatments in the volatile collection experiments and field trap catches (means of trapped beneficial insects) were compared by either Student's *t*-test or analyses of variance (ANOVA) followed by Fisher's protected least significant difference test (FPLSD).

RESULTS

Volatiles from Soybean Aphid-Infested Soybean Leaves. In total, 19 volatile compounds were tentatively identified from emissions of both infested, uninfested and artificially damage soybean leaves by comparing their retention indices and mass spectra with those of synthetic standards (Figure 1). Most volatiles released from the soybean plants were common plant volatiles. Further quantitative GC and GC-MS analyses revealed consistent differences in emissions of D-limonene, methyl salicylate, and (*E*,*E*)-α-farnesene between the

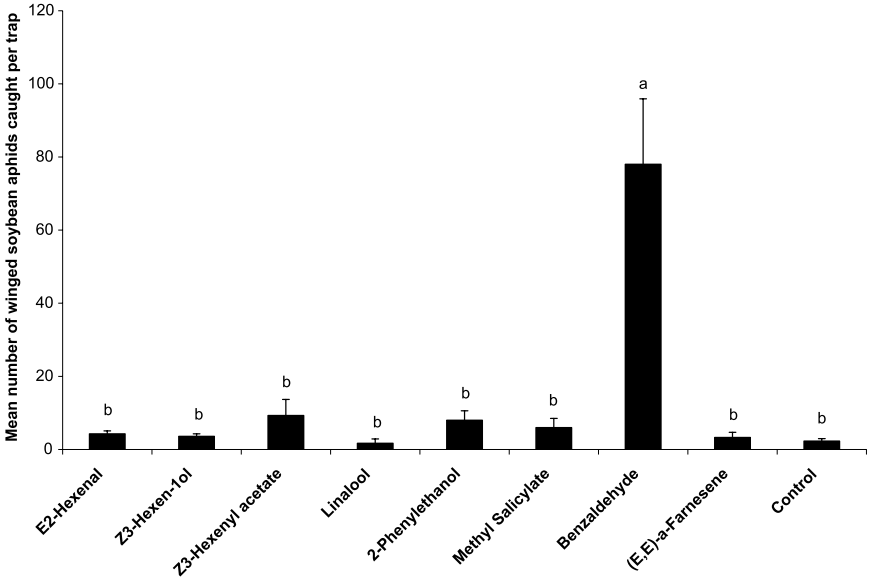

FIG. 5. Mean number of alate viviparous soybean aphids caught in traps baited with selected soybean associated volatile compounds (100 mg) in the soybean field (line indicates standard error). Columns with no letters in common are significantly different ($N = 10$, ANOVA followed by FPLSD test, $P < 0.05$).

aphid-infested and the undamaged, uninfested plants at the two growth stages (Figure 2). Among these three compounds, methyl salicylate was the only compound emitted in significantly higher quantities from aphid-infested plants at both V1 and V2 stages, relative to the undamaged, uninfested control. Significantly more D-limonene was released from the aphid-infested plants at the V1 stage, whereas more (*E*,*E*)-α-farnesene was produced by V2-stage plants. The amounts of methyl salicylate released from aphid-infested plants were significantly higher than those emitted from undamaged, uninfested plants, and amounts increased to maximum levels on d 12 of the collection period (Figure 3). No volatile compounds were found from extracts of soybean aphids only, except a trace of (*E*)-β-farnesene was identified.

Antennal Responses of Seven-Spotted Lady Beetles to Volatiles from Aphid-Infested Soybean Plants. Only one compound of the headspace of aphid-infested plants elicited a significant EAD response in *C. septempunctata.* The

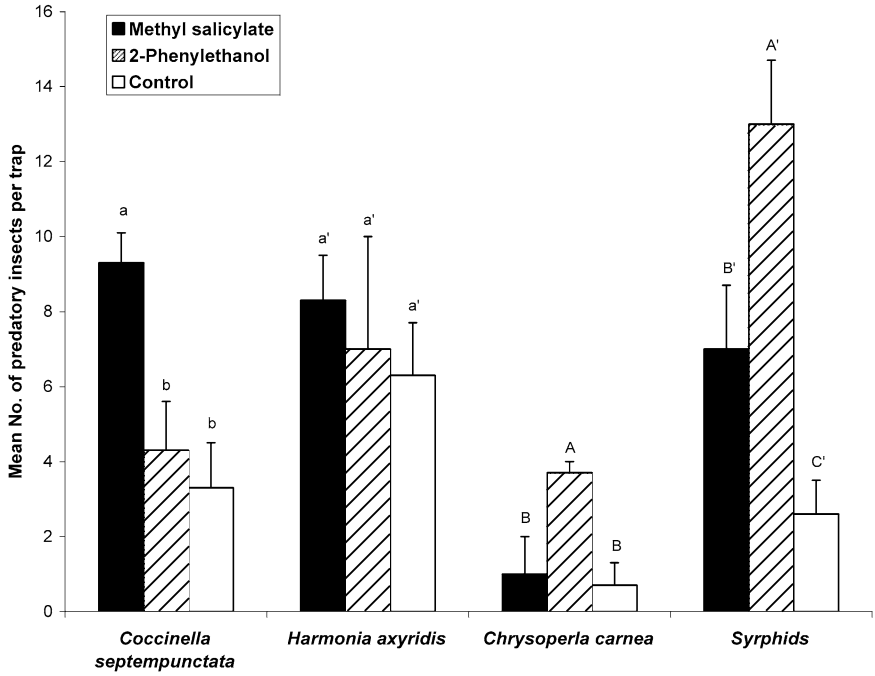

FIG. 6. Mean number of predatory insects (both sexes) caught in traps baited with 100 mg of methyl salicylate and 2-phenylethanol, and the control from an Iowa soybean field in 2003. Columns with no letters in common in four different categories are significantly different ($N = 10$, ANOVA followed by FPLSD test, $P < 0.05$).

retention time of this compound matched that of synthetic methyl salicylate (Figure 4). Further GC-MS analyses of the same extract confirmed its chemical identity as methyl salicylate.

Attraction of Aphids and Their Predators to Soybean Leaf Volatiles. Results from field-trapping tests using Rebell-type yellow sticky traps baited with either soybean leaf volatiles identified from this study or with previously reported leaf volatiles (Liu et al., 1989) showed that among eight common soybean plant-associated volatiles tested, benzaldehyde was the only compound attractive to alate soybean aphids (Figure 5). Relatively high numbers of four predatory insect species were captured in traps baited with 100 mg of methyl salicylate and 2-phenylethanol (Figure 6). Adults of *C. septempunctata* were significantly attracted only to methyl salicylate, whereas both adults of *C. carnea* and syrphid flies were attracted to 2-phenylethanol. However, there were no differences in catches of *H. axyridis* among traps baited with either of the two tested attractant compounds, compared with the control. A subsequent dose–response test with the synthetic methyl salicylate demonstrated that the highest attraction to adult *C. septempunctata* was at doses from 100 to 300 mg (Figure 7). A significant reduction in trap catch was observed at the highest tested dose of this compound.

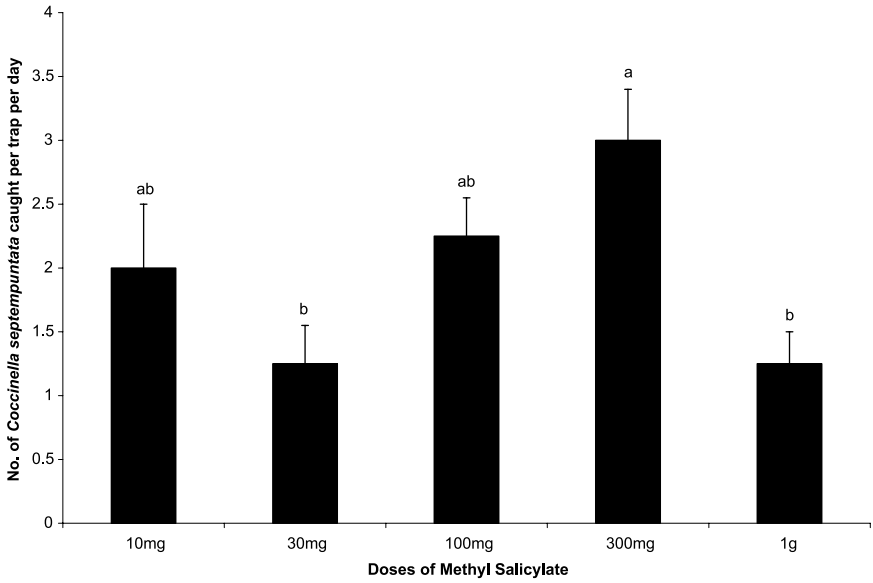

FIG. 7. Mean catches ±SE of *C. septempunctata* adults in traps baited with different doses of the synthetic methyl salicylate. Different letters on top of columns indicate significant differences ($N = 10$, ANOVA followed by Fisher FPLSD test, $P < 0.05$).

DISCUSSION

The analyses show that volatiles collected from soybean plants in their early growth stages (V1–V2) consist of aliphatic esters, aldehydes, alcohols, and ketones as well as some halogenated hydrocarbons and terpenoids. Some of these volatiles are similar to those that have been reported from a previous study (Liu et al., 1989), even though different cultivars of soybean were selected and different growth stages of plants were tested. Du et al. (1994) reported that alate viviparous soybean aphids are attracted or arrested by volatiles from soybean plants, and electroantennographic tests (EAG) have shown that these aphids show significant antennal responses to some volatiles from young leaves. Our field trapping experiments using the most common soybean volatile compounds also showed that alate viviparous soybean aphids are highly attracted to benzaldehyde, a component present in the headspace of soybean, and that this compound elicits significant EAG responses (Du et al., 1995; Zhu, unpublished data).

Despite large variation in the amounts of volatiles released from the plants we tested, methyl salicylate was consistently released at a significantly higher rate from aphid-infested plants compared with undamaged V1- and V2-stage plants or from artificially damaged leaves. This finding demonstrates that the infestation of aphids induces the emission of methyl salicylate, which is a volatile derivative of the plant hormone salicylic acid (Shulaev et al., 1997; Ozawa et al., 2000). Methyl salicylate has been identified in herbivore-induced plant volatile blends from at least 10 plant species infested with mites, aphids, beetles, and caterpillars (Walling, 2000; James, 2003; De Boer and Dicke, 2004; Van den Boom et al., 2004). This volatile plays an important role in the activation of plant defense responses (Yang et al., 1997), which also has been reported from tobacco plants inoculated with tobacco virus (Shulaev et al., 1997). The temporal emission patterns of two other volatile compounds, D-limonene and (*E*,*E*)-α-farnesene, differ between infested and undamaged plants at the two growth stages. Even though these two terpenes have been reported as herbivore-induced volatiles from several plant species (Paré and Tumlinson, 1999), it remains to be tested whether their emissions are triggered by soybean aphid infestation, or differ with plant growth stage.

Previous studies have shown that several parasitoids and predatory mites locate their hosts by using volatile semiochemicals emitted from their hosts and from the plants infested by their hosts (Vet and Dicke, 1992; Dicke and Vet, 1999). Recently, De Boer and Dicke (2004) and Van den Boom et al. (2004) showed that one of the major soybean volatiles induced by the herbivorous mite *Tetranychus urticae* is methyl salicylate, and that this compound is also attractive to its predatory mite *Phytoseiulus persimilis*. During the Iowa soybean growing season, we observed that a variety of insect predators attack soybean aphids. The complex of these natural enemies seems to play a key role in

regulating aphid populations. *C. septempunctata* dominates early in the growing season, when heavy aphid infestation is first observed in the field. Both larva and adults of this beetle can consume up to 90 aphids per day (Han, 1997). Our GC-EAD analyses showed that antennae of this lady beetle species exclusively responded to methyl salicylate, which may be induced by the infestation of soybean plants with aphids. Field trapping tests conducted in soybean fields further confirmed that synthetic methyl salicylate-baited traps are highly attractive to *C. septempunctata*. Methyl salicylate also has been demonstrated to be attractive to predatory mites *P. persimilis* and predatory bugs *Anthocoris nemoralis* as well as minute pirate bugs, *Orius tristicolor*; bigeyed bugs, *Geocoris pallens*, and lady beetle *Stethorus punctum* (Dicke and Sabelis, 1988; Drukker et al., 2000; James, 2003). In addition, several studies have shown that methyl salicylate can act as a repellent to several aphid species when they leave their overwintering places to colonize host plants in spring (Lösel et al., 1996; Ninkovic et al., 2003).

Relatively higher numbers of syrphid flies are attracted to traps baited with methyl salicylate compared with non-methyl salicylate-baited traps, which coincides with a previous report (James, 2003). However, James (2003) has reported that the catches of coccinellids in traps baited with methyl salicylate are not significantly different from those of control traps. The discrepancy in trap catches of coccinellids with methyl salicylate between the current study and that of James' field experiment could be attributed to geographical variation in populations of these coccinellids, different trap designs, and different doses of methyl salicylate. In the field experiment, James (2003) used ~1 g methyl salicylate (~1 ml), a dose that has been shown in our field trapping experiment to reduce attraction of *C. septempunctata.* Similar examples have been documented for several parasitic wasp species that are attracted to low doses of green leaf volatiles but are repelled by high doses (Whitman and Eller, 1992). One of the most abundant lady beetle species, *H. axyridis*, which accounts for most of the soybean aphid feeding during the late growing season in Iowa, was not attracted to methyl salicylate. The number of *H. axyridis* caught in traps baited with methyl salicylate was not statistically different from that in the control traps. A minor soybean volatile, 2-phenylethanol, identified from both aphid-infested and undamaged soybean plants is highly attractive to adults of *C. carnea,* which is similar to findings of Zhu et al. (1999).

The use of natural enemies as biological control agents for soybean aphids has great potential to suppress aphid populations. However, the biggest concern for the success of biological control is how to recruit predaceous insects into natural or damaged soybean fields and synchronize their presence with the targeted aphid pest, thereby increasing their predatory efficacy. The present study provides a base to help further development of enhanced biological control strategies via applications of attractants of predatory insects that attack aphids

early in the growing season, which is considered as an alternative to suppress their populations below the economic threshold level. The identification of methyl salicylate as a soybean aphid-induced plant volatile and the demonstration of its attractiveness to one of the most abundant lady beetle species (and to syrphid flies that also attack soybean aphids) may provide a tool to enhance the biological control of these aphids. This compound also has been reported to attract some *Orius* species, of which *O. insidiosus* has been considered as another key predator for soybean aphid biological control in the Midwest. The application of beneficial insect attractants such as methyl salicylate and 2-phenylethanol has been tested in soybean fields to enhance biological control of aphids. Although our preliminary results have shown positive impact on increasing predatory insect presence and on suppressing soybean aphid populations (Zhu et al., unpublished data), detailed further studies are necessary to implement such strategies. Negative effects caused by factors such as excessively high concentrations of attractants could repel predatory insects, and host deficiency might result in reduction of predation or parasitism due to relatively low-density prey in the natural habitat (Dicke et al., 1990).

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COMPONENTS OF HONEYBEE ROYAL JELLY AS DETERRENTS OF THE PARASITIC *Varroa* MITE, *Varroa destructor*

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Abstract—The parasitic mite *Varroa destructor* Anderson & Trueman reproduces on the immature stage of the honeybee, *Apis mellifera* L. Mites are found more often on drone brood than worker brood and only infrequently on queen brood. We investigated the chemical basis for the low incidence of mites on queen brood. *V. destructor* mites were deterred by a crude extract of royal jelly, a glandular secretion produced by nurse bees and fed to queen larvae. Bioassay-driven fractionation of the crude extract via column chromatography resulted in one active fraction that was as active as the crude extract. Compounds in the active fraction were identified using gas chromatography (GC) and coupled gas chromatography/mass spectrometry (GC-MS). Before injection, compounds were esterified with MeOH/sulfuric acid, followed by silylation of any hydroxyl groups present. The active fraction contained at least 22 compounds, all fatty acids, several of which contained an additional hydroxyl group on the alkyl chain. Synthesis of some of these compounds that are not commercially available is described. A synthetic mixture containing most of the compounds in the active fraction was as active as the active fraction in the bioassay.

Key Words—Honey bee, host location, royal jelly, parasitic mite, deterrent, *Apis mellifera*, *Varroa destructor*.

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INTRODUCTION

Varroa destructor Anderson & Trueman is a parasitic mite of the honeybee *A. mellifera* L. and the greatest threat to beekeeping worldwide. Mites require the immature stage of their host for reproduction (reviewed in De Jong et al., 1982; De Jong, 1990). Those reproducing on drone brood (males) average 2.2–2.6 female offspring per host, whereas those reproducing on worker brood (females) average 1.3–1.4 female offspring per host (Schulz, 1984; Fuchs and Langenbach, 1989). Mites do not reproduce on queen brood (also females) (Romaniuk et al., 1988; Rehm and Ritter, 1989; Harizanis, 1991; Santillan-Galicia et al., 2002). The rate of infection of immature honeybees varies with their sex and caste, reflecting the opportunity each type of host affords the mite for reproduction. Mites are found more often on drone brood than worker brood, with average differences ranging between 5- and 12-fold (Grobov, 1977; Sulimanovic et al., 1982; Schulz, 1984; Issa and Goncalves, 1984; Fuchs, 1990, 1992; Boot et al., 1991; Calderone and Kuenen, 2001). Mites are found infrequently on queen brood (Harizanis, 1991; Calderone et al., 2002; Santillan-Galicia et al., 2002).

Numerous studies have sought to identify the mechanisms responsible for the different rates at which worker and drone brood are parasitized. Both chemical and physical factors appear to be involved (De Jong and Morse, 1988; Le Conte et al., 1989; Rickli et al., 1992, 1994; Goetz and Koeniger, 1993; Donzé et al., 1998; Kuenen and Calderone, 1998, 2000; Beetsma et al., 1999; Calderone and Kuenen, 2003; Calderone and Lin, 2001; Nazzi et al., 2001). However, the exact role of host kairomones and/or allomones has not been clearly established (Calderone and Lin, 2001).

The low incidence of mites on queen brood has received less attention. Calderone et al. (2002) found that the rate at which queen brood was infected was independent of the rate of infection of worker brood in the same colony. In addition, eliminating potential worker hosts from the nest had no effect on the incidence of mites in queen cells. However, Santillan-Galicia et al. (2002) and Harizanis (1991) reported that the absence of alternate hosts slightly increased the incidence of infection of queen brood. Trouiller et al. (1994) reported that queen larvae produce about half as much of three putative attractants as worker larvae. They proposed that these differences might account for the low frequency of *V. destructor* on queen brood. Supporting that hypothesis, Calderone et al. (2002) reported that mites in a bioassay were arrested at higher rates by crude extracts of worker larvae than by crude extracts of queen larvae. However, crude extracts of royal jelly (glandular secretions from adult worker bees fed to queen larvae) deterred mites (Calderone et al., 2002), and the larval extracts of the queen larvae may have been contaminated with small quantities of royal jelly, thereby reducing their attractiveness relative to extracts

from worker larvae. This possibility arises from the fact that queen larvae, unlike worker larvae, develop in a pool of royal jelly provided by worker bees in large amounts. In this study, we continue to explore the deterrent activity of royal jelly. We use a bioassay-driven fractionation of a crude extract of royal jelly to identify active fractions and their constituent compounds with deterrent activity.

METHODS AND MATERIALS

Royal Jelly Fractions. Royal jelly was collected from queen cells with larvae between 0 and 12 hr before cell capping. A crude extract of the royal jelly was obtained by extracting it with dichloromethane (four times with 2 ml per gram royal jelly) and then with dichloromethane:MeOH (five times with 1 ml per gram royal jelly) as previously described (Calderone et al., 2002). Bioassay-driven fractionation was performed as outlined in Figure 1.

Step 1: Crude extract equivalent to four gram of fresh royal jelly (4 g RJeq) was applied to a 10.5 × 300 mm open column of 10 g silica gel 60 (70–230 mesh, E. Merck). In the initial fractionation, hexane:ethyl ether at ratios of 2:1 and 1:1 yielded fractions F1 and F2, and ethyl ether and ethyl ether:MeOH (1:1) were used to elute fractions F3 and F4, respectively. Each fraction was ca. 50 ml.

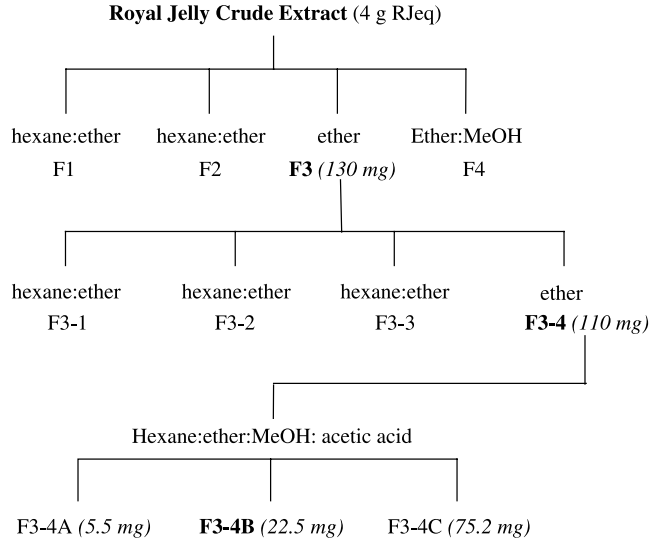

FIG. 1. Scheme for the bioassay-driven fractionation of royal jelly crude extract.

Step 2: Fraction F3, which retained nearly all of the deterrent activity (see below), was further fractionated on an open column of silica gel [10.5 × 300 mm, 10 g silica gel 60 (70–230 mesh, E. Merck)] with hexane:ethyl ether at ratios 2:1, 1:1, 1:2, and finally with 100% ethyl ether. Of the four resulting fractions (each 45 ml), fraction F3-4 eluted with ethyl ether retained deterrent activity (see below).

Step 3: Fraction F3-4 was further fractionated on an open silica gel column [10.5 × 300 mm, 10 g silica gel 60 (70–230 mesh, E. Merck)] with hexane:ether:MeOH (10:10:2) and 1% acetic acid as an eluent. Each 4–5 ml fraction was collected and analyzed with thin-layer chromatography (TLC) using the same eluent. Compounds were visualized using MeOH/sulfuric acid as a charring agent. Three groups of compounds were identified: F3-4A, F3-4B, and F3-4C.

In all steps of the fractionation process, each fraction was concentrated under N_2 to 1 g RJeq per ml CH_2Cl_2 for use in the deterrent contact bioassay. When acetic acid was used in the solvent system, recovered samples were dried under reduced pressure with added water and 2-propanol to remove acetic acid by azeotroping before use in the bioassay.

Chemical Analysis of the Royal Jelly Fractions. Fractions F3-4A, F3-4B, and F3-4C were analyzed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC-MS) after methylation with methanol–sulfuric acid followed by silylation of the hydroxyl groups with bis(trimethylsilyl)trifluoroacetamide (BSTFA) (see below). The GC was an HP6890 (Agilent Technologies) with a flame ionization detector and an HP-5 column (30 m × 0.32 mm i.d., film thickness 0.25 μm). Injector and detector temperatures were 250°C. The oven was programmed at 40°C (2 min hold) then 4°C/min to 300°C.

GC/MS analysis was performed on an HP6890 GC interfaced to an HP6890 mass selective detector, equipped with an HP-1 column (30 m × 0.32 mm i.d., film thickness 0.25 μm). The oven was programmed at 70°C (2 min hold) then 4°C/min to 300°C (10 min hold). The mass spectrometer was operated at 70 eV, scanning from 40 to 500 amu at 1.5 scans/sec.

Fatty acids were identified as their methyl ester derivatives, and hydroxy fatty acids were identified as their methyl esters and trimethylsilyl (TMS) ethers. Identification of compounds in the extracts was carried out by GC/MS using retention times and mass spectra from reference compounds or with mass spectra from the NIST Mass Spectral Library.

Derivatizations. Methylation was carried out by evaporating all the solvent and then adding a solution of 2% sulfuric acid in MeOH (approx. 1 ml per 1 mg dried extract), and heating at 50°C for 2 hr. The reaction was quenched with deionized water and extracted three times with ether after which the combined organic layers were dried with anhydrous Na_2SO_4 and analyzed by GC and/or GC-MS. Where necessary, BSTFA was used as silylation reagent. Thus, the methyl ester extract was concentrated and BSTFA (100-fold excess) was added.

The reaction was carried out at 50°C for 30 min and directly analyzed with GC and GC-MS.

Chemicals. The following standards were purchased from Sigma-Aldrich Company (St. Louis, MO, USA): 3-hydroxyoctanoic acid, 8-hydroxyoctanoic acid, 3-hydroxydecanoic acid, sebacic acid, 10-hydroxydecanoic acid, 12-hydroxydodecanoic acid, 2-dodecenedioic acid, erucic acid [(*Z*)-13-docosenoic acid], oleic acid [(*Z*)-9-octadecenoic acid], nervonic acid [(*Z*)-15-tetracosenoic acid], (*Z*)-11-eicosenoic acid, (*Z*)-10-heptadecenoic acid, and (*E*)-12-octadecenoic acid.

The (ω-1)-hydroxy fatty acids (9-hydroxydecanoic acid, 11-hydroxydodecanoic acid, 13-hydroxytetradecanoic acid, 15-hydroxyhexadecanoic), the (ω-2)-hydroxy fatty acids (10-hydroxydodecanoic acid and 12-hydroxytetradecanoic acid), and (*E*)-2-decenedioic acid were synthesized as described below. 10-Hydroxy-(*E*)-2-decenoic acid was available in one of our laboratories (J. Kochansky). All solvents were distilled before use and all chemical reagents were of analytical reagent quality.

Syntheses. The (ω-1)- and (ω-2)-hydroxy fatty acids were synthesized in two steps. In the first step, an unsaturated fatty acid was ozonized and reduced, forming an oxo-fatty acid. The second step employed a Grignard reaction with an alkyl magnesium bromide.

General Method for the Ozonolysis. Approximately 100 mg of the unsaturated fatty acid [erucic acid, oleic acid, nervonic acid, (*Z*)-11-eicosenoic acid, (*Z*)-10-heptadecenoic acid, or (*E*)-12-octadecenoic acid] was dissolved in 8–10 ml dichloromethane. A small amount of 2-propanol was added to dissolve the longer fatty acids such as nervonic acid. The solution was stirred at −78°C while ozone was circulated through the reaction vessel until the solution turned blue. Excess ozone was then purged with nitrogen. The ozonide was reduced by adding 2 eq. dimethylsulfide and stirring for 8 hr at room temperature. Thereafter, the solution was concentrated on a rotary evaporator. The mixture was used directly in the Grignard reaction.

General Method for the Grignard Reaction. Ozonolysis products were dissolved in 10 ml dry ether per 100 mg starting material (from the unsaturated fatty acid). The solution was cooled to −30°C and 3–4 eq. of the Grignard reagent (ca. 0.5–0.6 ml of a 3 M solution) were slowly added [methyl magnesium bromide to get (ω-1)-hydroxy fatty acids and ethyl magnesium bromide to get (ω-2)-hydroxy fatty acids] and stirred for 1 hr. After removing the mixture from the dry ice/acetone bath, it was acidified with 1 N HCl to pH 3–4 and then extracted with ether and dried with anhydrous Na_2SO_4. Solvent was evaporated to yield an oily/solid substance, depending on the hydroxy fatty acid synthesized.

The (ω-1)-hydroxy fatty acids were purified with open column chromatography on silica gel [10.5 × 300 mm, 10 g silica gel 60 (70–230 mesh, E. Merck)] eluting with hexane:ethyl acetate (1:1) and 1% acetic acid. The (ω-2)-

hydroxy fatty acids were eluted with hexane:ether (1:1) and 1% acetic acid. Fractions of 4–5 ml were collected and visualized with TLC. Fractions containing the hydroxy acids were pooled and dried on a rotary evaporator. All hydroxy acids were obtained in >95% purity.

Synthesized compounds were identified by GC/MS and nuclear magnetic resonance (NMR). NMR spectra were recorded at 298 K with a Varian Unity+ (500 MHz proton; 126 MHz carbon) spectrometer. Chemical shifts are reported in parts per million (ppm) relative to the residual $CHCl_3$ peak in solvent $CDCl_3$ at 7.26 ppm unless noted otherwise.

9-Hydroxydecanoic acid: ^{1}H NMR ($CDCl_3$): δ 1.18 (d, 3H, J = 6.2 Hz), 1.25–1.5 (m, 10H), 1.62 (q, 2H, J = 7.3 Hz), 2.33 (t, 2H, J = 7.5 Hz), 3.8 (sextet, 1H, J = 6.1 Hz). ^{13}C NMR: δ 23.45, 24.84, 25.78, 29.12, 29.33, 29.53, 34.23, 39.27, 68.47, 179.55. MS (as TMS ether methyl ester): *m/z* (%) = 73 (51), 117 (100), 146 (8), 159 (11), 227 (13), 230 (13), 243 (2), 259 (7).

10-Hydroxydodecanoic acid: MS (as TMS ether methyl ester): 73 (65), 131 (100), 146 (6), 159 (11), 169 (14), 244 (11), 255 (14), 273 (46), 287 (2).

11-Hydroxydodecanoic acid: ^{1}H NMR ($CDCl_3$): δ 1.19 (d, 3H, J = 6.2 Hz), 1.23–1.5 (m, 14H), 1.62 (q, 2H, J = 7.4 Hz), 2.34 (t, 2H, J = 7.5Hz), 3.81 (sextet, 1H, J = 6.0 Hz). ^{13}C NMR: δ 23.49, 24.86, 25.88, 29.12, 29.36, 29.49, 29.65, 29.73, 34.25, 39.36, 68.51, 179.78. MS (as TMS ether methyl ester): 73 (40), 117 (100), 146 (6), 159 (16), 255 (19), 258 (17), 271 (4), 287 (8).

12-Hydroxytetradecanoic acid: ^{1}H NMR ($CDCl_3$): δ 0.94 (t, 3H, J = 7.5 Hz), 1.23–1.37 (m, 18H), 1.63 (q, 2H, J = 7.6 Hz), 2.34 (t, 2H, J = 7.5Hz), 3.54 (q, 1H, J = 4.7 Hz). ^{13}C NMR: δ 10.09, 24.57, 25.77, 29.17, 29.34, 29.50, 29.58, 29.69, 29.82, 30.23, 34.23, 37.00, 73.67, 179.64. MS (as TMS ether methyl ester): 73 (77), 131 (100), 146 (8), 159 (9), 272 (17), 283 (17), 301 (70).

13-Hydroxytetradecanoic acid: ^{1}H NMR ($CDCl_3$): δ 1.19 (d, 3H, J = 6.2 Hz), 1.22–1.5 (m, 18H), 1.63 (q, 2H, J = 7.4 Hz), 2.34 (t, 2H, J = 7.5Hz), 3.81 (sextet, 1H, J = 6.0 Hz). ^{13}C NMR: δ 23.56, 24.88, 25.91, 29.20, 29.37, 29.55, 29.66, 29.67, 29.74, 29.77, 34.23, 39.44, 68.53, 179.71. MS (as TMS ether methyl ester): 73 (34), 117 (100), 146 (6), 159 (12), 283 (12), 286 (10), 299 (2), 315 (2).

15-Hydroxyhexadecanoic acid: ^{1}H NMR ($CDCl_3$): δ 1.20 (d, 3H, J = 6.2 Hz), 1.24–1.35 (m, 22H), 1.64 (q, 2H, J = 7.4 Hz), 2.35 (t, 2H, J = 7.5Hz), 3.81 (sextet, 1H, J = 6.0 Hz). MS (as TMS ether methyl ester): 73 (24), 117 (100), 146 (8), 159 (11), 311 (13), 314 (9), 327 (2), 343 (4).

(E)-2-Decenedioic acid. The intermediate in the synthesis of (*E*)-2-decenedioic acid, 8-oxooctanoic acid, was synthesized from cyclooctene as described by Tolstikov et al. (1982). (*E*)-2-Decenedioic acid was then synthesized from 8-oxooctanoic acid based on a pathway described by Odinokov et al. (1983). A solution of 300 mg 8-oxooctanoic acid, 300 mg malonic acid, and 100 μl piperidine in 2 ml pyridine was kept at room temperature for 30 hr.

Thereafter, it was heated at 110–115°C for 2 hr, poured into a mixture of 3 ml concentrated HCl in 2.5 g of ice, and extracted three times with 3 ml ether per extraction. The ethereal extract was dried with anhydrous $MgSO_4$. After filtration, the ether was evaporated and a 10% solution of $NaHCO_3$ was added until a pH of 8–9 was reached. This solution was washed twice with 2 ml ether per wash. The aqueous layer was acidified with 10% HCl to pH 4, then extracted three times with 3 ml ether and dried with anhydrous Na_2SO_4, after which the solvent was evaporated. Purification of the dicarboxylic acid was carried out by recrystallization from hexane and ethyl acetate (1:1) at −20°C, and filtering at this temperature.

(*E*)-*2-Decenedioic acid:* ^{1}H NMR (acetone-d_6): δ 1.35–1.65 (m, 6H), 2.06 (quintet, 2H, J = 2.5 Hz), 2.24 (dq, 2H, J = 7.5, 1 Hz), 2.3 (t, 2H, J = 7.0 Hz), 5.83 (dt, 1H, J = 15.5, 1.7 Hz), 6.95 (dt, 1H, J =15.4, 6.9 Hz), 10.45 (s, 2H, –OH). MS (as dimethyl ester): 55 (83), 73 (46), 81 (77), 95 (73), 119 (54), 136 (100), 164 (66), 168 (57), 196 (22), 197 (20), 227 (1), 228 (0.1).

Deterrent Bioassay. Deterrent bioassays (Calderone et al., 2002) were conducted in a walk-in environmental chamber maintained at brood nest conditions (32–34°C and 50% RH). Extracts were presented on a 5.8 × 5.8 cm glass plate with one frosted surface. Glass plates were washed, heated to 175°C for 24 hr, soaked in acetone for at least 12 hr, and rinsed twice with CH_2Cl_2 before each test. A 5 × 5 cm pattern (Figure 2) was printed on paper and attached to the underside of the glass plate to serve as an area marker. An aliquot (2–10 µl) of extract was applied evenly to the frosted surface in each of the treatment lanes ("T" in Figure 2) and 10 µl dichloromethane (or ether, as required) were applied for control runs. After air-drying, the plate and pattern were placed on a moistened filter paper in a 9-cm Petri dish. Each bioassay

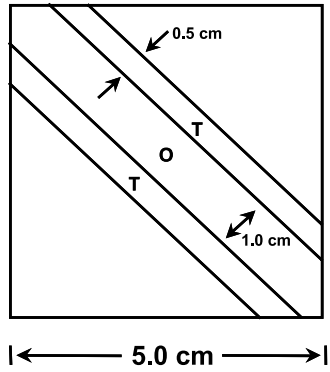

FIG. 2. Diagram of test pattern used to evaluate deterrent activity of extracts in the bioassay. See Methods and Materials section for details.

TABLE 1. CHARACTERISTIC IONS PRODUCED BY FATTY ACID METHYL ESTERS AND TMS ETHERS OF HYDROXY FATTY ACID METHYL ESTERS FOUND IN FRACTION F3-4B AFTER GC/MS ANALYSIS

				Fragments (*m/z*)						Other (*m/z*)								
Peak[a]	Compound	ID[b]	M^+	M-15 ($-CH_3$)	M-29 ($-C_2H_5$)	M-31 ($-CH_3O$)	M-44 ($-C_2H_4O$)	M-47 ($-CH_3 \rightarrow -CH_3OH$)	M-58 ($-C_3H_6O$)	73	89	103	117	131	133	146	159	175
	Diacid																	
6	Sebacic acid (decanedioic acid)[c]	Auth	230			199												
9	Dodecanedioic acid[c]	Auth	258			227												
8	2-Decenedioic acid[c]	Auth	228			197												
17	2-Dodecenedioic acid[c]	Auth	256			225												
	β-Hydroxy acid																	
1	3-Hydroxyoctanoic acid[c]	Auth	246	231		215		199		•	•				•		•	•
4	3-Hydroxydecanoic acid[c]	Auth	274	259		243		227		•	•				•		•	•
	ω-Hydroxy acid																	
3	8-Hydroxyoctanoic acid[c]	Auth	246	231		215		199			•	•					•	
7	10-Hydroxydecanoic acid[c]	Auth	274	259		243		227		•	•	•					•	
16	12-Hydroxydodecanoic acid[c]	Auth	302	287		271		255		•		•				•	•	
9	10-Hydroxy-(*E*)-2-decenoic acid[c]	Auth	272	257		241		225		•	•	•						
18	12-Hydroxy-2-dodecenoic acid	Litt	300	285		269		253		•								

	(ω-1)-Hydroxy acid													
5	9-Hydroxydecanoic acid[c]	Auth	274	259		243	230	227		•	•		•	•
12	11-Hydroxydodecanoic acid[c]	Auth	302	287		271	258	255		•	•		•	•
20	13-Hydroxytetradecanoic acid[c]	Auth	330	315		299	286	283		•	•		•	•
22	15-Hydroxyhexadecanoic acid[c]	Auth	358	343		327	314	311		•	•		•	•
15	11-Hydroxydodecenoic acid	Litt	300	285		269	256	253		•	•		•	
21	13-Hydroxytetradecenoic acid	Litt	328	313		297	284	281		•	•		•	
	(ω-2)-Hydroxy acid													
11	10-Hydroxydodecanoic acid	Auth	302	287	273	271		255	244	•		•		•
19	12-Hydroxytetradecanoic acid[c]	Auth	330		301			283	272	•		•		•
14	10-Hydroxydodecenoic acid	Litt	300	285	271	269		253	242	•		•		

[a] Number corresponds with peak number in Figure 7.
[b] Auth: These compounds were identified by comparison of spectral and chromatographic data of authentic standards. Litt: These were identified according to spectral data from literature or comparing data from analogues (synthesized or commercially available).
[c] Compounds used in the synthetic mixture resembling the F3-4B fraction.

consisted of testing the response of 10–12 mites to a specific treatment, and each treatment was replicated 2–12 times, depending on the specific test.

Mites were obtained by using established methods (Kuenen and Calderone, 1997, 1998; Calderone and Lin, 2001). The bioassay was performed by first removing the Petri dish cover, introducing a single mite to the central lane at the point designated "O," and replacing the dish cover. Each test period lasted ≤3 min. A finding that a mite would not cross into or through either of the treatment lanes during the test period was used as evidence of a deterrent response. Therefore, a mite was scored as deterred if it moved off the plate while staying within the central lane or if it remained in the central lane for the entire 3-min period, moving and making returns when it contacted either of the treatment lanes. A mite was scored as not deterred if it failed to avoid either of the treatment lanes by moving into or through either of the treatment lanes during the 3-min observation period.

Fractions Evaluated. The royal jelly crude extract was evaluated at three doses (5, 10, and 20 mg RJeq). Fractions F1, F2, F3, and F4 and fractions F3-1, F3-2, F3-3, and F3-4 were each evaluated at 10 mg RJeq. These quantities are smaller than the 200–300 mg of royal jelly normally available to a queen larva in a queen cell (Schmidt and Buchmann, 1992).

Fractions F3-4A, F3-4B, and F3-4C were each evaluated at three doses: 5, 10, and 20 mg RJeq. The crude royal jelly extract in this test was evaluated at 10 mg RJeq because previous studies (Calderone et al., 2002) have found the response at this level to be equivalent to the response at both 5 and 20 mg RJeq.

A synthetic mixture resembling F3-4B (the most active fraction of the F3-4 fractions) was constituted by combining commercially available standards or synthesized versions of the 15 most prominent compounds identified in that fraction (Table 1). Ether was used as a solvent. Although 10-hydroxydodecanoic acid was synthesized and correctly identified by GC/MS, the yield was very low after purification due to unknown reasons. Therefore, this compound could not be included in the synthetic mixture, and no NMR data could be collected. GC analysis was used to ensure that the quantities and ratios of compounds in the synthetic blend were the same as the corresponding quantities and ratios in the original fraction. The activity of the synthetic mixture (Syn-F3-4B) was compared to that of the native fraction (F3-4B). The amount of the native active fraction (F3-4B) used was 10 mg RJeq. The equivalent dose of the synthetic mixture used was 52 μg/lane. Ether was used as a control.

Statistical Analysis. Arcsine square root transformed data were analyzed using PROC MIXED (SAS, 1996) with fraction modeled as a fixed effect and mite source and the interaction between fraction and mite source as random effects. The Tukey–Kramer (SAS, 1988) test was used to compare means.

RESULTS

Royal Jelly Fractions. Bioassay of the royal jelly crude extract at three concentrations (5, 10, and 20 mg RJeq) yielded the same results as previously described by Calderone et al. (2002) (data not shown). There were significant differences among the activities of the four fractions produced in Step 1 of the fractionation process (Figure 3; $F_{4,7} = 30.78$, $P < 0.001$). The deterrent activity of fraction F3 (yield of 130 mg from 4 g RJeq crude extract) was 96.15 ± 0.05%, which was greater than that of the control and the other three fractions (Figure 3; Tukey–Kramer, $P < 0.001$ for each significant comparison) and equivalent to the activity observed for the original crude extract (Calderone et al., 2002).

There were significant differences among the activities of the four fractions produced in Step 2 (Figure 4; $F_{4,10} = 15.33$, $P < 0.001$). The deterrent activity of fraction F3-4 (yield of 110 mg) was 89.40 ± 0.08%, which was greater than the activity of the control and the other three fractions (Figure 4; Tukey–Kramer, $P < 0.05$ for each significant comparison).

Fraction F3-4 was further fractionated into three fractions: F3-4A (yield of 5.5 mg), F3-4B (22.5 mg), and F3-4C (75.2 mg). Each fraction was evaluated at three doses (5, 10, and 20 mg RJeq). There were significant differences in deterrent activity among these fractions (Figure 5; $F_{10,67} = 18.73$, $P < 0.001$). The activity of each fraction at each dose was greater than the activity of the control (except F3-4A at 5 mg RJeq) (Figure 5; Tukey–Kramer, $P < 0.05$ for

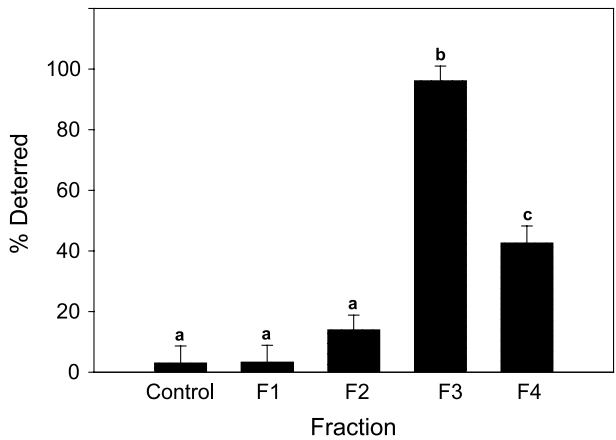

FIG. 3. Deterrent activity of the first four fractions of the royal jelly crude extract produced in Step 1 of the fractionation process. Each fraction was evaluated at 10 mg RJeq. Dichloromethane was used as a control. Bars with different letters are significantly different from each other (Tukey–Kramer, $P < 0.05$).

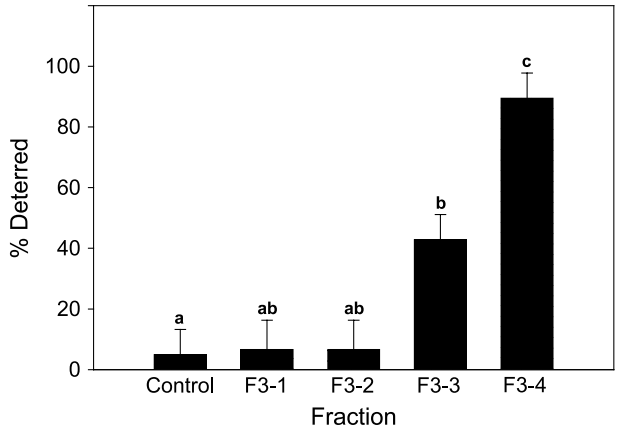

FIG. 4. Deterrent activity of the four fractions derived from fraction F3 in Step 2 of the fractionation process. Each fraction was evaluated at 10 mg RJeq. Dichloromethane was used as a control. Bars with different letters are significantly different from each other (Tukey–Kramer, $P < 0.05$).

each significant difference). The activities of fraction F3-4A and F3-4C at each dose were less than that of the crude royal jelly extract (RJ CE) (Figure 5; Tukey–Kramer, $P < 0.05$ for each comparison). The activity of F3-4B at each dose tested was equivalent to the activity of the original crude extract (RJ CE) (Figure 5; Tukey–Kramer, $P > 0.20$ for each comparison).

There were significant differences in the activities of the original F3-4B fraction, the synthetic F3-4B blend, and the control (Figure 6; $F_{2,40} = 171.19$, $P < 0.001$). The activity of the original F3-4B fraction and the synthetic F3-4B blend were both greater than that of the control (Tukey–Kramer, $P < 0.001$ each comparison) but not from each other (Tukey–Kramer, $P > 0.75$).

Chemical Analysis. Figure 7 shows the chromatogram of fraction F3-4B analyzed by gas chromatography after methylation and silylation. The compounds present are listed in Table 1. All the compounds identified were fatty acids; more specifically, the extract contained normal fatty acids, diacids, and β-, ω-, (ω-1)-, and (ω-2)-hydroxy fatty acids. Fraction F3-4A had a similar profile as F3-4B, except that the concentration of the compounds was much lower. The last fraction, F3-4C, was only analyzed on GC. It contained not only some of the compounds found in F3-4B, but also many larger molecules, according to their retention times.

The main reason for derivatization before injecting the samples in the GC was that previous experiments in our laboratory (data not published) as well as earlier studies (Brown et al., 1961; Lercker et al., 1981, and references therein;

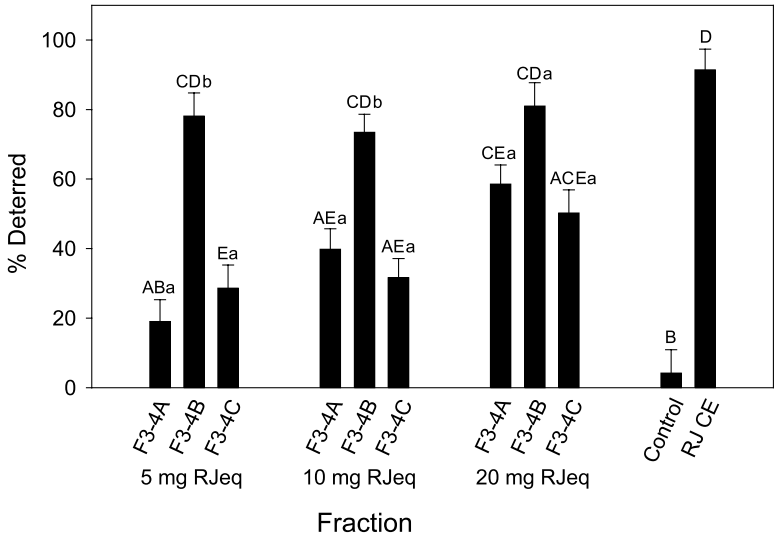

FIG. 5. Deterrent activity of the three fractions derived from fraction F3-4 in Step 3 of the fractionation process. Each fraction was evaluated at three doses: 5, 10, and 20 mg RJeq. Dichloromethane was used as a control and the response to crude extract of royal jelly (RJ) was included for comparison. Results of multiple comparison procedures are shown in lower and uppercase letters. Lowercase letters are valid for comparing responses to the three fractions within a single dose. Uppercase letters are valid for comparing the response of any fractions at one dose with the response to any fractions at the other two doses and to the control and crude extract. Bars with different letters are significantly different from each other (Tukey–Kramer, $P < 0.05$).

Lercker et al., 1982) showed that the fractions might contain hydroxy fatty acids. To confirm that these fatty acids were not present as their methyl esters in the original fraction, we carried out an experiment in which the active fraction was silylated with BSTFA without prior methylation. Analysis of this silylated extract revealed that only the TMS esters and TMS ethers of the acids were present; no methyl esters of the fatty acids were found.

The silylated β- and ω-hydroxy fatty acid methyl esters were identified by mass spectrometry according to an extensive study by Eglinton et al. (1968) and Capella and Zorzut (1968). The typical fragments of these fatty acids are given in Table 1. The ω-hydroxy esters all had characteristic peaks at M-15 and M-47 with a smaller peak at M-31. Other peaks present, such as $m/z = 73$ and 89, were all according to Eglinton et al. (1968). The β-hydroxy esters were identified with the same M-15, M-47, and M-31 peaks, but the relative intensities were different from the corresponding ions of the ω-hydroxy esters. Characteristic for

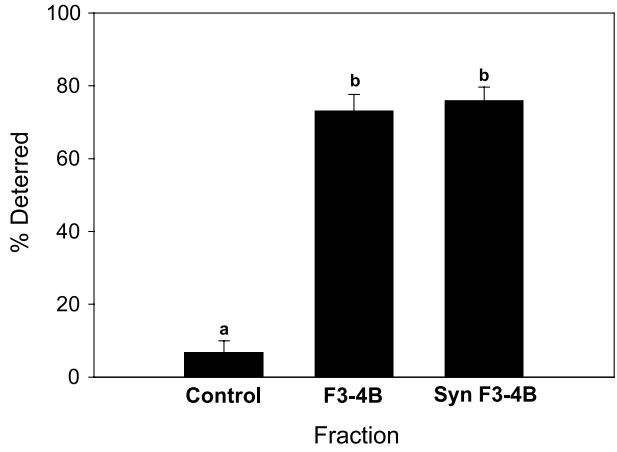

FIG. 6. Deterrent activity of the synthetic blend (Syn-F3-4B) and the original fraction (F3-4B). The amount of the original active fraction (F3-4B) used was 10 mg RJeq. The equivalent dose of the synthetic mixture used was 52 μg/lane. Ether was used as a control. Bars with different letters are significantly different from each other (Tukey–Kramer, $P < 0.05$).

these hydroxy esters was the loss of the alkyl chain adjacent to the hydroxy group, resulting in a loss of 56 amu (C_4H_8) for β-hydroxyoctanoic acid and a loss of 84 amu (C_6H_{12}) for β-hydroxydecanoic acid.

All the (ω-1)- and (ω-2)-hydroxy esters had the M-15, M-47, and M-31 peaks present. In addition to these peaks, an M-44 peak was present in the

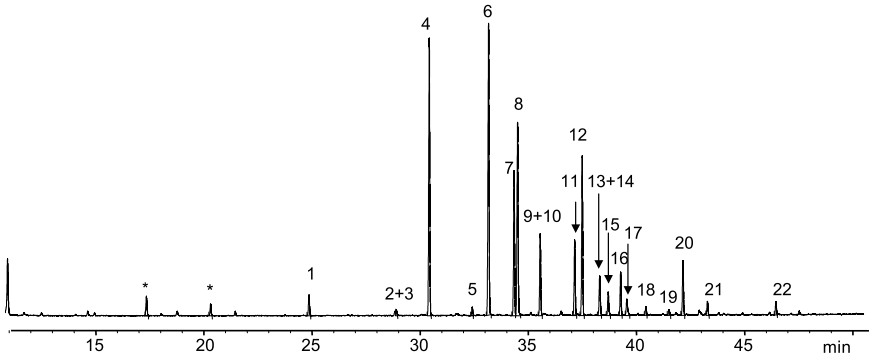

FIG. 7. Gas chromatogram of fraction F3-4B after methylation and silylation. For details on compounds, see Table 1. *Denotes compounds from the derivatization reaction.

(ω-1)-hydroxy esters and an M-58 peak was present in the (ω-2)-hydroxy esters. This M-44 (or M-58) peak corresponds to a shift of the TMS group from the hydroxy group to the carboxyl group followed by the elimination of $C_xH_{2x}O$. In the (ω-2)-hydroxy esters the M-31 peak was small and another peak, M-29 (loss of C_2H_5), was present in this type of ester.

Four unsaturated hydroxy fatty acids, that is, 10-hydroxydodecenoic acid, 11-hydroxydodecenoic acid, 12-hydroxydodecenoic acid, and 13-hydroxytetradecenoic acid, were not synthesized but were tentatively identified by comparison of their mass spectra with those of similar compounds (synthesized or commercially available). Each of these compounds had a similar fragmentation pattern to its analogue. Position and geometry of the double bond were not established in these compounds.

DISCUSSION

Varroa mites were deterred by crude extracts and fractions of crude extracts of royal jelly. Fraction F3-4B exhibited activity equivalent to that of the crude extract, and the activity of the synthetically reconstituted F3-4B fraction was equal to that of the native fraction. The tested amounts of crude extract, active fraction, or synthetically reconstituted fraction were all within the same range and thus comparable to each other. Compared to what a mite would encounter in a queen cell, the amounts used in the bioassay were ca. 10 times lower. However, the amounts per unit area were probably similar. Therefore, we think that the *in vivo* and *in vitro* responses would be similar. From studying the behavior of the mites in the bioassays, it appeared that the mites had to make contact with the extract before responding. However, whether contact is needed or whether being very close is sufficient to elicit a response still has to be investigated.

These results provide a possible mechanism to explain the low frequency of female *V. destructor* in capped queen cells. However, the difference in cell orientation between queen and worker/drone cells, as a possible contributing factor, has not been examined. In addition, our findings do not exclude a role for extractable compounds present on cuticles of worker and queen larvae (Trouiller et al., 1994; Calderone et al., 2002).

Most of the royal jelly compounds identified in this study have been reported previously (Brown et al., 1961; Lercker et al., 1981, and references therein; Lercker et al., 1982). Lercker et al. (1981) identified a large number of fatty acids and hydroxy fatty acids from royal jelly, and we have now identified several hydroxy esters listed as “unknown” by these authors. The composition of the active fraction F3-4B closely resembled that of the secretion of the mandibular glands of the workers (Simon et al., 2001). This is not surprising

because royal jelly is produced by the mandibular and hypopharyngeal glands of nurse bees. No 9-oxodecanoic acid (9-ODA, or queen substance) was found in the active fraction. Also, other components of the queen mandibular pheromone (QMP), such as the two enantiomers of (*E*)-9-hydroxy-2-decenoic acid, and 4-methyl-3-methoxyphenylethanol were not found. Only a small amount of methyl 4-hydroxybenzoate was found.

The next step in this research will be to determine the contribution of each of the 15 compounds in the synthetic blend to the deterrent activity exhibited by the native fraction. Field tests will be required to provide data on the possible application of these compounds for managing mite levels in honeybee colonies.

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CUTICULAR HYDROCARBONS AS SEX PHEROMONE OF THE BEE *Colletes cunicularius* AND THE KEY TO ITS MIMICRY BY THE SEXUALLY DECEPTIVE ORCHID, *Ophrys exaltata*

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Abstract—Male *Colletes cunicularius* bees pollinate the orchid, *Ophrys exaltata*, after being sexually deceived by the orchid's odor-mimicry of the female bee's sex pheromone. We detected biologically active volatiles of *C. cunicularius* by using gas chromatographic–electroantennographic detection (GC-EAD) with simultaneous flame ionization detection. After identification of the target compounds by coupled gas chromatography–mass spectrometry (GC-MS), we performed behavioral tests using synthetic blends of the active components. We detected 22 EAD active compounds in cuticular extracts of *C. cunicularius* females. Blends of straight chain, odd-numbered alkanes and (*Z*)-7-alkenes with 21–29 carbon atoms constituted the major biologically active compounds. Alkenes were the key compounds releasing mating behavior, especially those with (*Z*)-7 unsaturation. Comparison of patterns of bee volatiles with those of *O. exaltata* subsp. *archipelagi* revealed that all EAD-active compounds were also found in extracts of orchid labella. Previous studies of the mating behavior in *C. cunicularius* showed linalool to be an important attractant for patrolling males. We confirmed this with synthetic linalool but found that it rarely elicited copulatory behavior, in accordance with previous studies. A blend of active cuticular compounds with

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linalool elicited both attraction and copulation behavior in patrolling males. Thus, linalool appears to function as a long-range attractant, whereas cuticular hydrocarbons are necessary for inducing short-range mating behavior.

Key Words — *Ophrys*, solitary bee, sex pheromone, pollination by sexual deception, alkane, alkene, floral mimicry.

INTRODUCTION

Colletes cunicularius (L.) (Hymenoptera: Colletidae) is a solitary oligolectic bee that nests in aggregations and mates soon after emergence. Males display a competitive mate-searching behavior based on olfactory cues. Females dig their nest cavities up to 1 m deep in sandy soil and line the brood cells with a secretion of the Dufour's gland (Cane, 1981). Males emerge first and patrol along the nest entrances in search of a mate. Using olfactory cues, males are able to accurately locate preemergent virgin females and distinguish them from mated females (Bergström and Tengö, 1978; Batra, 1980; Cane and Tengö, 1981). However, patrolling males are also attracted to preemergent males (Cane and Tengö, 1981). Females emerging from their nests stimulate scramble competition among large numbers of competing males (Müller, 1991). *C. cunicularius* is active in early spring from March to May when few bees or other insect species are in flight and at a time that restricts its primary pollen source to the early flowering *Salix* (Amiet et al., 1999). Males die during April/May, whereas females continue to provision nests until the end of May.

Previous studies on chemical communication in *C. cunicularius* concentrated on the effects of cephalic compounds, in particular linalool, because it is the major compound in the mandibular gland secretions of both sexes in all *Colletes* species examined (Bergström and Tengö, 1978; Hefetz et al., 1979; Cane and Tengö, 1981; Lindsley and Zavortink, 1997). In behavioral experiments, Cane and Tengö (1981) demonstrated that linalool acts as a pheromone that enhances attraction and directs local search behavior of patrolling males to emerging females. However, in these tests linalool rarely stimulated male excavating or pouncing (copulatory) behaviors. In contrast, head extracts of female *C. cunicularius* and even floral extracts of the orchid that mimics the female bees, *Ophrys arachnitiformis–sphegodes*, attracted more males, caused more local searching, and released more copulatory attempts than linalool alone (Cane and Tengö, 1981). The lack of full copulatory behavior and the occurrence of linalool in males as well as females led Cane and Tengö to suggest that the presence of linalool may function mainly as a long-range attractant or aggregation signal stimulating precopulatory behavior in males. They suggested that pouncing behavior and the ability of males to distinguish between virgin and mated females may involve other olfactory cues, such as

long-chain hydrocarbons that are present in *Colletes* and have subsequently been shown to be active in other bee genera such as *Lasioglossum* (Ayasse et al., 1999) and *Andrena* (Schiestl et al., 2000).

Recently, Borg-Karlson et al. (2003) showed enantiometrically pure (*S*)-(+)-linalool to be the main mandibular gland constituent of males and females of *C. cunicularius*, although both enantiomers induced electrophysiological responses from male antennae. Virgin females and males contain similar amounts of (*S*)-(+)-linalool (mean 71% of cephalic pentane extracts), whereas mated females had reduced amounts (mean 47%) (Borg-Karlson et al., 2003). Synthetic (*S*)-(+)-linalool attracted the highest number of male bees, followed by the racemate and (*R*)-(–)-linalool. Thus, (*S*)-(+)-linalool, present in *C. cunicularius*, was shown to be an important component of the mate attractant pheromone. The decrease in (*S*)-(+)-linalool in mated females was suggested to account for the loss of attractiveness of mated females to patrolling males. However, the role that other compounds might play in eliciting *C. cunicularius* mating behavior was not further discussed. The apparent simplicity in the chemical communication between sexes was posited to be a response to the low interspecific competition in chemical signals and to the harsh meteorological environment of the early European spring.

The mandibular glands of females are an important source of sex pheromones in eusocial stingless bees (Engels et al., 1990; Engels, 1993), bumble bees (van Honk et al., 1978), and carpenter bees (Gerling et al., 1989). However, recent studies indicate that male attractant pheromones can involve multiple compounds from cuticular as well as other glandular secretions (Ayasse et al., 2001). Studies on *Andrena* (Schiestl et al., 1999), *Lasioglossum* (Wcislo, 1987; Ayasse et al., 1999), *Osmia* (Ayasse and Dutzler, 1998), and *Nomia* species (Wcislo, 1992) have detected female sex pheromones consisting of compounds localized on the surface of the cuticle. In *Lasioglossum zephryum* (Halictidae), macrocyclic lactones produced by the Dufour's gland appear to function as a sex pheromone (Smith et al., 1985). However, behavioral tests using synthetic lactones did not elicit copulatory behavior. Examination of the related *L. malachurum* revealed that the Dufour's gland was not the sole source of sex pheromone activity (Ayasse et al., 1993). Rather, hydrocarbons, isopentenyl esters, and unsaturated macrocyclic lactones localized on the cuticle induced copulation attempts by males.

Cuticular hydrocarbons, although known to have a major function in desiccation resistance (Hadley, 1981), are also used as sex pheromones in many insect taxa, especially among Diptera (Howard, 1993; Singer, 1998). In *Andrena* bees (Andrenidae), species-specific blends of cuticular hydrocarbons function as sex pheromones attracting males (Schiestl et al., 2000; Schiestl and Ayasse, 2002). Chemical analysis of the floral odors of the orchid, *Ophrys sphegodes*, showed hydrocarbon patterns similar to those found in females of the pollinator

species, *Andrena nigroaenea* (Schiestl et al., 2000). Behavioral tests confirmed that *O. sphegodes* precisely mimics the female bee's olfactory cues and sexually deceives the pollinating *Andrena* males and thereby avoids the provision of floral rewards.

In this study, we reexamined the sex pheromone of *C. cunicularius*, the pollinator of *Ophrys exaltata* Tenore (*O. arachnitiformis* species group), a sexually deceptive orchid species from southern Italy (Paulus and Gack, 1990a,b; Delforge, 1994). Our objectives were to (1) identify all components of the female sex pheromone of *C. cunicularius*, (2) investigate differences in the patterns of volatiles between virgin and mated females, (3) examine the importance of different physiologically active compounds for male attraction, and (4) compare the pattern of physiologically active compounds in *C. cunicularius* with the volatiles of its orchid mimic, *O. exaltata*.

METHODS AND MATERIALS

Sample Collection. Virgin *C. cunicularius* females were collected during the early flight season in March at Neuhausen (Switzerland). Wherever a cluster of males with an attractive female in the center was detected, the female was separated from the males as fast as possible, placed in a Perspex vial, and kept in a chilled box. Mated females were collected after the disappearance of males in late April at Neuhausen. All sampled bees were freeze-killed on the day on which they were caught. A total of 56 virgin and 22 mated females as well as 10 males were collected. For cuticle extracts, the whole body was extracted in 400 μl hexane for 1 min., whereas for head extracts, the head was extracted in 150 μl hexane for 24 hr.

For the orchids, 69 samples of *O. exaltata* subsp. *archipelagi* Gölz and Reinhard, representing a subsample of those individuals sampled by Mant et al. (2005) were collected at two nearby populations in Monte Gargano (southeastern Italy). The labella were extracted in 200 μl hexane for 1 min. All samples were stored at −20°C.

Gas Chromatographic Analysis with Electroantennographic Detection (GC-EAD). Aliquots of 1 μl of the cuticle extracts of virgin females were injected splitless at 50°C (1 min) into an Agilent 6890 N gas chromatograph (Agilent Palo Alto, CA, USA) followed by programming to 300°C at 10°C min^{-1}. The gas chromatograph (GC) was equipped with an HP-5 column (30 m × 0.32 mm diam × 0.25 μm film thickness) and a flame ionization detector (FID). Helium was used as carrier gas. A GC effluent splitter (SGE International Pty Ltd, Sydney, Australia) was used, and a portion of the eluate was added to a purified and humidified air stream, directed over the excised antenna of a male

bee. The tip of the antenna was cut off, and the antenna was mounted between two electrodes using electrocardiograph gel. The electrode holding the base of the antenna was grounded. The distal end of the antenna was connected via an interface box (Syntech, Hilversum, the Netherlands) for signal transfer to a personal computer. EAD signals and FID responses were simultaneously recorded. Twelve GC-EAD runs were performed with antennae from six males to check the reproducibility of antennal responses.

Structure Elucidation. The structures of compounds that elicited GC-EAD responses were identified by coupled gas chromatographic–mass spectrometric (GC-MS) analysis and coinjection with authentic standards. Extracts were analyzed with an HP-5970 (Hewlett-Packard) gas chromatograph equipped with a DB-5 capillary column (30 m $\times$ 0.32 mm diam) operated at 120°C for 30 sec, then 4°C min^{-1} to 280°C. Structure elucidation of individual compounds was based on GC-MS analysis (VG70/250 SE instrument, Vacuum Generators, Manchester, England, UK, linked to an HP-5890 GC; conditions as mentioned above). Mass spectra (70 eV) were compared with those reported in the literature (McLafferty and Stauffer, 1989) and with those of authentic reference samples. Gas chromatographic retention times were checked by coinjection. Double bond positions in mono- and diunsaturated compounds were assigned according to Buser et al. (1983) and Dunkelblum et al. (1985). The stereochemistry of double bonds was determined by comparison of retention times using corresponding reference samples, including dimethyldisulfide (DMDS) derivatives, as the erythro- and threo-adducts could be separated by GC.

Synthetic Compounds. The following compounds were purchased from Aldrich: linalool, decanol, dodecanol, hexadecanol, eicosanol, tetradecanoic acid, hexadecanoic acid, oleic acid, linoleic acid, tetracosanoic acid, saturated hydrocarbons, (9*Z*)-tricosene, 1-alkynes. Aldehydes were prepared from commercially available alcohols by using Swern oxidation (Mancuso and Swern, 1981). Mass spectra of synthetic compounds matched the expected patterns (McLafferty and Stauffer, 1989) and GC retention times. Esters were prepared form the corresponding acid chlorides and the appropriate alcohols according to standard laboratory methods. Mass spectra of synthetic compounds matched the expected patterns (Francke et al., 2000) and GC retention times. Alkenes were prepared by Lindlar hydrogenation of the corresponding alkynes. In a typical synthesis, a 1-alkyne was coupled to a 1-iodoalkane according to Buck and Chong (2001). The crude product was purified by column chromatography (Merck silica 60, 120–400 mesh, hexane). Lindlar catalyst (440 mg) was added to a solution of 0.7 ml (5.8 mmol) freshly distilled quinoline in 10 ml hexane. After addition of 1.87 mmol of the alkyne in 5 ml hexane, hydrogenation was carried out for 4 hr at room temperature and atmospheric pressure. Subsequently, the catalyst was filtered off, and the solvent was removed *in vacuo*. Chromatography on silica provided the pure (*Z*)-alkene in over 95% yield and

99% chemical purity. Alkadienes were prepared from the corresponding alkadiynes by Lindlar hydrogenation. As an example, the synthesis of (8*Z*, 20*Z*)-nonacosadiene is described. The corresponding reaction scheme is shown in Figure 1.

2-(10′-Bromodecyloxy)-tetrahydropyrane ***1***

A solution of 1.47 ml (16.3 mmol) 2,3-dihydro-4*H*-pyrane in 10 ml dichloromethane was added to a solution of 2.5 ml (13.6 mmol) 10-bromodecan-1-ol **1** (Aldrich) and 51 mg (0.27 mmol) *p*-toluene-sulfonic acid hydrate in 100 ml dichloromethane, which was cooled to −15°C. The reaction mixture was stirred for 1 hr, and subsequently warmed to room temperature. After the addition of 75 ml hexane, 75 ml of a saturated aqueous sodium carbonate solution were added. After separation, the aqueous layer was extracted three times with 50 ml hexane. The combined organic layers were washed with brine, dried over magnesium sulfate, and concentrated *in vacuo*. The crude product was chromatographed on silica using hexane: ethyl acetate 15:1 (v:v). As a result, 3.25 g (10 mmol, 74%) of 2-(10′-bromodecylocy)-tetrahydropyrane were obtained.

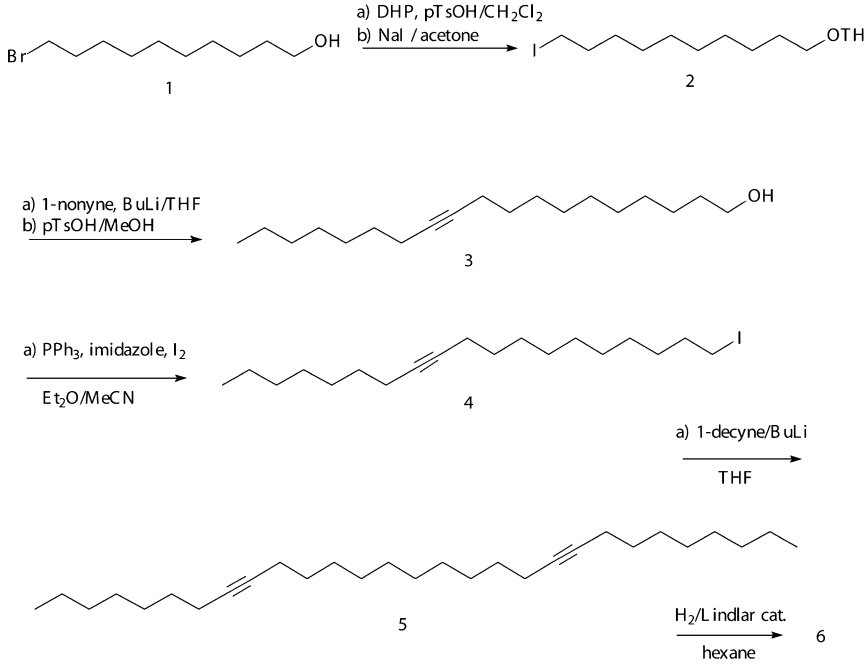

FIG. 1. Synthesis of (*Z*8,*Z*20)-nonacosadiene.

^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] = 1.27–1.72 (m, 20H, H-3-H-5/H-2′-H-8′), 1.84 (tt, 2H, *J* = 7.12 Hz, H-9′), 3.40 (t, 2H, *J* = 6.86 Hz, H-10′), 3.48–3.55 (m, 1H, H-6a), 3.63 (t, 2H, *J* = 6.61 Hz, H-1′), 3.82–3.89 (m, 1H, H-6b), 4.49–4.53 (m, 1H, H-2).

^{13}C NMR (101 MHz, $CDCl_3$): δ [ppm] = 19.80/25.84/26.13/28.56/29.14/29.77/29.87/30.94/33.16/33.23/34.41 (t, C-3-C-5/C-2′-C-10′), 62.36/63.38 (t, C-6/C-1′), 100.12 (d, C-2).

MS (70 eV): *m/z* [%] = 322 (0.74, M^+ ($C_{15}H_{29}O_2{}^{81}Br$)), 320 (0.72, M^+($C_{15}H_{29}O_2{}^{79}Br$)), 102 (8), 101 (8), 97 (10), 87 (25), 86 (96), 85 (100), 84 (11), 83 (18), 71 (9), 69 (21), 68 (8), 67 (10), 58 (11), 57 (42), 56 (30), 55 (46), 43 (32), 42 (17), 41 (52).

2-(10′-Iododecyloxy)-tetrahydropyrane ***2***

Sodium iodide [2.23 g (15 mmol)] was added to a solution of 3.25 g (10 mmol) 2-(10′-bromodecyloxy)-tetrahydropyrane in 40 ml acetone. The mixture was stirred for 12 hr at room temperature. Subsequently, the solvent was removed *in vacuo*, and the residue was partitioned between 100 ml water and 100 ml of a 1:1 mixture of hexane and ethyl acetate. The organic layer was dried over magnesium sulfate, concentrated *in vacuo*, and directly used for the next step.

^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] = 1.26–1.72 (m, 20H, H-3-H-5/H-2′-H-8′), 1.82 (tt, 2H, *J* = 7.63 Hz, H-9′), 3.19 (t, 2H, *J* = 7.12 Hz, H-10′), 3.48–3.55 (m, 1H, H-6a), 3.63 (t, 2H, *J* = 6.61 Hz, H-1′), 3.82–3.89 (m, 1H, H-6b), 4.49–4.53 (m, 1H, H-2).

^{13}C NMR (101 MHz, $CDCl_3$): δ [ppm] = 7.63 (t, C-10′), 19.77/25.85/26.13/26.62/28.91/29.74/29.77/30.17/30.89/30.94/33.17 (t, C-3-C-5/C-2′-C-9′), 62.36/63.40 (t, C-6/C-1′), 100.20 (d, C-2).

MS (70 eV): *m/z* [%] = 368 (1.69, M^+), 183 (6), 102 (10), 101 (17), 97 (11), 87 (14), 86 (69), 85 (100), 84 (12), 83 (26), 71 (6), 69 (24), 68 (5), 67 (10), 58 (7), 57 (33), 56 (26), 55 (48), 43 (23), 42 (11), 41 (40).

Nonadec-11-yne-1-ol ***3***

A total of 6.7 ml (10.7 mmol) of a 1.6 M solution of *n*-butyl lithium in hexane were added dropwise to a solution of 1.94 ml (11.7 mmol) non-1-yne in 100 ml absolute THF, cooled to −78°C. The mixture was warmed to room temperature within 4 hr, and 3.59 g (9.74 mmol) of **2**, dissolved in 10 ml hexane, were added. The mixture was refluxed for 12 hr. After cooling, 100 ml of saturated aqueous ammonium chloride solution were added. After separation of the layers, the aqueous phase was extracted three times with 50 ml of diethyl

ether. The combined organic solutions were washed with brine, dried over magnesium sulfate, and concentrated *in vacuo*. The residue was dissolved in 50 ml absolute methanol, and 0.5 g *p*-toluene sulfonic acid hydrate were added. The mixture was stirred for 20 min at 50°C. After cooling to room temperature, the solvent was removed *in vacuo*, and the residue was partitioned between 100 ml diethyl ether and 100 ml water. Subsequently, the aqueous layer was extracted three times with 50 ml diethyl ether. The combined organic solutions were washed with brine and concentrated *in vacuo*. Purification was carried out by column chromatography using silica and hexane:ethyl acetate 10:1 (v:v). As a result, 2.06 g (7.35 mmol, 75%) of nonadec-11-yne-1-ol **3** were obtained.

^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] = 0.89 (t, 3H, *J* = 6.61 Hz, H-19), 1.28–59 (m, 26H, H-2-H-9/H-14-H-18), 2.13 (t, 4H, *J* = 6.86 Hz, H-10/H-13), 3.63 (t, 2H, *J* = 6.61 Hz, H-1).

^{13}C NMR (101 MHz, $CDCl_3$): δ [ppm] = 14.48 (q, C-19), 19.17/23.03/26.15/29.24/29.57/29.82/29.88/29.97/32.19/33.20 (t, C-2-C-10/C-13-C-18), 63.45 (t, C-1), 80.55/80.68 (s, C-11/C-12).

MS (70 eV): *m*/*z* [%] = 280 (0.07, M^+), 138 (23), 135 (7), 124 (6), 123 (9), 121 (13), 111 (7), 110 (28), 109 (28), 108 (6), 107 (12), 98 (7), 97 (14), 96 (50), 95 (61), 94 (11), 93 (23), 91 (10), 85 (5), 83 (21), 82 (56), 81 (95), 80 (19), 79 (41), 77 (11), 70 (6), 69 (37), 68 (49), 67 (100), 66 (9), 65 (8), 57 (13), 56 (11), 55 (82), 54 (46), 53 (14), 43 (44), 42 (10), 41 (70).

*Nonadec-11-ynyliodide **4***

A total of 2.12 g (8.09 mmol) triphenyl phosphane and 0.55 g (8.09 mmol) imidazole were dissolved in a mixture of 60 ml absolute diethyl ether and 20 ml absolute acetonitrile. The mixture was cooled to 0°C, and 2.05 g (9.09 mmol) iodine were slowly added. The mixture was stirred for an additional 20 min at 0°C and 2 hr at room temperature. After cooling to 0°C, 2.06 g (7.35 mmol) of the ynol **3** were added. After additional stirring for 2 hr at room temperature, 100 ml water were added. After separation of the layers, the aqueous phase was extracted three times with hexane. The combined organic layers were washed with brine and dried over magnesium sulfate. Purification was carried out by column chromatography using silica and hexane:ethyl acetate 15:1 (v:v). As a result, 2.15 mg (5.5 mmol, 75%) nonadec-11-ynyliodide **4** were obtained, which were used for the next step.

^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] = 0.89 (t, 3H, *J* = 6.87 Hz, H-19), 1.20–1.51 (m, 24H, H-3-H-9/H-14-H-18), 1.82 (tt, 2H, *J* = 7.12 Hz, H-2), 2.14 (t, 4H, *J* = 6.87 Hz, H-10/H-13), 3.19 (t, 2H, *J* = 7.12 Hz, H-1).

^{13}C NMR (101 MHz, $CDCl_3$): δ [ppm] = 7.67 (t, C-1), 14.49 (q, C-19), 19.17/23.04/28.94/29.23/29.52/29.56/29.59/29.78/29.84/30.91/32.19/33.98 (t, C-2-C-10/C-13-C-18), 80.61/80.70 (s, C-11/C-12).

MS (70 eV): *m/z* [%] = 390 (1.28, M^+), 196 (6), 155 (9), 137 (6), 124 (5), 123 (19), 111 (5), 110 (11), 109 (29), 97 (12), 96 (16), 95 (54), 93 (12), 91 (11), 83 (21), 82 (23), 81 (83), 79 (37), 77 (15), 69 (42), 68 (20), 67 (100), 65 (12), 57 (13), 56 (11), 55 (66), 54 (23), 53 (16), 52 (7), 43 (65), 42 (14), 41 (96).

Nonacosa-8,20-diyne ***5***

A solution of 0.28 ml (1.54 mol) 1-decyne in 30 ml absolute THF was cooled to −78°C, and 0.8 ml (1.41 mmol) of a 1.6 M solution of *n*-butyl lithium in hexane was added. The stirred solution was warmed to room temperature within 3 hr. Subsequently, 0.5 g (1.28 mmol) nonadec-11-ynyliodide **4** was added, and the mixture was refluxed for 12 hr. After cooling to room temperature, 50 ml saturated aqueous ammonium chloride solution were added. After separation of the layers, the aqueous solution was extracted three times with 30 ml hexane. The combined organic layers were washed with brine, dried over magnesium sulfate, and concentrated *in vacuo*. Purification was carried out by column chromatography using silica and hexane. As a result, 312 mg (0.78 mmol, 61%) of nonacosadiyne **5** were obtained.

^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] = 0.88 (t, 6H, *J* = 6.86 Hz, H-1/H-29), 1.21–1.40 (m, 30H, H-2-H-5/H-12-H-16/H-24-H-28), 1.45–1.52 (m, 8H, H-6/H-11/H-18/H-23), 2.10–2.17 (m, 8H, H-7/H-10/H-19/H-22).

^{13}C NMR (101 MHz, $CDCl_3$): *δ* [ppm] = 14.32/14.48 (q, C-1/C-29), 19.24/23.10/23.18/29.01/29.21/29.26/29.32/29.71/29.98/30.02/30.05/32.18/32.25 (t, C-2-C-7, C-10-C-19/C-22-C-28), 80.42/80.72 (s, C-8/C-9/C-20/C-21).

MS (70 eV): *m/z* [%] = 400 (0.10, M^+), 301 (6), 287 (6), 203 (5), 189 (7), 175 (7), 161 (6), 149 (6), 147 (8), 135 (13), 133 (9), 123 (8), 122 (5), 121 (21), 119 (12), 110 (6), 109 (21), 108 (8), 107 (21), 106 (5), 105 (17), 97 (9), 96 (12), 95 (52), 94 (15), 93 (32), 92 (5), 91 (19), 83 (17), 82 (18), 81 (78), 80 (18), 79 (48), 77 (12), 71 (8), 70 (6), 69 (34), 68 (18), 67 (100), 66 (6), 65 (9), 57 (33), 56 (14), 55 (91), 54 (35), 53 (18), 52 (5), 43 (68), 42 (11), 41 (82).

(8Z,20Z)-Nonacosadiene ***6***

Sixty milligrams Lindlar catalyst (Lancaster) and 125 mg (0.31 mmol) nonacosa-8,20-diyne **5** were added to a solution of 0.09 ml (0.75 mmol) freshly distilled quinoline in 5 ml hexane. Hydrogenation was carried out at room temperature and atmospheric pressure. After filtration, the solvent was removed *in vacuo*. Purification was carried out by column chromatography using silica and hexane. As a result, 117 mg (0.29 mmol, 94%) (8*Z*,20*Z*)-nonacosadiene were obtained.

^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] = 0.88 (t, 6H, J = 7.12 Hz, H-1/H-29), 1.20–1.45 (m, 38H, H-2-H-6/H-11-H-18/H-23-H-28), 1.98–2.05 (m, 8H, H-7/H-10/H-19/H-22), 5.32–5.37 (m, 4H, H-8/H-9/H-20/H-21).

^{13}C NMR (101 MHz, $CDCl_3$): δ [ppm] = 14.50 (q, C-1/C-29), 23.08/25.74/27.62/29.63/29.68/29.73/29.97/30.19/32.29/32.32/32.88 (t, C-2-C-7, C-10-C-19/C-22-C-28), 130.25/130.32 (d, C-8/C-9/C-20/C-21).

MS (70 eV): *m*/*z* [%] = 404 (0.87, M^+), 152 (5), 138 (10), 137 (7), 125 (5), 124 (16), 123 (12), 111 (12), 110 (23), 109 (20), 97 (35), 96 (63), 95 (35), 84 (5), 83 (48), 82 (62), 81 (43), 80 (6), 79 (6), 71 (7), 70 (13), 69 (55), 68 (26), 67 (43), 57 (31), 56 (23), 55 (100), 54 (28), 53 (5), 43 (56), 42 (9), 41 (53).

Quantitative Analyses of GC Data. Gas chromatography was used for quantitative analysis of extracts of individual female bees and orchids, using the same parameters as for GC-EAD. For quantitative analysis, *n*-octadecane was added as an internal standard. Absolute amounts were calculated by dividing the peak area of each compound by the peak area of the internal standard and multiplied with the internal standard amount (head extracts 0.5 μg, cuticle extracts 1 μg). Means and standard deviations were calculated for all compounds within each group of samples (cuticle of virgin females, head of virgin females, cuticle of mated females, cuticle of males, labella of *O. exaltata*).

Behavioral Tests. Head extracts and cuticle extracts of ten bees were tested for their attractiveness during the 2003 season. All behavioral tests were performed at Neuhausen, Switzerland, except for tests with linalool, which were performed at a second *C. cunicularius* population site at Fussach, in western Austria. Dead male bees, made odorless by extraction with a mixture of hexane and dichloromethane for 24 hr, were used as dummies. For each 3-min test, 70 μl of the head extracts and 200 μl of the cuticle extracts were applied to a dummy. After the solvent had evaporated, the dummy was placed in a male patrolling area. Behavioral responses of *C. cunicularius* males were classified into two categories: (1) approach, a zig-zagging or undulating approach towards the scented source and (2) contact, either a short pouncing contact with the scented source or a longer contact involving copulatory behavior. To control for the effect of visual and tactile stimuli alone, odorless dummies were tested after every fifth test.

Bioassays with synthetic compounds were performed during the 2004 season using cylindrical, black plastic beads as dummies rather than dead, odorless males, but otherwise with unchanged conditions. A subtractive design was chosen to test the relative importance of various mixtures of active compounds. However, mixtures of only the 12 most abundant compounds were used because not all compounds were available as synthetic substances. The following mixtures were prepared and their attractiveness tested in comparison to controls: (1) 12 active compounds, (2) active alkenes, (3) active alkanes,

(4) active (*Z*)-7-alkenes. Synthetic blends were tested that matched the mean relative amounts of compounds found in virgin females at the Neuhausen *C. cunicularius* population. The absolute amount used corresponded to that found in one individual female bee. One hundred microliter solutions of each blend were applied to the dummies. In addition, to investigate the behavioral effects of linalool, the attractiveness of linalool alone was tested and in a mixture including all 12 active compounds, using synthetic racemic linalool (1 μg).

Statistical Analysis. To compare the relative amounts of odor compounds among groups Kruskal–Wallis tests were employed because variances were not homogeneous among groups. Mann–Whitney *U* tests were used for *a posteriori* multiple comparison with a Bonferroni correction and the level of significance set to $P = 0.05$ divided by the number of comparisons. To test for differences in male bee responses in the different behavioral experiments, either the *t*-test or Kruskal–Wallis test followed by Mann–Whitney *U* tests with a Bonferroni correction were used, as for the GC data. All calculations, tests, and graphics were performed with the statistical package SPSS 11 (Brosius, 2002).

RESULTS

Attractiveness of Virgin Female Extracts. Head extracts and cuticle extracts elicited similar numbers of approaches by male bees (Figure 2). However, cuticle extracts were more attractive than head extracts as they elicited significantly more "contacts" than head extracts.

Electrophysiology (GC-EAD). Because head extracts elicited few contacts in the behavioral tests, GC-EAD was performed only with cuticle extracts. Using GC-EAD, we detected 22 electrophysiologically active peaks representing 24 compounds in the cuticle extracts of virgin *C. cunicularius* females (Figure 3 and Table 1). Two GC peaks that elicited antennal responses consisted of more than one compound: hexadecanal and isopropyl tetradecanoate (peak 2), and dodecyl tetradecanoate and decyl hexadecanoate (peak 17). As a conservative approach, we treated both compounds found within each peak as active. The most abundant of the 22–24 electrophysiologically active compounds found in the cuticle were straight chain, odd-numbered alkanes and alkenes with 21 to 29 carbon atoms. Other compounds included two esters (compound numbers 2 and 17) three aldehydes (numbers 2, 5, and 13), and a terpene, linalool. The active compound A, which occurred in relatively small amounts, remained unidentified. The strongest reactions from male antennae were elicited by (*Z*)-9 and (*Z*)-7-alkenes and alkanes (Figure 3).

Differences in Volatiles among Female and Male C. cunicularius. Cuticle extracts of virgin and mated females differed with respect to the quantities of several bioactive compounds (Table 1, Figure 4). Virgin female cuticle extracts

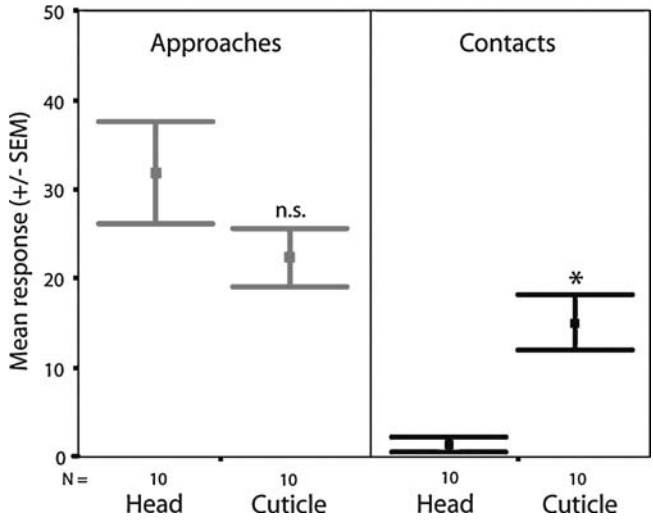

FIG. 2. Mean behavioral responses of *C. cunicularius* males exposed to head and cuticle extracts of virgin *C. cunicularius* females. Error bars are standard errors of means. Cuticle extracts elicited a similar number of approaches as head extracts ($t_{18} = 1.44$, $P = 0.17$), but cuticles elicited more contacts than head extracts ($t_{10,4} = -4.3$, $P < 0.01$).

had significantly more linalool, (*Z*)-7-heneicosene, heptacosane, hexadecanal, and eicosanal than mated female cuticles. Virgin females had less (*Z*)-9-heneicosene, dodecyl tetradecanoate, and decyl hexadecanoate.

Quantities of many compounds that did not elicit antennal responses also differed significantly between virgin and mated female cuticles. Three of these occurred in increased amounts in mated female cuticle extracts, namely, oleic acid (virgin cuticle 0.8% vs. mated female 27%), linoleic acid (0.6% vs. 8.6%), and the unidentified compound U3 (1.2% vs. 12%). These three compounds did not elicit EAD responses, but it should be noted that they were present only in small amounts in the virgin cuticle extracts used in the GC-EAD experiments. As for the cuticle extracts, head extracts of virgins contained significantly more linalool and (*Z*)-7-heneicosene than head extracts of mated females.

In male cuticles, the most abundant compounds were alkanes with 21 to 29 carbon atoms and (*Z*)-7-heneicosene. In particular, the EAD-active compounds heneicosane, tricosane, and (*Z*)-7-heneicosene occurred in relatively high amounts (Table 1, Figure 4). Other EAD-active compounds occurred in extracts from males in only minor amounts.

Differences in Odor between C. cunicularius and O. exaltata. All 22 compounds detected in cuticle extracts of *C. cunicularius* that elicited EAD

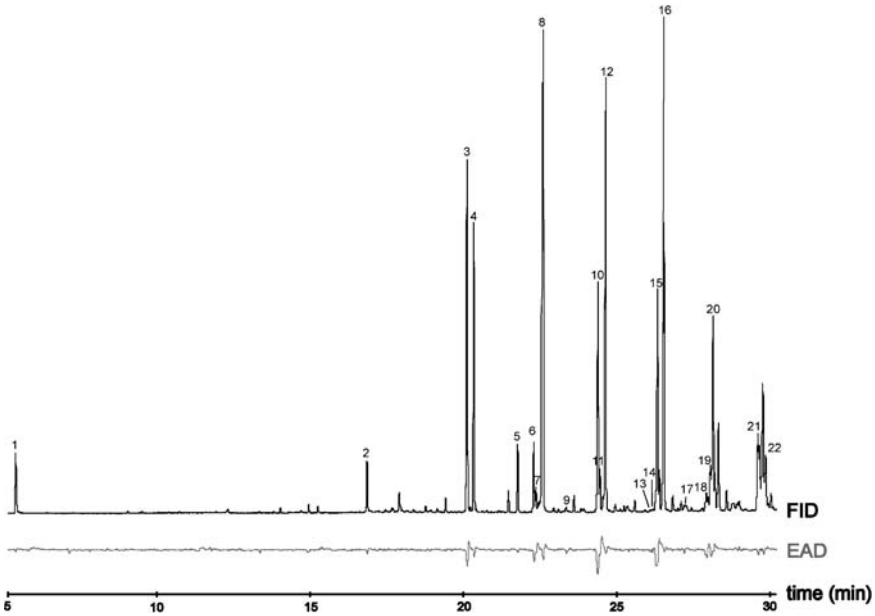

FIG. 3. Gas chromatographic analysis with electroantennographic detection (GC-EAD) of a cuticle extract of a virgin *C. cunicularius* female. Flame ionization detector and EAD responses were simultaneously recorded using an antenna of a *C. cunicularius* male. Numbered peaks correspond to compounds eliciting EAD responses. Compounds are listed in Table 1. Twelve GC-EAD runs were carried out with six males and the reproducibility of all responses at the same retention times was confirmed.

responses were found in the labellum extracts of *O. exaltata* subsp. *archipelagi* (Table 1, Figure 5). However, most of the compounds differed in their relative amounts between orchid and bee extracts (Figure 5). In particular, the (*Z*)-7-alkenes (C21, C23, C25) occurred in higher amounts in *O. exaltata* than in *C. cunicularius* females and, to a lesser extent, eicosanal and tetracosanal.

Behavioral Tests Using Synthetic Compounds. The first bioassay showed that linalool attracts patrolling males without eliciting corresponding contacts (pounces or attempted copulations) with the artificial odor source (Figure 6) (all tests using Mann–Whitney *U* test, with $P < 0.01$). The blend of 12 EAD-active odor compounds attracted a similar number of males to linalool alone, but elicited more contacts. When linalool was combined with the blend of active compounds, the number of males attracted was not different from the number attracted by linalool alone, but the number of contacts was higher than with the blend or linalool alone.

TABLE 1. MEAN RELATIVE AMOUNTS (± SD) OF COMPOUNDS IN EXTRACTS OF *C. cunicularius* AND *O. exaltata*[1]

			Female					
			Cuticle		Head		Male	*Ophrys*
		Source[2]	Virgin	Mated	Virgin	Mated	Cuticle	Labellum
Active compounds[3]								
1*	Linalool	A	1.46 ± 1.63[a]	0.04 ± 0.03[b]	29.35 ± 19.3[c]	13.21 ± 9.16[d]	0.79 ± 0.72[a]	0.10 ± 0.60[e]
2	Hexadecanal and isopropyl tetradecanoate	C	0.59 ± 0.28[a]	0.18 ± 0.26[b]	0.10 ± 0.05[b]	0.05 ± 0.03[d]	1.27 ± 1.55[c]	0.97 ± 0.67[c]
3*	(*Z*)-7-Heneicosene	D	6.79 ± 2.82[a]	0.11 ± 0.09[b]	1.45 ± 0.92[d]	0.43 ± 0.37[e]	38.59 ± 21.50[c]	9.14 ± 5.93[a]
4*	Heneicosane	A	6.86 ± 2.75[a]	7.16 ± 5.76[ab]	1.89 ± 0.93[d]	2.87 ± 2.07[bd]	24.63 ± 10.51[c]	4.48 ± 2.25[b]
5	Eicosanal	B	1.19 ± 0.37[a]	0.31 ± 0.58[b]	0.92 ± 0.61[d]	1.38 ± 0.93[acde]	2.49 ± 1.46[c]	2.66 ± 1.89[e]
6*	(*Z*)-9-Tricosene	A	2.62 ± 2.69[a]	4.61 ± 3.21[b]	1.34 ± 1.26[d]	2.12 ± 1.23[ab]	0.14 ± 0.13[c]	1.08 ± 0.79[d]
7*	(*Z*)-7-Tricosene	D	0.92 ± 0.85[a]	1.27 ± 1.16[a]	0.53 ± 0.40[ac]	0.64 ± 0.30[ac]	0.30 ± 0.09[c]	15.04 ± 8.18[e]
8*	Tricosane	A	27.63 ± 6.16[a]	28.16 ± 6.33[a]	5.27 ± 3.32[c]	7.04 ± 3.45[c]	16.44 ± 5.64[b]	14.76 ± 6.44[b]
9	(*Z*)-9-Tetracosene	D	0.34 ± 0.21[a]	0.56 ± 0.46[ab]	2.01 ± 2.23[d]	0.50 ± 0.33[b]	0.06 ± 0.02[c]	0.30 ± 0.40[e]
10*	(*Z*)-9-Pentacosene	D	9.85 ± 5.50[a]	10.83 ± 7.80[ab]	4.16 ± 3.59[d]	5.55 ± 5.20[bd]	1.16 ± 0.52[c]	2.83 ± 2.50[d]
11*	(*Z*)-7-Pentacosene	D	2.13 ± 1.49[a]	3.73 ± 4.38[a]	0.92 ± 0.73[c]	0.95 ± 0.84[ac]	0.08 ± 0.10[b]	28.54 ± 16.70[d]
12*	Pentacosane	A	12.52 ± 3.73[a]	14.55 ± 5.60[a]	2.51 ± 1.44[c]	4.11 ± 1.97[d]	8.11 ± 2.91[b]	8.27 ± 5.20[b]
13	Tetracosanal[4]	B	0.12 ± 0.12[a]	0.11 ± 0.12[a]	0.23 ± 0.26[ab]	0.20 ± 0.34[abc]	0.06 ± 0.04[b]	1.03 ± 0.54[c]
14	A		0.04 ± 0.07[a]	0.05 ± 0.05[abc]	0.05 ± 0.09[abd]	0.13 ± 0.10[c]	0.00 ± 0.00[d]	0.10 ± 0.12[c]
15*	(*Z*)-9-Heptacosene	D	7.40 ± 4.36[a]	7.02 ± 4.31[ab]	3.02 ± 2.71[de]	3.36 ± 2.28[be]	0.44 ± 0.20[cd]	3.05 ± 2.99[e]
16*	Heptacosane	A	7.45 ± 3.07[a]	5.31 ± 2.23[b]	1.80 ± 0.81[c]	1.51 ± 0.83[c]	4.90 ± 1.83[b]	3.38 ± 3.31[d]
17	Dodecyl tetradecanoate and decyl hexadecanoate[5]	C	0.35 ± 0.21[a]	0.50 ± 0.24[b]	0.39 ± 0.36[abd]	0.31 ± 0.32[ad]	0.04 ± 0.04[c]	0.35 ± 0.47[d]
18	(8*Z*, 20*Z*)-Nonacosadiene[6]	E	0.71 ± 0.44[a]	0.98 ± 0.86[a]	0.79 ± 0.54[a]	0.60 ± 0.55[ac]	0.07 ± 0.05[b]	0.67 ± 1.28[c]
19	(*Z*)-11-Nonacosene	D	1.54 ± 3.42[a]	2.71 ± 3.14[a]	0.49 ± 0.56[c]	1.04 ± 1.25[abc]	0.03 ± 0.02[b]	0.65 ± 1.24[c]
20*	(*Z*)-9-Nonacosene	D	5.13 ± 2.69[a]	5.89 ± 4.06[ab]	2.17 ± 1.52[d]	2.83 ± 2.22[bde]	0.15 ± 0.18b[c]	1.23 ± 1.64[e]
21*	(8*Z*, 20*Z*)-Hentriacontadiene	E	2.73 ± 1.46[a]	2.97 ± 1.51[a]	1.99 ± 1.11[a]	1.69 ± 0.93[a]	0.09 ± 0.08[b]	0.81 ± 1.79[c]
22	(*Z*)-9-Hentriacontene	D	1.62 ± 1.95[a]	2.93 ± 2.42[ab]	1.14 ± 0.93[ad]	1.71 ± 1.67[abd]	0.15 ± 0.07[c]	0.57 ± 1.07[e]

Nonactive compounds[7]								
23	Docosane	A	5.22 ± 2.00^{a}	3.82 ± 1.71^{ab}	1.29 ± 0.79^{d}	2.32 ± 1.43^{bde}	6.58 ± 1.06^{c}	3.72 ± 1.84^{be}
24	(*Z*)-5-Tricosene	D	2.18 ± 0.90^{a}	1.16 ± 0.73^{b}	0.20 ± 0.26^{c}	0.26 ± 0.22^{cd}	2.12 ± 0.39^{a}	0.79 ± 3.31^{d}
25	(*Z*)-3-Tricosene	D	4.18 ± 2.82^{a}	3.02 ± 7.94^{b}	0.39 ± 0.61^{c}	0.21 ± 0.15^{cd}	1.97 ± 0.23^{b}	0.45 ± 0.27^{c}
26	Tetracosane	A	3.90 ± 1.36^{a}	2.52 ± 0.83^{b}	0.23 ± 0.12^{c}	0.31 ± 0.15^{c}	4.14 ± 0.67^{a}	6.43 ± 3.22^{d}
27	(*Z*)-5-Pentacosene	D	7.04 ± 8.77^{a}	0.25 ± 0.67^{b}	0.33 ± 0.25^{d}	0.06 ± 0.15^{b}	0.81 ± 0.37^{c}	10.02 ± 21.98^{e}
28	Hexacosane	A	2.82 ± 0.82^{a}	1.67 ± 0.85^{b}	0.21 ± 0.12^{c}	0.24 ± 0.15^{c}	3.06 ± 0.35^{a}	1.54 ± 1.20^{b}
29	(*Z*)-7-Heptacosene	D	14.16 ± 8.14^{a}	14.07 ± 13.86^{ab}	0.69 ± 0.53^{c}	1.07 ± 1.19^{c}	3.55 ± 2.25^{b}	22.76 ± 15.56^{b}
30	Octacosane	A	1.74 ± 0.89^{a}	0.81 ± 0.51^{b}	0.47 ± 0.35^{d}	0.29 ± 0.38^{d}	2.23 ± 0.21^{c}	1.39 ± 0.95^{e}
31	(*Z*)-7-Nonacosene	D	7.86 ± 3.73^{a}	6.32 ± 5.40^{ab}	0.37 ± 0.30^{c}	0.69 ± 0.45^{c}	3.42 ± 1.68^{b}	6.61 ± 7.99^{b}
32	Nonacosane	A	27.53 ± 9.21^{a}	7.53 ± 3.08^{b}	1.29 ± 0.70 ^{d}	1.42 ± 0.83^{d}	44.85 ± 3.59^{c}	6.03 ± 5.08^{e}
33	Oleic acid	A	0.81 ± 2.71^{a}	26.96 ± 14.0^{b}	4.27 ± 13.88^{c}	12.74 ± 12.^{b}	0.29 ± 0.11^{a}	0.14 ± 0.18^{d}
34	Linoleic acid	A	0.64 ± 1.07^{a}	8.63 ± 4.43^{b}	1.55 ± 1.12^{c}	5.56 ± 3.89^{b}	1.20 ± 0.29^{c}	1.15 ± 2.62^{d}
35	U3		1.15 ± 1.67^{a}	12.07 ± 5.68^{b}	0.26 ± 0.15^{cd}	2.79 ± 4.21^{a}	0.13 ± 0.16^{c}	0.62 ± 0.58^{d}
36	B		0.54 ± 0.69^{a}	0.30 ± 0.54^{b}	0.12 ± 0.08^{c}	0.06 ± 0.07^{d}	0.39 ± 0.08^{a}	1.91 ± 1.22^{e}
37	C		4.77 ± 2.42^{ab}	1.00 ± 0.51^{c}	0.50 ± 0.24^{d}	0.18 ± 0.08^{e}	5.76 ± 1.01^{a}	3.87 ± 1.71^{b}
38	D		2.43 ± 1.14^{a}	3.47 ± 1.69^{b}	0.59 ± 0.27^{c}	1.17 ± 1.22^{c}	4.93 ± 2.29^{b}	22.88 ± 16.17^{d}
39	E		1.59 ± 0.99^{a}	0.89 ± 0.43^{b}	0.33 ± 0.33^{d}	0.37 ± 0.22^{d}	2.17 ± 0.29^{c}	5.63 ± 3.71^{e}
40	F		1.43 ± 0.58^{a}	0.45 ± 0.39^{b}	0.29 ± 0.23^{bd}	0.56 ± 0.28^{b}	9.94 ± 2.78^{c}	0.60 ± 0.33^{b}
41	G		1.84 ± 0.80^{a}	1.33 ± 1.37^{b}	0.21 ± 0.25^{d}	0.20 ± 0.12^{d}	0.37 ± 0.09^{c}	0.33 ± 0.36^{c}
42	H		1.46 ± 0.82^{a}	1.20 ± 0.69^{a}	0.24 ± 0.18^{c}	0.45 ± 0.27^{bc}	0.58 ± 0.36^{b}	0.58 ± 1.65^{c}
43	I		4.24 ± 1.87^{a}	1.10 ± 0.49^{b}	0.50 ± 0.65^{c}	0.24 ± 0.21^{c}	0.91 ± 0.66^{b}	0.48 ± 0.53^{c}
44	J		2.48 ± 1.28^{a}	1.43 ± 1.33^{b}	0.17 ± 0.18^{c}	0.15 ± 0.19^{c}	0.59 ± 0.34^{b}	2.06 ± 4.43^{b}

[1] Different letters indicate significant differences between groups as calculated by a Mann–Whitney *U* test, $P < 0.003$.
[2] Letters refer to the sources of reference compounds as described in Methods and Materials. Peak numbers 2 and 17 represent two compounds, with the second mentioned compound occurring in trace amounts.
[3] Compounds arranged according to retention time. *Indicates compounds used for bioassays.
[4] Corresponding peak in cuticle of virgin females is not tetracosanal.
[5] In postcopulatory females: hexadecyl decanoate (and decyl hexadecanoate).
[6] Identified in *Ophrys*, in mated females: mix of (7,15)-, (7,17)-, (7,20)-nonacosadienes (geometry of double bonds not determined).
[7] A selection of abundant nonactive compounds found in the extracts of *C. cunicularius*.

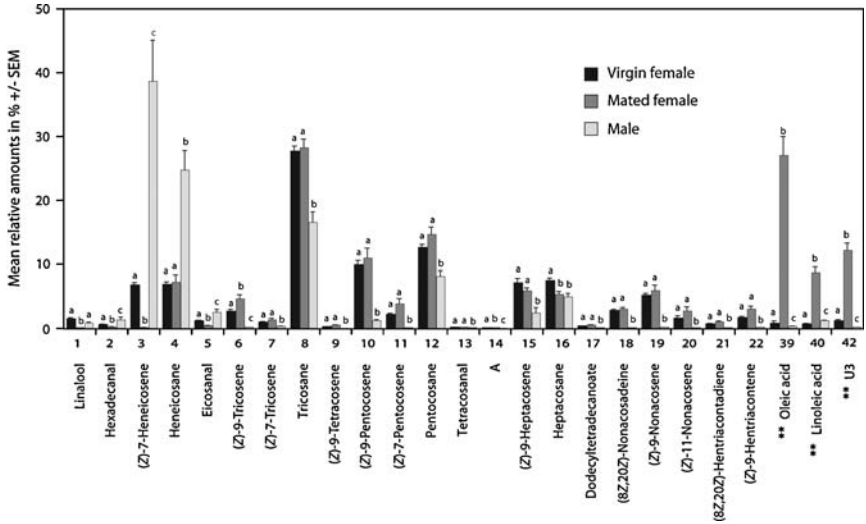

FIG. 4. Relative mean amounts of EAD-active compounds in cuticle extracts of males and virgin and mated females of *C. cunicularius*. Error bars are standard errors of means. Significant differences between groups for one compound are indicated by different letters according to Mann–Whitney *U* test. For compounds numbers 2 and 17 please compare Table 1 for details.

Bioassays using various synthetic blends (Table 2) showed that the mixture of all 12 EAD-active hydrocarbons and the (*Z*)-7-alkenes induced similar numbers of attractions and contacts (Mann–Whitney *U* test, with $P < 0.005$, Figure 7). Behavioral responses to the mixture containing only alkenes were not different from the responses shown to (*Z*)-7-alkenes, but elicited fewer attractions or contacts than the 12-compound blend. Responses to alkanes were equivalent to responses to controls.

DISCUSSION

This study has shown that sex pheromone components of *C. cunicularius* are associated with the cuticle of virgin females and are also present in head extracts. GC-EAD experiments demonstrated that electrophysiologically active compounds include linalool and straight-chain, odd-numbered alkenes and alkanes with 21 to 29 carbon atoms. The attraction of males to a subset of these compounds was demonstrated in tests using synthetic compounds, confirming their function as attractant pheromones.

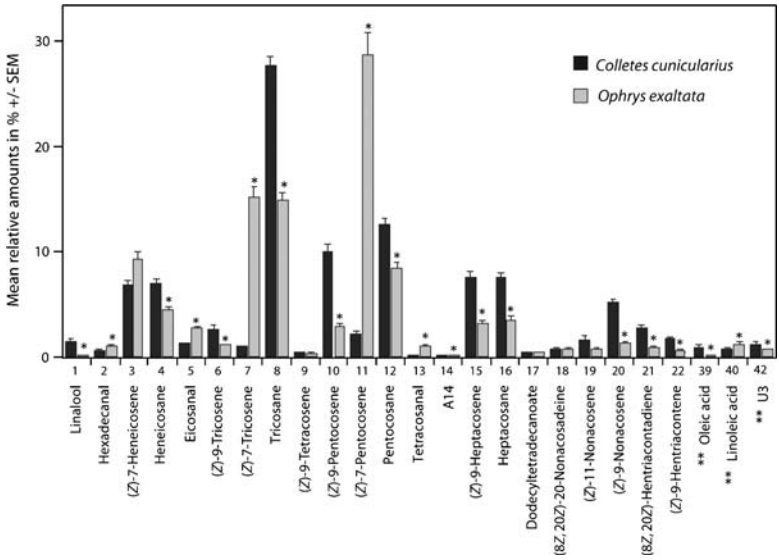

FIG. 5. Relative mean amounts of EAD-active compounds in cuticle extracts of virgin *C. cunicularius* females and labellum extracts of *O. exaltata*. Error bars are standard errors of means. Asterisk indicates significantly different relative amounts (see Table 1).

Linalool has previously been shown to have a sex pheromone function in *C. cunicularius* by its stimulation of an immediate increase in flight activity among patrolling males (Bergström and Tengö, 1978; Cane and Tengö, 1981; Borg-Karlson et al., 2003). Our study confirmed these earlier results by showing the attraction of patrolling males to synthetic linalool and to head extracts that contained high relative amounts of linalool. However, our behavioral tests also indicated that linalool alone rarely releases full mating behavior in males, consistent with previous findings (Cane and Tengö, 1981; Borg-Karlson et al., 2003). Head extracts containing high amounts of linalool elicited few contacts with the odor source, whereas this was frequently induced by cuticular extracts. Yet, more contacts were recorded with a mix of linalool and the blend of 12 active hydrocarbons than recorded with the blend of hydrocarbons or linalool alone. Presumably, the high amount of linalool in head extracts attracts patrolling males, whereas the relatively low amounts of other physiologically active compounds fail to trigger full mating behavior. These results lead us to support the contention of Cane and Tengö (1981) that linalool functions as a long-range sex attractant that enhances the onset of mating, whereas the presence of physiologically active cuticular hydrocarbons is necessary to induce copulatory behavior.

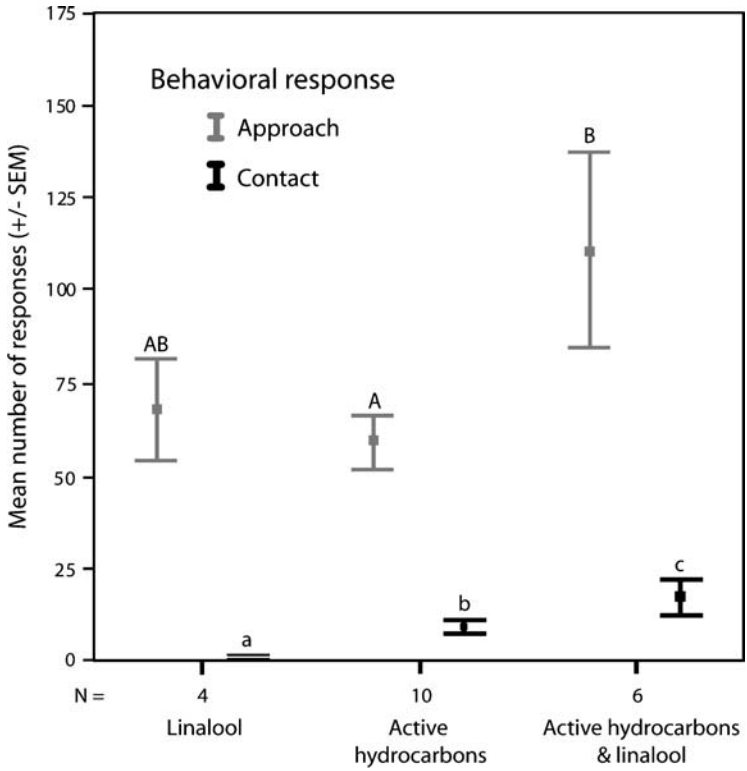

FIG. 6. Behavioral responses of *C. cunicularius* males to racemic linalool and a blend of EAD-active alkanes and alkenes (see Table 2 for blend composition). Different letters indicate significant differences among the test groups for "approaches" and "contacts" separately.

The behavioral tests with various subsets of the EAD-active compounds showed considerable differences in attractiveness to males. The most complete mixture of 12 compounds and the (*Z*)-7-alkenes were most attractive, followed by the blend of alkenes. The mixture of alkanes was not attractive (Figure 7). Interestingly, the mixture of 12 active compounds was more attractive than the full alkene blend, which suggests a synergistic effect among alkanes and alkenes, because the alkanes alone were not attractive. However, in our tests, there was a trend for the blend containing only (*Z*)-7-alkenes to be more attractive than the full alkene blend, suggesting that certain alkenes may inhibit the behavioral activity of the (*Z*)-7-alkenes. More behavioral tests are needed to confirm this. Generally, our results are similar to those from behavioral tests

TABLE 2. ABSOLUTE AMOUNTS (μg) OF SYNTHETIC COMPOUNDS USED FOR BIOASSAYS

Compound	*Colletes*[a]	12-Compound blend	(*Z*)-7-Alkenes	All alkenes	All alkanes
1. (*Z*)-7-Heneicosene	0.53	0.50	0.51	0.31	–
2. Heneicosane	0.53	0.60	–	–	0.51
3. (*Z*)-9-Tricosene	0.20	0.18	–	0.12	–
4. (*Z*)-7-Tricosene	0.08	0.09	0.08	0.18	–
5. Tricosane	2.29	2.37	–	–	1.77
6. (*Z*)-9-Pentacosene	0.80	0.86	–	0.52	–
7. (*Z*)-7-Pentacosene	0.18	0.21	0.20	0.10	–
8. Pentacosane	1.02	1.01	–	–	0.84
9. (*Z*)-9-Heptacosene	0.60	0.63	–	0.36	–
10. Heptacosane	0.58	0.64	–	–	0.54
11. (*Z*)-9-Nonacosene	0.41	0.35	–	0.21	–
12. (*Z*)-8-(*Z*)-20-Hentriacontadiene	0.21	0.12	–	0.12	–

[a] Virgin female

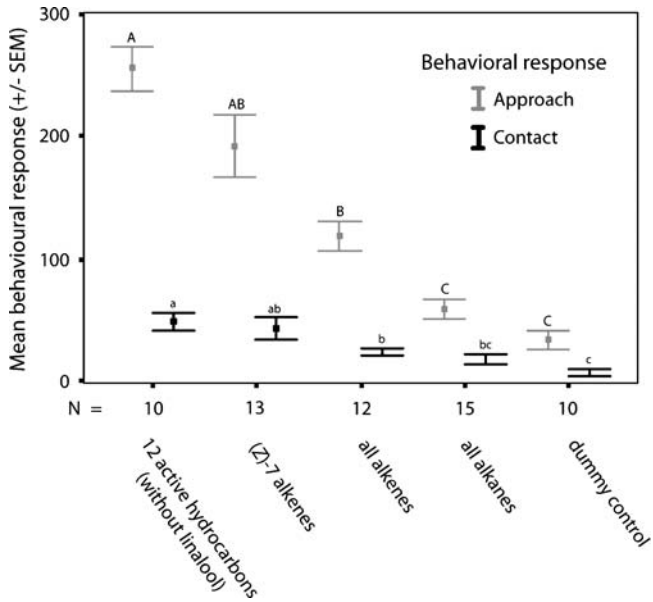

FIG. 7. Behavioral responses of *C. cunicularius* males to different blends of EAD-active compounds (see Table 2 for blend compositions). Different letters indicate significant differences among the synthetic mixtures for "approaches" and "contacts" separately.

with *A. nigroaenea* in which blends of alkenes triggered approaches and contacts, whereas blends of alkanes were unattractive (Schiestl et al., 2000).

Avoidance of mated females by males has been suggested to be mediated by olfactory cues (Cane and Tengö, 1981). In the solitary bee, *A. nigroaenea*, the avoidance of mated females by patrolling males is due to an increase in farnesyl hexanoate, an antiaphrodisiac compound released from the Dufour's gland of mated females (Schiestl and Ayasse, 2000). The repellent compounds are also produced in pollinated flowers of *O. sphegodes* and may function to guide pollinators to unpollinated flowers within an inflorescence, thus increasing the reproductive fitness of the plant (Schiestl et al., 1997; Schiestl and Ayasse, 2001). In *C. cunicularius*, three compounds that increased markedly in cuticle extracts of mated females may also function as repellent or deterrent compounds, namely, oleic acid, linoleic acid, and the unidentified U3 (Figure 4). However, behavioral tests with these compounds have yet to be performed, and we did not detect male antennal responses to them.

The drop in male response to mated females could also be due to a reduction in the compounds that comprise the sex pheromone. Of the physiologically active compounds in cuticle extracts, linalool, (*Z*)-7-heneicosene and eicosanal, and to a lesser extent, hexadecanal and isopropyl tetradecanoate, decreased in mated females. Of these compounds, only linalool and (*Z*)-7-heneicosene showed a strong decrease also in head extracts of mated females in comparison to virgin ones. Thus, our behavioral tests, by highlighting the relative importance of (*Z*)-7-alkenes and linalool (Figures 6 and 7), support the suggestion that a reduction in (*Z*)-7-heneicosene and linalool contributes to a reduction in responses of males to mated females. Of note is that these two compounds are also present among the volatiles of males, with linalool being the most abundant compound in head extracts of males (Borg-Karlson et al., 2003) and (*Z*)-7-heneicosene the most abundant compound in cuticle extracts of males (Table 1). The observation that patrolling males were attracted to preemergent males as well as to preemergent females (Cane and Tengö, 1981) may be due to the production of (*Z*)-7-heneicosene and linalool by males.

Behavioral observations have shown that the orchid *O. exaltata* mimics the sex pheromone of *C. cunicularius* in a sexual deceit that leads to effective pollination by male bees (Kullenberg, 1961; Paulus and Gack, 1990b; Mant et al., 2005). Patrolling males are attracted to and attempt copulation with hexane extracts of the orchid labellum on dummies (Cane and Tengö, 1981; Mant et al., 2005). Our comparison of the labellum extracts of *O. exaltata* subsp. *archipelagi* and the cuticle extracts of virgin female *C. cunicularius* revealed all physiologically active compounds to be present in both organisms. This result is in accord with those obtained with the sexually deceptive *O. sphegodes* and its pollinator, *A. nigroaenea* (Schiestl et al., 2000). Both systems are consistent with the expectation of chemical mimicry in *Ophrys* pollination.

However, the relative amounts of 19 of the 22 active compounds differed significantly between our *O. exaltata* and *C. cunicularius* extracts. In particular, *O. exaltata* showed higher relative amounts of (*Z*)-7-heneicosene, (*Z*)-7-tricosene, and (*Z*)-7-pentacosene, which we have shown to be key components of the sex pheromone of female *C. cunicularius* (Figure 5). The odor differences in the present study may be due to the geographic distance between the sampled populations of *Colletes* and *Ophrys*, the former from northern Switzerland, the latter from southern Italy (Mant et al., 2005). However, the pattern is also indicative of directional selection for higher production of key odor components, leading to a supernormal stimulus by the orchid, as suggested by Schiestl (2004) and Ayasse et al. (2003).

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INTERACTIONS BETWEEN ACETOIN, A PLANT VOLATILE, AND PHEROMONE IN *Rhynchophorus palmarum*: BEHAVIORAL AND OLFACTORY NEURON RESPONSES

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Abstract—Aggregation of *Rhynchophorus palmarum* weevils on host plants is mediated by a male pheromone (rhynchophorol: R) and host-plant volatiles (PVs) acting in synergy. Synthetic PV blends synergizing pheromone contain acetoin (A) and ethyl acetate (EtAc). R, A, and EtAc are detected by specialized olfactory receptor neurons (ORNs). In addition, particular types of ORNs are tuned to both A and R. To specify the role played by acetoin in pheromone perception, we recorded the responses of ORNs to 100 ng of A or R presented either separately or mixed. Behavioral responses to R, A, and EtAc were studied in a four-armed olfactometer and by field trapping. We screened 59 R-, A-, and AR-tuned ORNs by recording specific responses to odors presented either separately or mixed. Stimulations by blends elicited complex response profiles from the three ORN types: some gave synergistic responses, others were inhibited, and the remainder responded as though both odors were detected independently. Several gave either a weak or no response to a first stimulation by R, but responded clearly to a second stimulation after an intercalary stimulation by A. In the olfactometer, both sexes were more attracted to a blend of A + R (1 + 0.01 ng/sec) than to pure compounds, whereas EtAc did not enhance response to R. Pheromone-baited traps (1 mg/day) containing PV blends (650 mg/day) based on an ethanol/EtAc blend (1:1), plus either 5 or 10% A, or a more complex reference blend, or sugarcane (natural pheromone synergist), caught similar numbers of weevils and about twice as many insects as a control ethanol/EtAc blend. Traps with only pheromone caught about 10 times fewer insects. Behavioral results support the role of acetoin as a

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pheromone synergist for *R. palmarum*, and electrophysiological data provide evidence of modulation of peripheral sensory responses to pheromone by acetoin. Sexual dimorphism was observed neither at the ORN nor at the behavioral levels.

Key Words—Coleoptera, *Rhynchophorus*, aggregation pheromone, acetoin, ethyl acetate, synergy, mixture effect, single sensillum recording, EAG, olfactometer, field trapping.

INTRODUCTION

Detection of pheromones and plant odors by insects is generally considered to operate through independent pathways in the olfactory system (Mustaparta, 1984; Hansson et al., 1989, 1999). However, the modulation of behavioral responses to pheromones by plant compounds has been reported in an increasing number of species (Landolt and Phillips, 1997). Thus, the distinction between processing signals from host plants and from conspecifics might be neither ecologically nor physiologically relevant.

Palm weevil, *Rhynchophorus palmarum*, males emit a volatile aggregation pheromone, rhynchophorol: (4*S*)-2-methyl-(5*E*)-hepten-4-ol (Rochat et al., 1991; Oehlschlager et al., 1993). However, in the field, attraction to traps baited with pure rhynchophorol is low, whereas the addition of plant material increases catches considerably (Oehlschlager et al., 1993). The identification of natural volatiles emitted by host plants has resulted in the development of multicomponent synthetic blends that also increase trap catches when mixed with rhynchophorol (e.g., blend G; Rochat et al., 2000) and may be used instead of plant material. Synergy between pheromone and plant volatiles (PVs) was confirmed by analyzing the locomotory responses of *R. palmarum* in a four-choice olfactometer (Saïd, 2003), and field trials identified the components in blend G that were necessary for the synergy with the pheromone (Morin and Ollivier, 2001).

To understand how these compounds were encoded by the olfactory system, the sensory structures in the antennae of *R. palmarum* were characterized morphologically by using scanning and transmission electron microscopy. In addition, the sensitivity and specificity of olfactory receptor neurons (ORNs) to rhynchophorol and related compounds, as well as to components of two PV reference blends, were determined (Saïd et al., 2003). The authors characterized 17 functional types of ORNs common to both sexes, according to their response spectra, and found certain ORNs with narrow tuning and high sensitivity to rhynchophorol. There were also ORNs with specific responses to certain components of PVs, particularly acetoin (3-hydroxy-2-butanone) and ethyl acetate that are released by fermenting sugarcane. Certain

sensilla simultaneously responded to PVs and rhynchophorol with different spike amplitudes, suggesting that they may cohouse ORNs tuned both to the pheromone and to PVs. Furthermore, 9% of the ORNs exhibited more complex responses, either coactivated by rhynchophorol and acetoin, or activated by rhynchophorol while inhibited by blend G, a mix of PVs.

In this study, we investigated the interaction between plant and insect volatiles using behavioral and neurophysiological approaches. We compared the responses of single ORNs to either rhynchophorol or acetoin as well as different blends of the two. In addition, we conducted assays to examine the responses of the insects to correlate the peripheral sensory coding of the signals with the synergistic effect on behavior. We focused on acetoin for two reasons: it was present in PV blends that synergized with the pheromone in field trials, and some of the ORNs that detected acetoin were also coreceptive to rhynchophorol.

METHODS AND MATERIALS

Insects. Adult *R. palmarum* were caught in French Guyana using traps baited with synthetic rhynchophorol and sugarcane. Males and females were separated and maintained, with a supply of sugarcane, at 23–28°C, RH 75–90%, 13:11-hr light–dark period. Unless otherwise stated, the number of males and females used in any given experiment was similar, as both sexes respond similarly to the aggregation pheromone and the PVs (Rochat et al., 1991, 2000; Saïd, 2003).

Chemicals. Chemicals used in the laboratory and field studies were purchased from the following sources: (1) a racemic blend (99% purity) of rhynchophorol (R) from EGNO-Chimie (Tancarville, France); (2) acetoin (A), a blend of enantiomers and *meso* form (97%) from Fluka (Switzerland); (3) ethyl acetate (EtAc) (99%) from Merck (Germany); and (4) 96° ethanol (99% purity) from Carlo Erba Reagenti (Italy). A PV blend (blend G) was prepared following the recipe of Rochat et al. (2000). All chemicals were stored below 8°C before use.

A and R were diluted in high-grade hexane. A + R blends (10, 25, 50, 75, and 90% R at a total concentration of 100 ng/μl) were prepared for single sensillum recordings. Decadic dilutions of a 50:50 A + R blend were prepared for electroantennography (EAG). Solutions were kept at −18°C between experiments.

Electroantennography. Excised antennae were fixed on a Plexiglas plate, and the EAGs were measured using glass microelectrodes filled with saline solution and connected to chloridized silver wires. The reference and recording electrodes were inserted into the scape and antennal club, respectively. Prior to stimulation, 1 μl of one stimulus was applied to a filter paper strip in a Pasteur

pipette. The pipette was connected to the airstream tube using an intermediary Teflon piece (6 cm long, one per pipette) to reduce contamination by odors. A new stimulus pipette was prepared before each experiment, and one with pure hexane was used as the control stimulus.

Antennae of six weevils were stimulated (0.5 sec; 20 ml/min) with pure air and solvent, A, R, and a 50:50 A + R blend, over a range of decadic doses from 1 pg to 1 μg. For a given dose, the compounds were delivered in a random order.

Data Analyses. For each antenna, amplitudes were normalized relative to the mean response in the series. Then, the mean relative responses to A + R were compared with that of pure R as well as to the arithmetic sum of the responses to half doses of A and R (interpolated from the experimental dose–response curves) using paired *t*-tests.

Single Cell Recordings. The activity of ORNs was recorded with tungsten microelectrodes electrically sharpened in a 10% KOH–$NaNO_2$ solution (Saïd et al., 2003). The indifferent electrode was inserted into the scape. The recording electrode was brought randomly into contact with the surface of the antenna club and gently pressed with a micromanipulator until action potentials were recorded. As in previous experiments, we used excised antennae rather than taking *in situ* recordings, as the movement of live insects impeded stable recordings of ORN activity. A flow of humidified, charcoal-filtered, air (40 ml/min) was directed constantly onto the antenna. When recording of firing activity was stable, the antenna was stimulated by puffing air (0.5 sec, 20 ml/min) into the permanent airflow through a Pasteur pipette loaded with the stimulus. There was >1 min between stimuli, during which a puff of fresh air was applied, so that neurons could return to basal activity. In some cases, when an unexpected response was recorded, stimulations were replicated to verify the repeatability or reversibility of the observed phenomena (see Results).

Signals were amplified (×1000), filtered (low pass 10 Hz; high pass 15 kHz) and digitized at 10 kHz, 12 bits. Acquisition, display, and analysis were performed with AWAVE, an application hosted by Microsoft Windows, and developed in visual C++ (Marion-Poll, 1995, 1996). Olfactory receptor neuron responses were expressed as numbers of action potentials, sorted according to shape and amplitude, emitted during 1 sec after the onset of the stimulation.

First Series. Olfactory receptor neurons were stimulated successively with 100 ng R (R_i: initial stimulation), 100 ng A, 100 ng A plus 100 ng R (obtained by applying 1 μl of 100-ng/μl solutions of each on the same filter paper), and finally, 100 ng R (R_f: final stimulation).

Second Series. Olfactory receptor neurons were stimulated successively with 10 ng R, 100 ng A, and then with 100 ng of 90:10, 75:25, 50:50, 25:75, and 10:90 A/R blends. Finally, the preparation was stimulated with a puff of 100 ng R. All preparations were stimulated in this same order. The sex ratio of this series was 2:1 male/female.

Data Analyses. The number of spikes fired during 1 sec before (r_0) and 1 sec after (r_{stim}) the onset of stimulation was measured, and the response of a cell was expressed as the difference: $r_{stim} - r_0$. Thus, there could be negative values. In both series of treatments, if a response to a blend differed by >20% from the maximum response observed with a single compound, then it was classed as a mixture interaction, whereas anything less was not. In addition, in the first series, a linear regression model: $rR_f = arR_i$, was used to evaluate the relationship of the initial (rR_i) and final (rR_f) responses to rhynchophorol, testing the hypothesis: $a > 1$, to validate that rR_f was greater than rR_i.

Behavioral Responses in Olfactometer. The responses of male and female *R. palmarum* were tested in a four-armed olfactometer (55 × 55 × 5.5 cm; Laucoin s.a., Thoiry, France), coupled to an olfactory stimulator delivering constant concentrations (R at 10 pg/sec; A and EtAc at 1 ng/sec) of each stimulus from diluted aqueous solutions (Bartelt and Zilkowski, 1998; Saïd, 2003). In the first assay ($N = 20$), the responses to A, R, A + R, or odorless air were compared, whereas in the second ($N = 18$), the stimuli were R, EtAc, EtAc + R, or odorless air. In the third ($N = 22$), the choices were A + R, EtAc + R, 0.5A + 0.5EtAc + R, or odorless air. The complete mixture was positioned between the simple odors in the olfactometer. A weevil was placed in the center of the olfactometer, and its locomotory behavior was monitored for 10 min to determine the first odor source chosen (first arm entered) and the time spent in each arm. All assays were run during the first to fourth hr of the scotophase.

Statistical Analyses. Binomial tests compared the numbers of first choices for each arm to random choice ($P = 0.25$). One-way ANOVAs (insects as blocks), followed by Newman–Keuls tests (multiple comparisons, $\alpha = 0.05$; Dagnelie, 1975), compared the mean time spent in each arm.

Field Trapping. These experiments were carried out on a coconut plantation in Municipio de Santo Amaro (Sergipe, Brazil) between October 24 and December 19, 2003. Trap (≈10 × 60 cm: diam × h) cylinders, with a 10 × 25 cm opening, were made of plastic soda bottles (PET trap: Ferreira et al., 2001). Traps were hung on trees or shrubs about 1 m above ground and 100 m apart. The assay was a complete block design with six treatments that all included rhynchophorol: (1) R alone; (2) R with MAJ [a blend of ethanol and EtAc (1:1 v/v) that served as a solvent to release minor components]; (3) R + A5, a blend of 5% (v/v) acetoin in MAJ; (4) R + A10, a blend of 10% acetoin in MAJ; (5) R + blend G, a mixture of 11% minor components among which acetoin represented 4.3% (Rochat et al., 2000) in MAJ; and (6) R plus six halved 10-cm-long sugarcane pieces. The synthetic PV blends were dispensed from modified 50-ml polyethylene Kartell vials (Italy) with a braided cotton wick, whereas polyethylene sealed sachets were used as pheromone dispensers (Rochat et al., 2000). Each week, the insects were removed and counted; the sugarcane was renewed and traps were rerandomized in the blocks. The experi-

ment was replicated eight times. The mean weekly catches were first analyzed using an ANOVA [GLM procedure on $\ln(x + 1)$ transformed data] and then by Tukey multiple comparison tests at 5% (Minitab, v.12.2).

RESULTS

Electroantennography. Electroantennography responses were greater to rhynchophorol than to acetoin ($t = 4.74$, $df = 42$, $P < 0.001$), whereas the acetoin–pheromone blend gave similar responses as rhynchophorol alone (Figure 1). The dose–response curve to the 1:1 A + R blend was significantly

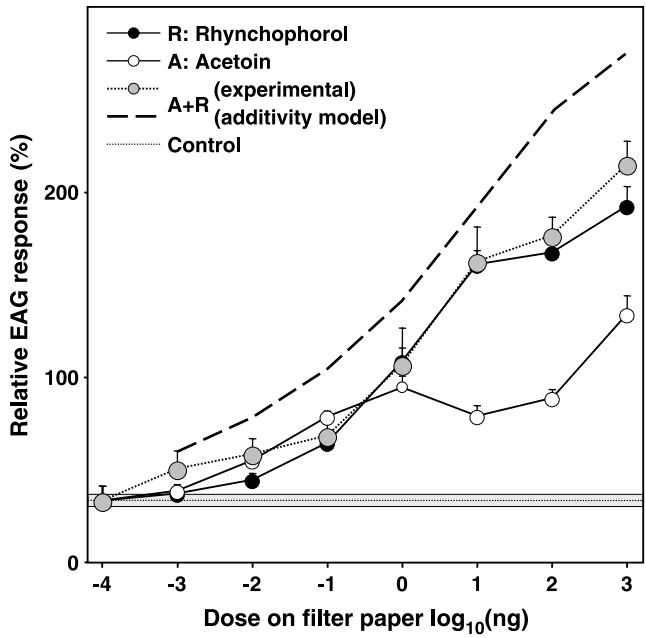

FIG. 1. Electroantennography (EAG) dose–response curves from eight *Rhynchophorus palmarum* antennae (six males + two females) to R (rhynchophorol: aggregation pheromone), A (acetoin: a host plant volatile), and A + R (a blend of both components), in relation to a pure air control and a theoretical response to A + R obtained under the hypothesis of simple additivity of EAG responses to R and to A. The EAG response (m + s.e.) is expressed as a relative EAG value, i.e., the percentage related to the mean response of the antenna to the series of stimulations.

lower than the theoretical curve calculated from the summed responses to the individual compounds (t = 6.79, df = 41, $P < 0.001$). This hypoadditivity suggests that rhynchophorol and acetoin are not detected independently but rather interact at the peripheral level.

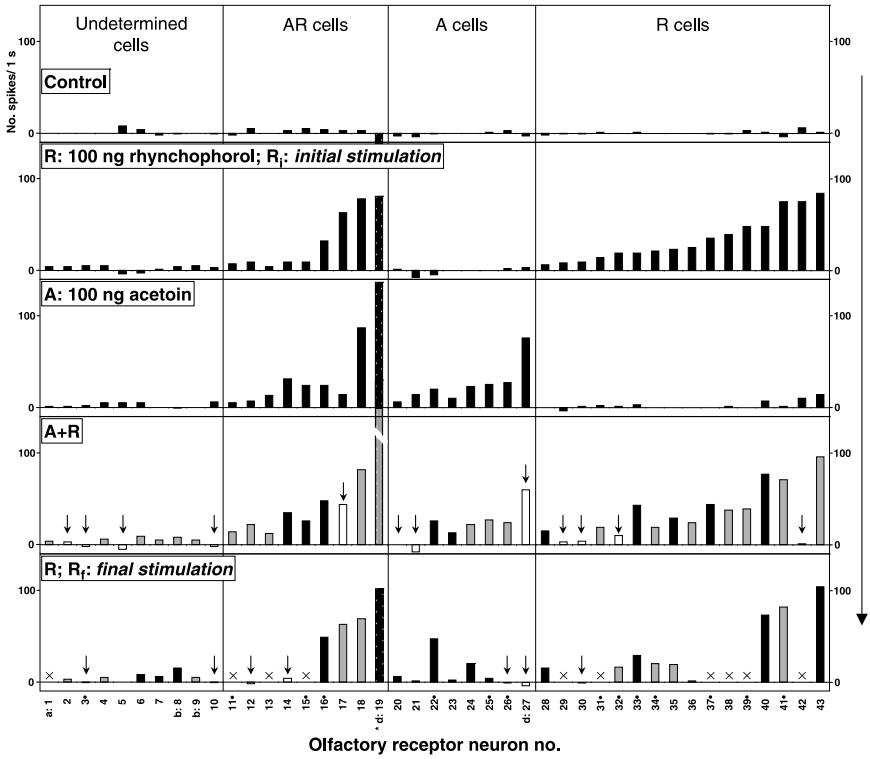

FIG. 2. Response profiles of *Rhynchophorus palmarum* olfactory neurons stimulated by 100 ng R (rhynchophorol: aggregation pheromone) at the beginning (R_i) and the end (R_f) of the series, 100 ng A (acetoin: plant volatile), and A + R (a blend of both components). *X*-axis: numbers of connected neurons. Dots: female antennae. The same letter associated with different neurons indicates firings obtained from the same recording and neurons probably housed in the same sensillum. Stimulations are given in chronological order from top to bottom (right arrow). The strong responses by neuron 19 (*) are shown at a three fourths scale. In the two lower profiles (A + R and R_f stimulations), black bars indicate greater (> 20%) and white bars plus arrow indicate lower (< 20%) neuron responses than the reference responses. Gray bars indicate responses within ±20% of the reference responses. *X* in the lowest profile: no recordings to R_f. The reference response to A + R was the maximum response to A and to R. The reference response to R_f was the response to R_i.

Single Cell Recordings. Of the 73 successful recordings, that showed the activity of one to three neurons, 75% were obtained from males. However, as we observed no sexual dimorphism in the entire set of responses in this or our previous work (Saïd et al., 2003), we present the data for both sexes combined.

Responses to acetoin and/or rhynchophorol were observed in 34 recordings (46%), and these were considered for further analysis (Figure 2). The spike amplitudes allowed us to recognized 43 different cells. The firing from the onset of stimulation ranged from −4 to 84 spikes. There was one exception, neuron 19, which exhibited a stronger activity, from −16 to 240 spikes. The most effective compound for each cell that fired >5 spikes upon stimulation was determined based on whether R or A induced the greatest response (r_{max}), when the relative difference between the responses to both molecules

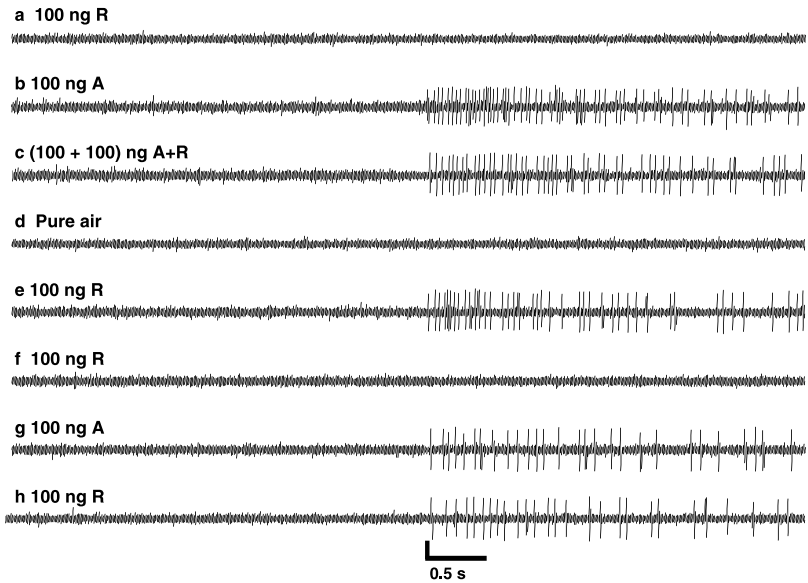

FIG. 3. Extracellular recordings of the activity of an olfactory receptor neuron of *Rhynchophorus palmarum* sensitized to pheromone stimulations (R) by acetoin (A: a plant volatile) in a reversible way. Successive stimulations by (a) R, (b) A, and (c) A + R elicited no response to R and a strong response to A and then to A + R. (d) The response to pure air (control) confirmed the absence of contamination of the preparation. (e) The second stimulation by R induced a response of the neuron similar to that induced by A. (f) The neuron did not respond to a third stimulation of 100 ng R, 3 min later. (g) Then it was resensitized by stimulation by A, and (h) it responded again to R. Scale: horizontal line, stimulation duration; vertical line, 1 mV.

was >25% ($r_{min} < 0.75r_{max}$). Using this criterion, we identified eight A cells and 16 R cells, whereas in nine others that responded to A and R, the relative differences were <25%. As neither the amplitude and shape of the action potentials nor the temporal pattern of firing allowed us to conclude that two different cells were present, we then checked their response to both stimuli using cross adaptation (Saïd et al., 2003). The results suggest that the same cells responded to both compounds and are referred to as AR cells. Neuron 19 was classified as an AR cell, although considering the level of response, two cells might have been involved (Figure 2). The remaining 10 cells were classified as "undetermined," as they fired ≤5 spikes following stimulation by either A or R.

In most recordings (30/34), cells responded to either A or R, indicating that acetoin and rhynchophorol are generally detected by receptor neurons housed in

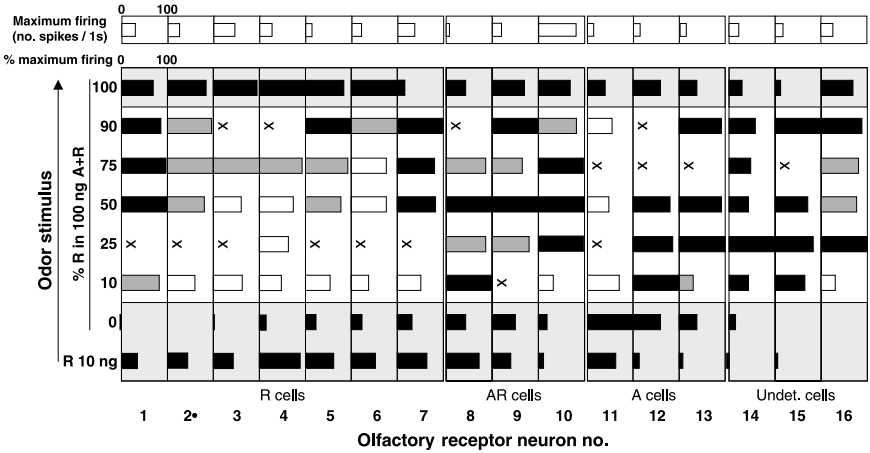

FIG. 4. Response profiles of *Rhynchophorus palmarum* olfactory neurons stimulated by 10 and 100 ng R, 100 ng A and 100 ng A + R, with relative proportions of the two compounds varying from 10 to 90%. The vertical arrow gives the chronology of stimulations. *X*-axis: numbers of connected neurons. Dot: female antenna. *X* in the central profiles: no recordings to the blend. Responses were quantified by the number of spikes emitted during 1 sec after the onset of the stimulation and are presented as the percentage of the highest neuron response (white bars in first row: number of spikes emitted during 1 sec). Central profiles (A + R stimulations): black bars indicate greater (>20%) and white bars plus arrow indicate lower (<20%) neuron responses than the reference responses. Gray bars indicate responses within ±20% of the reference responses. The reference response was the greatest response to 100 ng pure A, to 10 ng pure R, and to 100 ng pure R, shown with a gray background.

different sensilla. However, in four cases, A and R stimulated different cells, as attested by differences in spike amplitude or temporal pattern of firing, suggesting that some acetoin and rhynchophorol neurons may be colocalized in the same sensillum.

Responses to the A + R Blend (100 + 100 ng). Five R cells (nos. 28–43 in Figure 2) responded more to A + R than to rhynchophorol alone (rA + R > 1.2rR; black bars), whereas four responded less (rA + R < 0.8rR; white bars with arrow). The seven others gave similar responses to R and to A + R (0.8rR ≤ rA + R ≤ 1.2rR; gray bars).

Two of the eight A cells (20–27 in Figure 2) showed stronger responses to A + R than to acetoin (rA + R > 1.2rA), and three showed lower responses (rA + R < 0.8rA), whereas the remaining three responded equally to A + R and A (0.8rA ≤ rA + R ≤ 1.2rA). Three of the nine AR cells (11–19 in Figure 2) responded to A + R more than to acetoin or rhynchophorol alone [rA + R > 1.2max(rR, rA)], and one responded less [0.8max(rR, rA) < rA + R]. Responses to A + R by the remaining five cells to the blend were similar to that of the most active pure compound [0.8max(rR, rA) ≤ rA + R ≤ 1.2max(rR, rA)]. The 10 undetermined cells (1–10 in Figure 2) showed a similar ($N = 6$) or lower ($N = 3$) response to A + R than to either A or R alone.

Overall, the response to the second stimulation by rhynchophorol (R_f) after A + R ($N = 33$) was, on average, significantly greater than the first response (R_i) and is described by the following linear relationship: $rR_f = arR_i$ [$r^2 = 0.78$; $F(1, 31) = 198.6$, $P < 0.001$] with a significant slope of 1.091 ± 0.077 ($t = 15.25$,

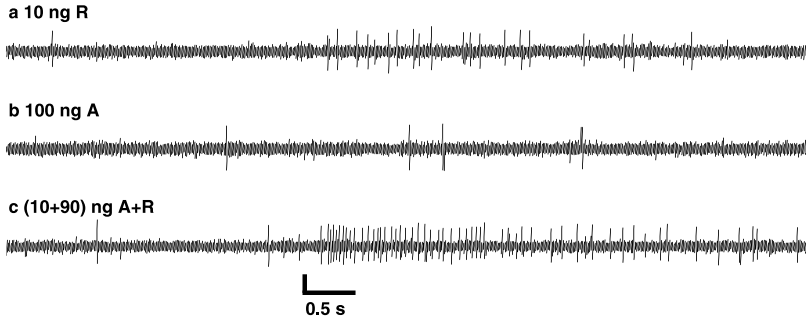

FIG. 5. Extracellular recordings of the synergistic response of *Rhynchophorus palmarum* olfactory receptor neurons to a blend of acetoin (A) and pheromone (R): (a) the high-spike-amplitude neuron responded feebly to 10 ng R and (b) did not respond to 100 ng A. (c) The blend of 10 ng R plus 90 ng A (A + R) induced a strong response of the high-spike-amplitude neuron. Scale: horizontal line, stimulation duration; vertical line, 1 mV. Responses are presented in chronological order.

df = 30, $P < 0.001$). This difference was seen in all cell types, and particularly the A cells (6/9). In 15 of the 33 cells, the second response was >20% higher than the first: $rR_f > 1.2rR_i$ (black bars, Figure 2). In two recordings, this effect was reversible, and the response to rhynchophorol returned to its initial value after a few minutes. However, after a second presentation of acetoin, response to rhynchophorol was restored (Figure 3).

Responses to Different A + R Blend Ratios. We were able to obtain an almost complete set of readings for the A + R blends from 16 neurons. The action potentials to the initial treatments with R (10 ng) and A (100 ng) alone ranged from −1 to 24, whereas the subsequent ones with the A + R blends were from −1 to 81. Using the same criteria mentioned above, we identified seven, three, three, and three R, A, AR, and undetermined type cells, respectively. Most responses to the blends showed mixture interaction (Figure 4). The

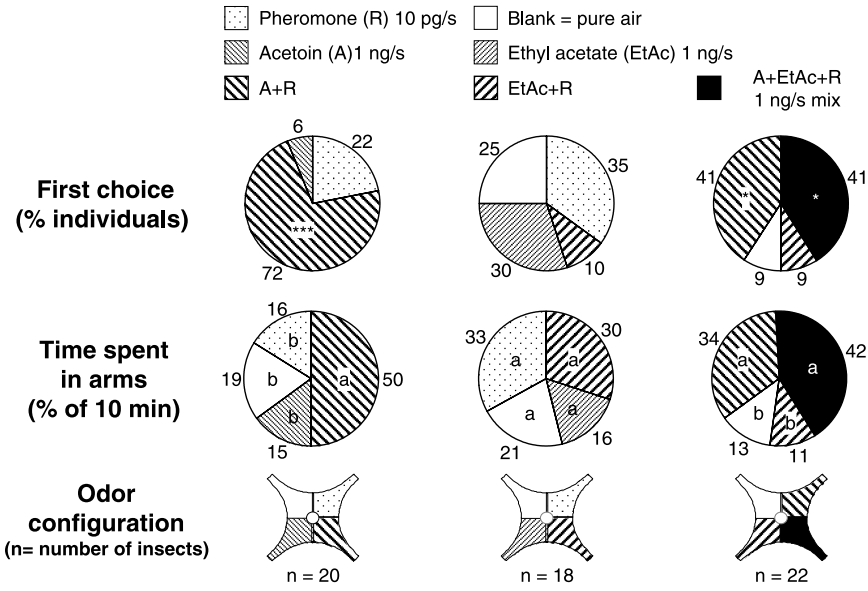

FIG. 6. Responses in a four-armed olfactometer of *Rhynchophorus palmarum* (ca. 1:1 sex ratio) stimulated simultaneously by the aggregation pheromone (R), a plant volatile: acetoin (A) or ethyl acetate (EtAc) and A + R, or by the three blends: A + R, EtAc + R, and A + EtAc + R. All tests included a blank arm. R and the plant compound(s) were delivered by an olfactory stimulator at 0.01 and 1 ng/sec, respectively. Two criteria were analyzed: (1) the arm chosen first [number of choices for a given arm was significant at $*P < 0.05$ or $***P < 0.001$ (binomial tests)] and (2) the time spent in each of the four arms (data with different letters differ statistically; Newman–Keuls tests, $P < 0.05$).

majority of R cells (1–7 in Figure 4) gave lower responses than expected with the blends, especially those containing ≤50% R. This suggests an inhibitory effect, as the response of four cells to the 90:10 A + R blend was lower than to 10 ng R alone, whereas the response to the 10:90 A + R blend was greater than to 100 ng pure R. All three AR cells (8–10 in Figure 4) gave maximum responses to the 50:50 A + R blend but not to pure A or R, whereas all the A and undetermined cells (11–13 and 14–16, respectively, in Figure 4) showed mixture interactions (Figure 4). All but one of these cells fired much more to the blends than A or R alone (Figure 5). A clear response to 100 ng R was also recorded from undetermined cell no. 16 that responded previously neither to 10 ng R nor to 100 ng A.

Behavioral Responses in Olfactometer. There was a significant preference, based both on first choice ($P < 0.001$; binomial tests) and time spent in a

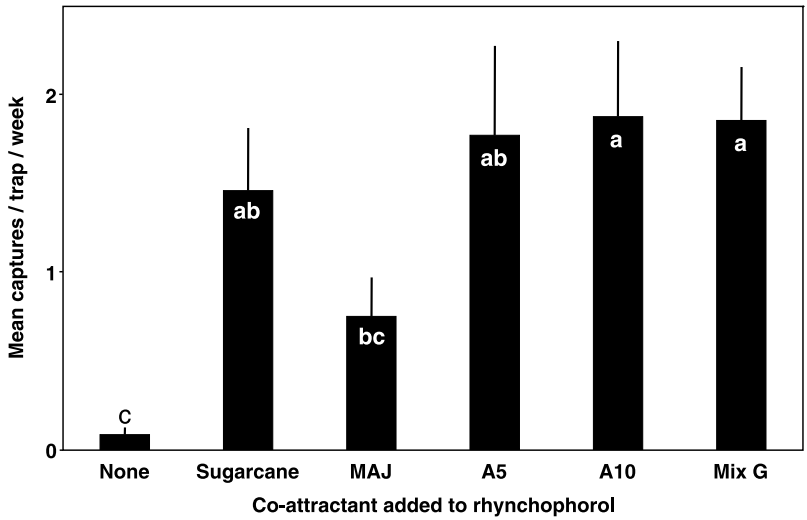

FIG. 7. Field catches of *Rhynchophorus palmarum* in traps baited with a dispenser of the aggregation pheromone, rhynchophorol (1 mg/day), and a co-attractant (either sugarcane or a synthetic blend emitted at 650 mg/day) (Municipio de Santa Amaro, SE, Brazil, October 20–December 15, 2003; $N = 6$ blocks). Four blends based on a 1:1 (v/v) blend of ethanol and ethyl acetate (EtAc) were evaluated: MAJ [pure ethanol/EtAc 1:1 (v/v) blend]; A5 (5% v/v acetoin in MAJ); A10 (10% v/v acetoin in MAJ), and blend (Mix) G (a mixture of 14 compounds representing 11% v/v minor fraction where acetoin accounts for 4.3% in MAJ, 89% v/v; Rochat et al., 2000). Catches associated with the same letter do not differ statistically [Tukey multiple comparison tests on $\ln(x + 1)$ transformed data].

given arm ($P < 0.05$, Newman–Keuls tests) for A + R than pure air, R, or A alone (Figure 6). In contrast, when offered a choice between EtAc, pure air, R, and a blend of EtAc + R, there were no significant preferences, either in first choice (binomial test: $P > 0.1$) or residence time [$F(3, 51) = 1.80$, $P > 0.15$]. In the assay where weevils had a choice between odorless air, A + R, EtAc + R, and A + EtAc + R, both indicators showed a significant but similar preference for A + R and A + EtAc + R over the other two air sources: EtAc + R and odorless (Figure 6; first choice: $P < 0.03$, binomial tests, and time spent in a given arm: $P < 0.05$, Newman–Keuls tests). Insects did not stay longer in the arm with A + EtAc + R (42% test duration) than with A + R (34% test duration).

Field Trapping. R with either A5, A10, or the complex pheromone synergist G made up in the same solvent (Rochat et al., 2000) performed equally well as R + sugarcane and captured significantly more than R alone (Figure 7, Tukey multiple comparison tests, $P < 0.05$). R + MAJ gave intermediate results and did not differ significantly from R alone or R with either sugarcane or A5 (Figure 7).

DISCUSSION

Acetoin, but not ethyl acetate, acted synergistically when mixed with the aggregation pheromone in the olfactometer at the dose used. Acetoin with ethyl acetate gave a somewhat higher response, and in the field, a mixture containing 5–10% acetoin in ethyl acetate was as effective a synergist with aggregation pheromone of *R. palmarum* as either a complex synthetic mixture or natural sugarcane odors. Thus, from a practical point of view, the simpler and cheaper synthetic pheromone synergist could be used instead of blend G for pest monitoring or control.

In the field, the synthetic plant odors included ethanol for homogeneity with past work and because of its solvent property for minor volatiles. In spite of its abundance in the synthetic blends or natural plant odors, the behavioral role of ethanol remains unclear in *R. palmarum*: only one specific ORN tuned to this compound was found in a study of 200 cells (Saïd et al., 2003), and its synergistic role with rhynchophorol appeared weak compared with ethyl acetate (Rochat et al., 2000).

Acetoin, a common volatile product of aerobic fermentation, is a pheromone compound of two scarab beetles: the dynast *Scapanes australis* (Rochat et al., 2002) and the chafer *Amphimallon solstitiale* (Tolasch et al., 2003). Numerous *R. palmarum* ORNs tuned to either acetoin or rhynchophorol have been found (Saïd et al., 2003), as one would expect in a system with independent pathways for pheromone and plant volatiles. In such a system, one would expect ORNs

with high specificity and sensitivity, as well as weak or no interactions between stimuli. However, while specificity and sensitivity of R and A cells have been confirmed, many recordings showed responses that did not agree with this model of specialist coding. First, some olfactory neurons responded at low doses (10 and 100 ng) to both chemicals that are neither structurally nor biologically related. Second, the level of responses to blends deviated from the level expected for independent detection of both compounds.

The pattern of ORN responses to blends of acetoin and rhynchophorol was complex, showing great variability in both the spontaneous activity of the receptor neurons and the responses to the odors. We cannot exclude that uncontrolled experimental factors have contributed to this variability. For example, stimulations were applied to the whole antenna so there may have been heterogeneity in the exposure of different sensilla to the odorized airflow. Some neurons may have been partly adapted (or sensitized) by preceding stimulations when we were recording from neighboring sensilla. However, given the correlations observed between types of responses to single compounds and to the blend (Figure 4), we believe this variability reflects intrinsic differences in sensitivity and specificity of neurons. Mixture suppression was observed mainly in neurons with high selectivity for acetoin and rhynchophorol, as well as in acetoin-tuned neurons. Generally, responses to A + R did not deviate from responses to R in neurons exhibiting high-level responses to rhynchophorol alone. Mixture enhancement was observed mainly in neurons responding to both A and R, as well as in neurons that only respond moderately to rhynchophorol. The latter result suggests a synergistic effect of acetoin on the concerned cells. The level of response to 100 ng was above the response thresholds of the R (0.01 ng) and A (1 ng) cells (Saïd et al., 2003), but the level of firing was not maximum. This situation, and the variability of the responses, made it difficult to establish the limit between additivity and synergy. To date, only one clear case of synergy between a pheromone component and a plant volatile has been reported. In *Helicoverpa zea*, stimulation with binary mixtures at a threshold dose of the pheromone component (*Z*)-11-hexadecenal and increasing doses of linalool or (*Z*)-3-hexenol increased the firing rate of pheromone-specific neurons compared to responses to the major component alone (Ochieng et al., 2002).

Considerable work has been devoted to understand and elucidate the mechanisms for encoding mixture quality by individual olfactory cells in lobsters, and different types of mixture interactions have been modeled (Ache et al., 1988; Derby et al., 1996; Cromarty and Derby, 1997, 1998). Such interactions were not expected to occur frequently in insects because of the narrow tuning of olfactory receptor neurons, especially those tuned to pheromone compounds. For instance, orientation to females by male *Agrotis segetum* moths results from the detection of a multicomponent blend, and responses of all types of the specific receptor neurons that are tuned to the individual sex pheromone

components are the same when stimulated with blends or specific excitatory compounds (Carlsson and Hansson, 2002). However, evidence of a contribution of mixture interactions at the receptor cell level to olfactory encoding in insects is now growing. Inhibition, or mixture suppression of receptor cells in response to blends compared to responses to pure odors, as reported in the American cockroach (Getz and Akers, 1997) and the Colorado potato beetle (De Jong and Visser, 1988), is the most commonly reported type of interaction. In *R. palmarum*, blends inhibited spontaneous firing (Saïd et al., 2003) or decreased responses (this study), mainly in A cells.

Sensitization by acetoin was repeatedly observed in R cells with low to moderate responses to rhynchophorol. Stimulation by acetoin, even a brief exposure (1 sec) to a nonsaturating dose, enhanced the sensitivity of certain of these neurons, resulting in increased firing when they received subsequent stimulations by rhynchophorol. This sensitization was reversible. Sensitization of pheromone responses by plant volatiles was also observed in two tortricid moths, *Argyrotaenia velutina* and *Choristoneura rosaceana*. The phenomenon was mediated by an increase of octopamine following the perception of plant odor (Stelinski et al., 2003). The behavior of *R. palmarum* following detection of acetoin and rhynchophorol relies on the interpretation of the entire sensory input provided by individual neurons. In spite of the variety of ORN responses, a global pattern of sensory input emerges. From a physiological perspective, the presence of acetoin increases sensitivity to rhynchophorol, whereas the detection of rhynchophorol decreases the level of detection of acetoin, both contributing to lower the global threshold of response when the two signals were presented simultaneously. From an ecological point of view, this modulation should improve detection of conspecifics in the presence of a plant signal. Conversely, the detection of pheromone should optimize the response to host plants odors (at least the acetoin), increasing the probability of *R. palmarum* localizing palm trees harboring conspecifics. Clearly, the synergistic response to the plant–pheromone message not only relies on the processing of sensory inputs in the insect brain, but also on interactions between the pheromone and a plant volatile at the receptor cell level. This property may reflect an adaptation of the olfactory system of insects exhibiting synergistic responses to "pheromone–kairomone" signals and should be searched in species from other families with similar chemical ecology, such as the Nitidulidae or Scolytidae.

In summary, our results about ORN outputs underline the absence of sexual dimorphism in the processing of host plant and pheromone cues by *R. palmarum*. This conclusion is supported by the behavioral responses reported previously (Rochat et al., 1991, 2000; Saïd, 2003). Contrary to other palm beetles, such as the dynasts *S. autralis* (Rochat et al., 2002) or *Oryctes elegans* (Rochat et al., 2004), *R. palmarum* aggregation pheromone appears to be more a male-produced signal for host-plant and trophic resources than a sexual signal.

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IDENTIFICATION OF THE SEX PHEROMONE OF THE SWEDE MIDGE, *Contarinia nasturtii*

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Abstract—Coupled gas chromatographic–electroantennographic detection analyses of ovipositor extracts of calling *Contarinia nasturtii* females revealed two compounds that elicited responses from antennae of male midges. Using synthetic reference samples, these components were identified by gas chromatography–mass spectrometry and enantioselective GC as (2*S*,9*S*)-diacetoxyundecane and (2*S*,10*S*)-diacetoxyundecane. In addition, trace amounts of 2-acetoxyundecane were found in ovipositor extracts, and the (*S*)-enantiomer was synthesized. When tested in the wind tunnel, a blend of 5 ng (2*S*,9*S*)-diacetoxyundecane and 10 ng (2*S*,10*S*)-diacetoxyundecane (mimicking the ratio found in the extracts) did not attract any of the males tested, but when 0.1 ng (*S*)-2-acetoxyundecane was added to the blend, 86.8% of the males were attracted to the bait. Three-component blends with lower or higher relative concentrations than 1% of (*S*)-2-acetoxyundecane [relative to (2*S*,10*S*)-diacetoxyundecane] were less attractive. In a field trapping experiment with released laboratory-reared *C. nasturtii* adults, traps baited with 500:1000:10 ng of (2*S*,9*S*)-diacetoxyundecane/(2*S*,10*S*)-diacetoxyundecane/(*S*)-2-acetoxyundecane applied to rubber septa or dental cotton rolls

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were tested. Traps without dispensers were used as controls. All three treatments were tested at 20 and 50 cm above ground. The estimated recapture rate was 30–50%, and 81.9% of the recaptured males were caught in traps positioned at 20 cm above ground, and 88.4% in traps with dental cotton rolls as dispensers.

Key Words—*Contarinia nasturtii*, swede midge, Cecidomyiidae, Diptera, sex pheromone, (2*S*,9*S*)-diacetoxyundecane, (2*S*,10*S*)-diacetoxyundecane, (*S*)-2-acetoxyundecane, GC-EAD, wind tunnel, field trapping.

INTRODUCTION

The swede midge, *Contarinia nasturtii* (Kieffer) (Diptera: Cecidomyiidae), occurs in many brassica-growing regions in Europe where it often causes serious yield losses, mainly in broccoli, cauliflower, and Brussels sprouts. Female midges oviposit in the shoots of host plants, where the larvae feed and develop. Feeding damage near the growing tips induces distortion and gall-like symptoms that prevent the plants from forming flower heads, or leave corky scars that render the heads unmarketable. Relatively recently, the occurrence of *C. nasturtii* was reported in Canada (Hallett and Heal, 2001), and it is now a quarantine pest in Canada (Canadian Food Inspection Agency, 2002).

One indirect reason for the high damage levels caused by *C. nasturtii* is the lack of a good monitoring method for determining the time of emergence of the adult midges, the life stage against which chemical control is most effective. Currently, the only available monitoring method is based on yellow pan traps filled with water and a detergent. These traps are not species specific, and the analysis of the trap contents is laborious and time-consuming. Furthermore, the trapping efficiency of yellow pan traps for *C. nasturtii* is low; the crop is frequently damaged by *C. nasturtii* even when no midges have been detected in traps. Temperature-based forecasting methods have been developed (Noll, 1959, 1961; Bouma, 1996) but have failed to give accurate field-specific prognosis. As long as monitoring or forecasting methods are not reliable, and damage thresholds are lacking, growers tend to rely on calendar-based insecticide sprays throughout the crop's growing period. As a consequence, supervised control (as opposed to scheduled spraying) against other important brassica pests, such as aphids and *Pieris* and *Mamestra* caterpillars, cannot be considered an option.

A successful pheromone-based monitoring system has been developed and commercialized for a closely related gall midge species, the pea midge, *Contarinia pisi* (Hillbur et al., 2000, 2001; Pillon and Hillbur, 2001; Biddle et al., 2002). Promising results with respect to pheromone-based monitoring have also been reported for two other cecidomyiids, the orange blossom wheat midge, *Sitodiplosis mosellana* (Gries et al., 2000), and the Douglas-fir cone gall

midge, *Contarinia oregonensis* (Gries et al., 2002; Morewood et al., 2002). Here, we report on the identification, synthesis, and testing of the sex pheromone of *C. nasturtii*.

METHODS AND MATERIALS

Insect Rearing. Larvae of *C. nasturtii* were collected in August 2000 from heavily attacked Brussels sprouts in a field near Kerzers, Canton Freiburg, Switzerland. Infested growing tips were placed on a peat–soil mixture in which larvae pupated when they had completed their development. Pots containing pupae were stored in cages, and from the emerging adults, a continuous laboratory culture was established. Similarly, larvae from a field near Ins, Canton Bern, Switzerland, were added to the culture in 2001.

Swede midge larvae were reared on cauliflower, *Brassica oleracea convar. botrytis*, cv. Candid Charm. Pairs of plants were grown in pots (Thermoform pots, 13-cm diam, 8-cm height, Desch Plantpak, The Netherlands) in a substrate consisting of 85% peat and clay (Floraton No. 5, Floragard, Oldenburg, Germany) in a greenhouse at 15–30°C with additional light supplied during winter. Plants were kept free from aphids and white flies by spraying, if necessary, once with pymetrozine (0.025% a.i.). Spraying was avoided during the last 3 wk before the plants were used for insect rearing. When plants had 8–10 true leaves and a tip diameter of 5–10 mm, they were placed in Perspex cages (50 × 50 × 50 cm) with two side walls consisting of fine-meshed screen. The cages contained 100–200 midges of both sexes and were transferred to environmental chambers with 16:8-hr light–dark period, 22°C, and RH 75%. The midges were allowed to mate and oviposit on the plants for 24 hr. The plants were then removed, replaced by the next batch, and incubated under the above-mentioned conditions. Every day, the tips of the plants, where larvae became visible 5–6 d after oviposition, were misted with tap water. Thirteen to fifteen d after oviposition, larvae left the plants and pupated in the peat substrate. Males emerged during the night, 18–20 d after oviposition. Females tended to emerge 1 d later, mostly during the night or the first hour of the photophase. In the cages, adult midges survived and oviposited for 3–5 d. Mating behavior was observed during the second to fourth hour of the photophase.

If larvae were used to supply the culture, aboveground plant parts were cut off on day 17 after oviposition. The pots with substrate containing the pupae were then placed in the oviposition cages where midges were allowed to emerge. If midges were to be used for electrophysiological experiments or for collection of pheromone gland extracts, aboveground plant parts were cut off on day 15 and the pots were sent by express mail from Wädenswil, Switzerland, to Alnarp, Sweden. Males for wind tunnel experiments were prevented from mating by

placing glass cylinders over pots. The morning after the night when the first midges had emerged, these cylinders, usually containing only males, were set aside before females started to emerge.

Extracts for Electrophysiological Analyses and Chemical Identification. Upon arrival in Alnarp, pots with soil containing *C. nasturtii* pupae were put in glass cages (28 × 28 × 33 cm) and kept at 18:6-hr light–dark period, 25°C, and RH 70% for the adults to emerge. After emergence, males and females were transferred to separate cages. Females were kept under the conditions mentioned, and males were kept at 18:6-hr light–dark period, 15°C, and RH 75%. Gland extracts were prepared by excising the ovipositors of females exhibiting calling behavior (extending their ovipositor; for a detailed description, see Harris and Foster, 1999). The ovipositors were collected in a vial kept in liquid nitrogen and then extracted for 1–2 min in redistilled hexane (LabScan). Extracts were kept in sealed glass capillaries at −18°C until use.

Extracts for Preliminary Wind Tunnel Assays. Calling females were collected from the laboratory culture and immersed in batches in hexane (p.a. grade, Fluka) for 15 min at room temperature. The supernatant was then decanted and concentrated by evaporating the hexane under N_2.

Electrophysiological Recordings. Antennal responses of males to ovipositor extracts were determined by gas chromatography with electroantennographic detection (GC-EAD; Arn et al., 1975), using a Hewlett-Packard 6890 GC (Palo Alto, CA, USA) with flame ionization detection and an Innowax column (30 m × 0.25 mm ID, H-P). The column was programmed from 80°C/2 min to 230°C at 10°C/min. Antennal recordings were made using a Plexiglas holder with two wells connected to an amplifier by gold wire electrodes (JoAC, Lund, Sweden; Zhang et al., 1997; Hillbur, 2001). Male insects were mounted in the holder as described by Hillbur et al. (2000). The antennal preparations were continuously exposed to a charcoal-filtered and humidified airstream (0.3 m/s) through a glass tube (8-mm diam.). Antennal signals were amplified (JoAC) before they were recorded and analyzed with ElectroAntennoGraphy software (Syntech, Hilversum, The Netherlands).

Chemical Analyses. Analyses were carried out by coupled gas chromatography–mass spectrometry (GC-MS). A Hewlett-Packard 5890 GC was linked to a VG 70/250 E double-focusing sector-field mass spectrometer (Vacuum Generators, Manchester, UK) operated at 70 eV. Alternatively, a quadrupole instrument (MD 800/GC 8060, Fisons, Ismaning, Germany) was employed. Using helium as carrier gas, separations were achieved with a 60 m × 0.25 mm ID, 0.25-μm film DB5-MS column (J & W Scientific, Folsom, CA, USA) under the following conditions: 50°C/2 min, then 5°C/min to 280°C. In addition, a 50 m × 0.25 μm ID, 0.27-μm film FFAP fused silica column (Macherey & Nagel, Düren, Germany) was used (50°C/3 min, then 3°C/min to 220°C). Separation of stereoisomers was carried out with a 25 m × 0.25 mm ID custom-

made fused silica column coated with a 1:1 mixture of heptakis-(6-*O*-*tert*-butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin and OV1701 (Hydrodex, Macherey & Nagel) using hydrogen as carrier gas and a temperature program of 80°C/3 min, then 3°C/min to 180°C.

Nuclear magnetic resonance (NMR) spectra of synthetic compounds were recorded with a Bruker AMX-400 instrument (Karlsruhe, Germany). Optical rotations were measured on a digital polarimeter at 589-nm (Jasco DIP-370, Tokyo, Japan).

Syntheses (see Figure 1). Chemicals and solvents were purchased from Aldrich or Merck and were of highest purity available. Purification of synthetic products was carried out by flash chromatography on silica gel (silica 32-63, 60 Å, ICN-Biomedicals, Eschwege, Germany) at 1.3 bar using mixtures of ethyl acetate and hexane. Tetrahydrofuran (THF) was dried before use. Extracts were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure.

*(S)-9-Decene-2-ol **2**.* Under an argon atmosphere, a Grignard reagent was prepared from 10.0 g (56.6 mmol) of 7-bromo-1-heptene **1** (Aldrich) and 2.06 g (84.8 mmol) freshly ground magnesium turnings in 50 ml dry THF at 60°C. After cooling to room temperature, the solution was added dropwise to 3.3 g (4 ml, 56.7 mmol) (*S*)-methyloxirane (Aldrich) and 1.1 g (5.7 mmol) CuI in 40 ml THF at −78°C. The solution was warmed to room temperature and stirred for 1 hr. After addition of 300 ml saturated aqueous NH_4Cl, the mixture was extracted with 200 ml ethyl acetate. The aqueous layer was separated and extracted three times with 200 ml of a 1:1 mixture of ethyl acetate and hexane. The combined organic extracts were dried and concentrated. Purification by flash chromatography (10% ethyl acetate in hexane) furnished 6.2 g (39 mmol, 70%) of (*S*)-9-decene-2-ol **2**. Analytical data were in accord with those reported in the literature (Trollsås et al., 1996).

*(S)-2-Benzyloxydec-9-ene **3**.* To a suspension of 3.2 g (133 mmol) sodium hydride (freed from paraffin) in 105 ml, ice-cold dry THF was added 6.2 g (39.5 mmol) (*S*)-9-decene-2-ol **2** under argon. After stirring for 2 hr, 470 mg (1.3 mmol) *tetra-n*-butylammonium iodide were added, followed by dropwise addition of 13.5 g (9.4 ml, 78.9 mmol) benzyl bromide, and stirring was continued for 12 hr. Then, 50 ml saturated aqueous NH_4Cl were added at 0°C followed by 50 ml hexane. The aqueous layer was separated and extracted four times with 100 ml of a 1:4 mixture of ethyl acetate and hexane. The combined organic layers were dried and concentrated. Flash chromatography (5% ethyl acetate in hexane) furnished 9.7 g (39.4 mmol, 100%) (*S*)-2-benzyloxydec-9-ene **3**. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 1.18 (d, 3H, J = 6.1 Hz, 1-H), 1.25–1.47 (m, 10H, 3-H to 7-H), 2.00–2.07 (m, 2H, 8-H), 3.49 (sext., 1H, J = 6.4 Hz, 2-H), 4.45 (d, 1H, J = 12 Hz, OC**H**$_a$-Ph), 4.56 (d, 1H, J = 12 Hz, OC**H**$_b$-Ph), 4.90–4.95 (m, 1H, 10-H$_b$), 4.95–5.02 (m, 1H, 10-H$_a$), 5.81 (ddt, 1H, $J_{10a,9}$ = 16.79 Hz, $J_{10b,9}$ = 10.17 Hz, $J_{9,8}$ = 6.61 Hz, 9-H), 7.23–7.34 (m, 5H, H$_{Ph}$); ^{13}C-

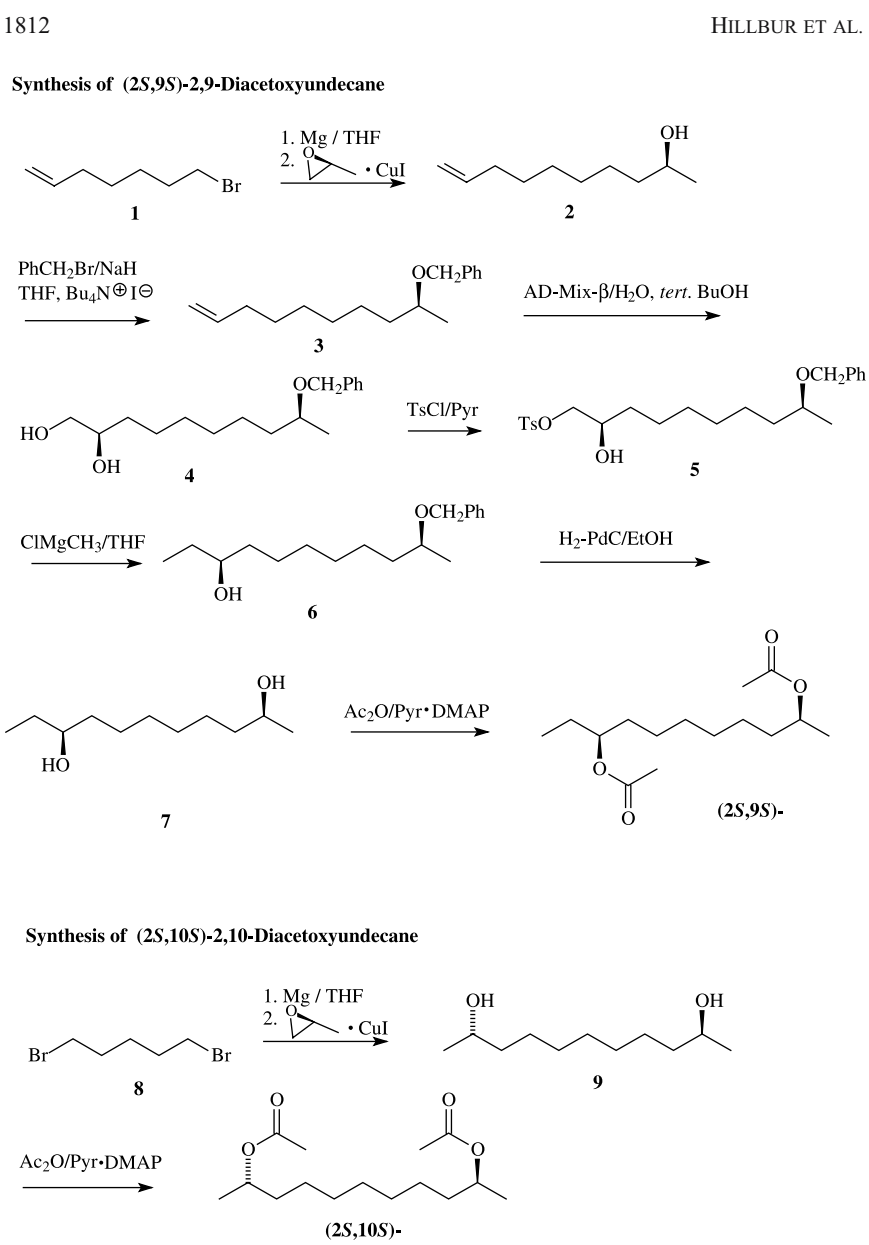

FIG. 1. Syntheses of (2*S*,9*S*)-2,9-diacetoxyundecane and (2*S*,10*S*)-2,10-diacetoxyundecane.

NMR (101 MHz, $CDCl_3$): δ (ppm) 19.63 (q, 1-C), 25.49/28.88/29.10/29.55/33.77 (t, 4-C to 8-C), 36.66 (t, 3-C), 70.28 (t, O**C**H_2-Ph), 74.90 (d, 2-C), 114.13 (t, 10-C), 127.32 (d)/127.60 (d, 2C)/128.73 (d, 2C)/139.18 (s) (C_{Ph}), 141.77 (d, C-9); MS (70 eV): *m*/*z* (%) 246 (0.2, M^+), 155 (0.5, $[M–Bn]^+$), 135 (12), 107 (18), 104 (6), 92 (16), 91 (100), 85 (7), 65 (7), 55 (10, $CH_2CHCH_2CH_2^+$), 43 (6), 41 (18), 39 (7).

(2R,9S)-9-Benzyloxydecane-1,2-diol ***4***. A vigorously stirred solution of 6.0 g (24.3 mmol) (*S*)-2-benzyloxydec-9-ene **3** in 300 ml of a 1:1 mixture of water and *tert*-butanol was cooled to −10°C. After addition of 29.2 g AD-Mix β (i.e., 1.2 g/mmol alkene), the mixture was stirred at 4°C for 40 hr. Sodium bisulfite (30.0 g, 158 mmol) was added, and the mixture was stirred at 20°C for another hour. The brownish suspension was extracted three times with 250 ml ethyl acetate. The organic layers were combined, dried, and concentrated. Flash chromatography (ethyl acetate and hexane 1:1) furnished 4.2 g (15 mmol, 62%) (2*R*,9*S*)-9-benzyloxydecane-1,2-diol **4** as a colorless oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.08 (d, 3H, J = 6.1 Hz, 10-H), 1.15–1.27 (m, 6H, 4-H to 6-H), 1.30–1.40 (m, 5H, 3-H, 7-H, and 8-H_a), 1.45–1.55 (m, 1H, 8-H_b), 1.76 (br.s, 2H, OH), 3.32 (dd, 1H, J = 11 Hz, 3.3 Hz, 1-H_a), 3.4 (sext., 1H, J = 6.4 Hz, 9-H), 3.54 (dd, 1H, J = 11 Hz, 3.0 Hz, 1-H_b), 3.57–3.64 (m, 1H, 2-H), 4.33 (d, 1H, J = 11.7 Hz, OC**H**$_a$-Ph), 4.46 (d, 1H, J = 11.7 Hz, OC**H**$_b$-Ph), 7.14–7.24 (m, 5-H, H_{Ph}); ^{13}C-NMR (101 MHz, $CDCl_3$): δ 19.62 (q, 10-C), 25.43/25.46/29.57 (2C)/33.17/36.62 (t, 5-C to 7-C/4-C/8-C/3-C), 66.83 (t, 1-C), 70.28 (t, O**C**H_2-Ph), 72.26 (d, 2-C), 74.88 (d, 9-C), 127.35 (d)/127.61 (d, 2C)/128.29 (d, 2C)/139.14 (s) (C_{Ph}); MS (70 eV): *m*/*z* 280 (0.1, M^+), 135 (2), 131 (3), 108 (7), 107 (17), 92 (10), 91 (100), 55 (7), 43 (6), 41 (7).

(2R,9S)-9-Benzyloxy-1-(p-toluenesulfonyloxy)-decan-2-ol ***5***. A solution of 2.9 g (15 mmol) *p*-toluenesulfonyl chloride in 20 ml dry pyridine was added to an ice-cold solution of 4.6 g (16.4 mmol) (2*R*,9*S*)-9-benzyloxydecane-1,2-diol **4** in 50 ml dry pyridine. Stirring was continued for 5 hr at 0°C and 12 hr at 4°C. Then, 150 ml ice water (brought to pH 5 with 0.1% hydrochloric acid) and 60 ml diethyl ether were added. After separation of layers, the aqueous layer was extracted four more times with 120 ml diethyl ether. The combined organic layers were washed three times with 100 ml of saturated aqueous $CuSO_4$ and twice with 100 ml brine. The organic layer was dried and concentrated. Flash chromatography (10% ethyl acetate in hexane) furnished 4.6 g (10.5 mmol, 64%) (2*R*,9*S*)-9-benzyloxy-1-(*p*-toluenesulfonyloxy)decan-2-ol **5**. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.08 (d, 3H, J = 6.1 Hz, 10-H), 1.15–1.35 (m, 11H, 3-H to 7-H and 8-H_a), 1.42–1.53 (m, 1H, 8-H_b), 1.77 (br.s, 1H, OH), 2.35 (s, 3H, CH_3-Tos), 3.39 (sext., 1H, J = 6.4 Hz, 9-H), 3.69–3.76 (m, 1H, 2-H), 3.78 (dd, 1H, J = 9.9, 2.5 Hz, 1-H_a), 3.93 (dd, 1H, J = 9.9, 2.8 Hz, 1-H_b), 4.34 (d, 1H, J = 11.7 Hz, OC**H**$_a$-Ph), 4.46 (d, 1H, J = 11.7 Hz, OC**H**$_b$-Ph), 7.15–7.28 (m, 7-H, H_{Ph}/Tol-H_b and Tol-$H_{b'}$), 7.70 (d, 2H, J = 8.4 Hz, Tol-H_a and Tol-

$H_{a'}$); ^{13}C-NMR (101 MHz, $CDCl_3$): δ 19.62 (q, 10-C), 21.63 (q, 4-C_{Tol}-**C**H_3), 25.15/25.39/29.37/29.49/32.61/36.60 (t, 3-C to 8-C), 69.48 (d, 2-C), 70.27 (t, O**C**H_2-Ph), 73.96 (t, 1-C), 74.82 (d, 9-C), 127.33 (d, C_{Ph}), 127.60 (2C, d, 3-C_{Tol} and 3′-C_{Tol}), 127.94 (d, 2C, C_{Ph})/128.27 (2C, C_{Ph}), 129.93 (2C, 2-C_{Tol} and 2′-C_{Tol}), 132.73 (s, 4-**C**$_{Tol}$-CH_3), 139.13 (s, C_{Ph}), 145.02 (s, 1-**C**$_{Tol}$-SO_3-); MS (70 eV): *m*/*z* (%) 171 (0.1, Tol-SO_3^+), 135 (2, BnOCHCH_3^+), 107 (18), 92 (10), 91 (100), 65 (6), 55 (6), 43 (5), 41 (11), 39 (5).

(3S,10S)-10-Benzyloxyundecan-3-ol ***6***. Dry THF (20 ml) was added to 11.8 ml of 20% methyl magnesium chloride (31.4 mmol) in THF. The solution was cooled to −78°C, and 595 mg (3.14 mmol) CuI were added. After stirring for 15 min, a solution of 4.55 g (10.5 mmol) (2*R*,9*S*)-9-benzyloxy-1-(*p*-toluenesulfonyloxy)decane-2-ol **5** in 10 ml dry THF was added over 2 hr. The solution was warmed to −30°C and stirred 1 hr, then 150 ml saturated aqueous NH_4Cl and 50 ml diethyl ether were added at 0°C. The aqueous layer was extracted three times with 150 ml ethyl acetate. The combined organic layers were dried and concentrated. Flash chromatography (16% ethyl acetate in hexane) furnished 2.6 g (9.4 mmol, 90%) (3*S*,10*S*)-10-benzyloxyundecan-3-ol **6**. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.93 (t, 3H, J = 7.3 Hz, 1-H), 1.18 (d, 3H, J = 6.1 Hz), 1.19–1.55 (m, 15H, 2-H/4-H to 9-H and OH), 3.45–3.54 (m, 2H, 3-H, and 10-H), 4.45 (d, 1H, J = 12 Hz, OC**H**$_a$-Ph), 4.56 (d, 1H, J = 12 Hz, OC**H**$_b$-Ph), 7.22–7.36 (m, 5-H, H_{Ph}); ^{13}C-NMR (101 MHz, $CDCl_3$): δ 9.84 (q, 1-C), 19.91 (q, 11-C), 24.28/25.47/25.59/29.67/30.13/36.63/36.92 (t, 4-C to 9-C and 2-C), 70.25 (t, O**C**H_2-Ph), 73.28 (d, 3-C), 74.87 (d, 10-C), 127.31 (d)/127.59 (d, 2C)/128.26 (d, 2C)/139.14 (s) (C_{Ph}); MS (70 eV): *m*/*z* (%) 260 (0.8, M^+–H_2O), 108 (9), 107 (31), 104 (10), 92 (15), 91 (100), 69 (6), 65 (6), 59 (11), 57 (6), 55 (12), 43 (9), 41 (14).

(2S,9S)-Undecane-2,9-diol ***7***. Pd/C catalyst (20 mg) was added to a solution of 1.95 g (7 mmol) (3*S*,10*S*)-10-benzyloxyundecan-3-ol **6** in 30 ml ethanol, and the mixture was held under H_2 atmosphere for 12 hr at 40 atm. After completion of the reaction, the mixture was filtered through silica gel, and the solution was concentrated *in vacuo*. Flash chromatography (ethyl acetate/hexane, 1:1) yielded 1.2 g (6.4 mmol, 92%) (2*S*,9*S*)-undecane-2,9-diol **7**. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.91 (t, 3H, J = 7.4 Hz, 11-H), 1.17 (d, 3H, J = 6.1 Hz, 1-H), 1.26–1.45 (m, 16H, 3-H to 8-H/10-H and OH), 3.45–3.52 (m, 1H, 9-H), 3.72–3.81 (m, 1H, 2-H); ^{13}C-NMR (101 MHz, $CDCl_3$): δ 9.84 (q, 11-C), 23.48 (q, 1-C), 25.56/25.67/29.57/29.63/30.15/36.89/39.31 (t, 3-C to 8-C and 10-C), 68.13 (d, 2-C), 73.28 (d, 9-C); MS (70 eV): *m*/*z* (%) 171 (0.2, [M^+– H_2O]), 155 (0.86, [M^+–H_2O–CH_3]), 141 (11, [M^+–H_2O–CH_2CH_3]), 123 (43), 112 (8), 110 (5), 98 (6), 97 (17), 96 (10), 95 (21), 85 (12), 84 (23), 83 (37), 82 (23), 81 (100), 77 (14), 71 (15), 70 (28), 69 (42), 68 (16), 67 (49), 63 (10), 59 (83, $CH_3CH_2CHOH^+$), 57 (44), 56 (35), 55 (87), 53 (5), 45 (88) (CH_3CHOH^+), 43 (54), 42 (19), 41 (63), 39 (12).

(2S,9S)-Diacetoxyundecane. A solution of 1.40 g (7.4 mmol) (2*S*,9*S*)-undecane-2,9-diol **7** in 20 ml dry pyridine was cooled to 0°C. After addition of a small amount of 4-dimethylaminopyridine, 2.3 g (2.1 ml, 22.3 mmol) acetic anhydride were added dropwise, and the mixture was stirred for 2 hr at room temperature. Usual workup followed by flash chromatography (7% ethyl acetate in hexane) furnished 1.9 g (6.9 mmol, 93%) (2*S*,9*S*)-diacetoxyundecane. $[\alpha]^{20}_{D}$ −2.85 (*c* 1.06, $CHCl_3$). Enantioselective gas chromatography showed an enantiomeric excess of > 98 %. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.95 (t, 3H, *J* = 7.4 Hz, 11-H), 1.17 (d, 3H, *J* = 6.4 Hz, 1-H), 1.20–1.32 (m, 8H, 4-H to 7-H), 1.40–1.60 (m, 6H, 3-H/8-H, and 10-H), 1.99 (s, 3H, COC**H**$_3$), 2.76 (s, 3H, COC**H**$_3$), 4.77 (quin., 1H, *J* = 6.6 Hz, 9-H), 4.85 (sext., 1H, *J* = 6.4 Hz, 2-H); ^{13}C-NMR (101 MHz, $CDCl_3$): δ 9.53 (q, 11-C), 19.92 (q, 1-C), 21.20 (q, CO**C**H$_3$), 21.34 (q, CO**C**H$_3$), 25.19/25.27/26.62/29.28/29.35/33.51/35.85 (t, 3-C to 8-C and 10-C), 70.95 (d, 2-C), 75.43 (d, 9-C), 170.73 (s, **C**O), 170.93 (s, **C**O); MS (70 eV): *m*/*z* (%) 209 (0.1, $[M–CH_3COOH]^+$), 148 (4, $[M–CH_3COOH–CH_3COOH_2]^+$), 141 (7), 123 (14), 110 (11), 103 (10), 97 (9), 96 (15), 95 (6), 83 (8), 82 (13), 81 (19), 69 (9), 68 (11), 67 (12), 57 (5), 55 (17), 43 (100, $COCH_3^+$), 41 (15).

For reference, a synthesis of the mixture of all four 2,9-diacetoxyundecanes was carried out. Racemic **3**, prepared from the bromide **1** and racemic methyloxirane, followed by protection of the hydroxyl group (see Figure 3), was oxidized with metachloroperbenzoic acid. The resulting epoxide was reacted with methylmagnesium bromide to yield 10-benzyloxyundecane-3-ol. After deprotection and acetylation, 2,9-diacetoxyundecane was obtained in good yield and high purity.

(2S,10S)-Undecane-2,10-diol ***9***. Under an argon atmosphere, a Grignard reagent was prepared from 2.5 g (10.9 mmol) 1,5-dibromopentane **8** (Aldrich), and 795 mg (32.6 mmol) freshly ground magnesium turnings in 25 ml dry THF at 60°C. After cooling to room temperature, the solution was added dropwise to 1.24 g (1.5 ml, 21.7 mmol) (*S*)-methyloxirane (Aldrich) and 420 mg (2.17 mmol) CuI, suspended in 10 ml dry THF at −78°C. Subsequently, the solution was warmed to room temperature and stirred for 1 hr. After addition of 250 ml of saturated aqueous NH_4Cl, the mixture was extracted with 100 ml ethyl acetate. The aqueous layer was separated and extracted three times with 150 ml of a 1:1 mixture of ethyl acetate and hexane. The combined organic extracts were dried and concentrated. Purification by flash chromatography (20% hexane in ethyl acetate) furnished 1.1 g (5.84 mmol, 54%) (2*S*,10*S*)-undecane-2,10-diol **9**. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.15 (d, 6H, *J* = 6.1 Hz, 11-H and 1-H), 1.21–1.49 (m, 14H, 3-H to 9-H), 1.58 (s, 2H, OH), 3.70–3.80 (m, 2H, 2-H and 10-H); ^{13}C-NMR (101 MHz, $CDCl_3$): δ 23.42 (q, 2C, 1-C, and 11-C), 25.68 (t, 2C)/29.51 (t, 3C)/39.28 (t, 2C), 68.06 (d, 2C, C-2, and C-10); MS (70 eV): *m*/*z* (%) 95 (6), 83 (6), 82 (6), 81 (8), 70 (8), 69 (12), 68 (6),

67 (8), 63 (7), 57 (9), 56 (12), 55 (18), 45 (100), 43 (24), 42 (6), 41 (21), 39 (7).

(2S,10S)-Diacetoxyundecane. A solution of 1.1 g (5.8 mmol) (2*S*,10*S*)-undecane-2,10-diol **9** in 15 ml dry pyridine was cooled to 0°C. After addition of a small amount of 4-dimethylaminopyridine, 1.79 g (1.66 ml, 17.5 mmol) acetic anhydride were added dropwise, and the mixture was stirred for another 2 hr at room temperature. Usual workup followed by flash chromatography (10% ethyl acetate in hexane) furnished 1.38 g (5.1 mmol, 87%) (2*S*,10*S*)-diacetoxyundecane. $[\alpha]^{20}_{D}$ −1.68 (*c* 1.07, $CHCl_3$). Enantioselective gas chromatography showed an enantiomeric excess of > 98%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.17 (d, 6H, J = 6.1 Hz, 1-H and 11-H), 1.20–1.57 (m, 14H, 3-H to 9-H), 1.90 (s, 6H, COC**H**$_3$), 4.85 (sext., 2H, J = 6.4 Hz, 2-H and 10-H); ^{13}C-NMR (101 MHz, $CDCl_3$): δ 19.92 (q, 2C, 1-C, and 11-C), 21.34 (q, 2C, CO**C**H$_3$), 25.33 (t, 6-C), 29.35 (t, 4C, 4-C/5-C, and 7-C/8-C), 35.87 (t, 2C, 3-C, and 9-C), 70.99 (d, 2C, 2-C, and 10-C), 170.73 (s, **C**O); MS (70 eV): *m/z* (%) 87 (13), 61 (5), 55 (11), 43 (100), 41 (12), 39 (5).

For reference, the sequence described for the synthesis of (2*S*,10*S*)-diacetoxyundecane was also carried out using racemic methyloxirane.

(S)-Undecan-2-ol. A Grignard reagent was prepared from 2.8 g (14.7 mmol) 1-bromooctane (Aldrich) and 900 mg (37.0 mmol) freshly ground magnesium turnings and reacted with 853 mg (1.03 ml, 14.7 mmol) (*S*)-methyloxirane (Aldrich). The preparation procedure and workup followed the protocol as described for the synthesis of (*S*)-9-decene-2-ol. After flash chromatography of the crude product (20% ethyl acetate in hexane), 2.0 g (11.6 mmol, 79%) (*S*)-undecan-2-ol were obtained. The compound has been described earlier. Analytical data of our product matched those reported in the literature (Nakamura and Matsuda, 1998).

(S)-2-Acetoxyundecane. Acetylation of 2.0 g (11.6 mmol) (*S*)-undecan-2-ol followed the protocol as described above for the acetylation of the undecanediols. Workup followed by flash chromatography (5% ethyl acetate in hexane) furnished 2.3 g (10.7 mmol, 93%) (2*S*)-acetoxyundecane. $[\alpha]^{20}_{D}$ +2.29 (*c* 1.22, $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.86 (t, 3H, J = 6.6 Hz, 11-H), 1.17 (d, 3H, J = 6.4 Hz, 1-H), 1.20–1.56 (m, 16H, 3-H to 10-H), 2.00 (s, 3H, COC**H**$_3$), 4.86 (sext., 1H, J = 6.1 Hz, 2-H); ^{13}C-NMR (101 MHz, $CDCl_3$): δ 14.07 (q, 1C, 11-C), 19.94 (q, 1C, 1-C), 21.36 (q, 1C, CO**C**H$_3$), 22.66/25.39/29.28/29.45/29.52 (2C)/31.87/35.93 (t, 3-C to 10-C), 71.07 (d, 1C, 2-C), 170.76 (s, **C**O); MS (70 eV): *m/z* (%) 87 (13), 70 (6), 69 (6), 61 (4), 57 (7), 56 (9), 55 (17), 43 (100), 41 (34), 39 (11).

Wind Tunnel. Wind tunnel experiments were performed in the second to fourth hour of the photophase at 22°C and RH 73% in a wind tunnel described in detail by Rauscher et al. (1984). Compounds were diluted in hexane (p.a. grade, Fluka). For the bioassays, 10 µl of the stimulus to be tested were applied

on a 1-cm^2 piece of filter paper (Chromatographiepapier "Schleicher & Schuell AG" Auer-Bittmann Soulié AG, Zürich, Switzerland, 69.2 g/m^2). The filter paper was placed in the wind tunnel 20 sec before the first male was tested. For each stimulus, a series of five males was tested individually, requiring a total of 5–10 min. For each series, a new piece of filter paper was loaded with stimulus.

Males were kept individually in glass tubes (10 cm, 15 mm ID) closed at one end with a screen (mesh size 1 × 1 mm) until tested. The tubes containing the males were transferred into the wind tunnel and placed horizontally 130 cm downwind from the odor source. The following behavioral steps were recorded: activation (expressed by wing fanning), takeoff, directed flight towards the odor source, flights closer than 30 cm from the source ("close in"), landing on the source, or landing elsewhere. The test was terminated when no activity or takeoff was registered for 2 min or when the insect landed elsewhere. When synthetic stimuli were tested, only the number of successful landings on the source was analyzed. Pairwise comparisons between different blends (for example, as shown in Figure 6) were based on Fisher's exact test, calculated with the software JMP 5.0.1 (SAS Institute Inc., Cary, NC, USA).

Field Assay. The blend with the highest attractiveness in the wind tunnel was tested in the field. Treatments tested were either dispensers consisting of red rubber septa (used as lids for serum bottles, VWR International, Sweden), dental cotton rolls (diam 14 mm, length 40 mm, IVF Hartmann AG, Neuhausen, Switzerland; Hillbur et al., 2000), or control traps without pheromone dispensers. Cotton rolls were cut in three, and one third was used per dispenser. Both dispenser types were loaded with a blend of 500 ng (2*S*,9*S*)-diacetoxyundecane, 1000 ng (2*S*,10*S*)-diacetoxyundecane, and 10 ng (*S*)-2-acetoxyundecane, diluted in 20 μl of hexane. Delta traps made of waxed cardboard were provided by PheroNet AB (Alnarp, Sweden). The traps had a height of 10 cm and were equipped with a 15.5 × 9 cm insert covered with a thin layer of Tangle Trap Insect Trap Coating (Tanglefoot, Grand Rapids, MI, USA). Dispensers were positioned 1–2 cm above the insert.

The field assay was performed in Wädenswil in a plot with broccoli (*B. oleracea convar. botrytis* var. *italica* cv. Fiesta) 2 wk before harvest. At this stage, plants were 50–70 cm high, the leaf canopy was closed, and many weeds were growing between the broccoli plants. On June 10, 2003, pots containing substrate with 150–250 pupae each of laboratory-reared midges were placed in the field. Two groups of five pots each were arranged in the center of the field, 8 m apart from each other. The pots were replaced by a new batch 7 d later. Groups of traps were placed at six locations between 3.5 and 5 m from the nearest release point. Each trap group consisted of two replicates of the three treatments, i.e., a total of six traps. Traps with the same treatment were attached to the same pole at 20 and 55 cm above ground. The distance between the poles

was 30–40 cm. The sticky inserts were replaced five times within the 10 d of the experimental period, and midges were counted using a binocular microscope.

RESULTS

Gas chromatographic–electroantennographic detection analysis of an ovipositor extract revealed two peaks that elicited a response from the male antenna (RT: 13.12 and 13.31 min) (Figure 2). The mass spectra of the antennal stimulatory peaks (Figure 3) were found to show similar fragmentation patterns to those of 2,11-diacetoxytridecane and 2,12-diacetoxytridecane (molecular masses 300), the major pheromone components of the pea midge, *C. pisi* (Hillbur et al., 1999). Signals in the higher mass region pointed to a molecular mass of 272 (detectable in the mass spectrum of one of the compounds that also showed *m*/*z* 243 = 272 − 29), strongly suggesting the target compounds to be the C11 analogs of the *C. pisi* diacetates: 2,9-diacetoxyundecane and 2,10-diacetoxyundecane. This was consistent with a diagnostic ion at *m*/*z* 152 representing an undecadiene fragment (loss of two molecules of acetic acid) and *m*/*z* 61 (CH_3COOH + H) as well as *m*/*z* 103 (for a diacetate). Key fragments at *m*/*z* 87 and *m*/*z* 101 revealed the positions of the acetoxy groups along the chain (see insert in Figure 3). Retention indices were found to be two carbon units lower than those of the *C. pisi* compounds.

The synthesis of an optically active stereoisomer of 2,10-diacetoxyundecane was straightforward (Figure 1). The *bis*-Grignard reagent of 1,5-dibromopentane was reacted with (2*S*)-2-methyloxirane to furnish (2*S*,10*S*)-undecane-2,10-diol.

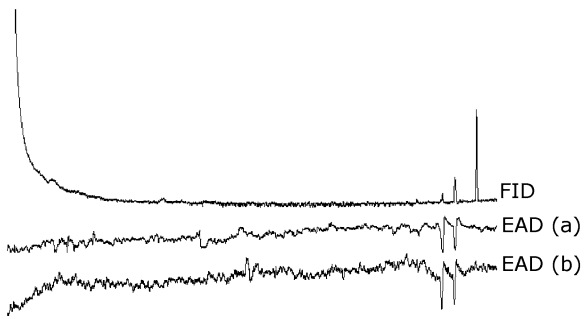

FIG. 2. Gas chromatogram with flame ionization detection (FID) and electroantennographic detector (EAD; a and b = separate male *Contarinia nasturtii* antennae) responses to a hexane extract of 30 *C. nasturtii* ovipositors (in both a and b). Repeatable antennal responses registered at 13.12 and 13.31 min.

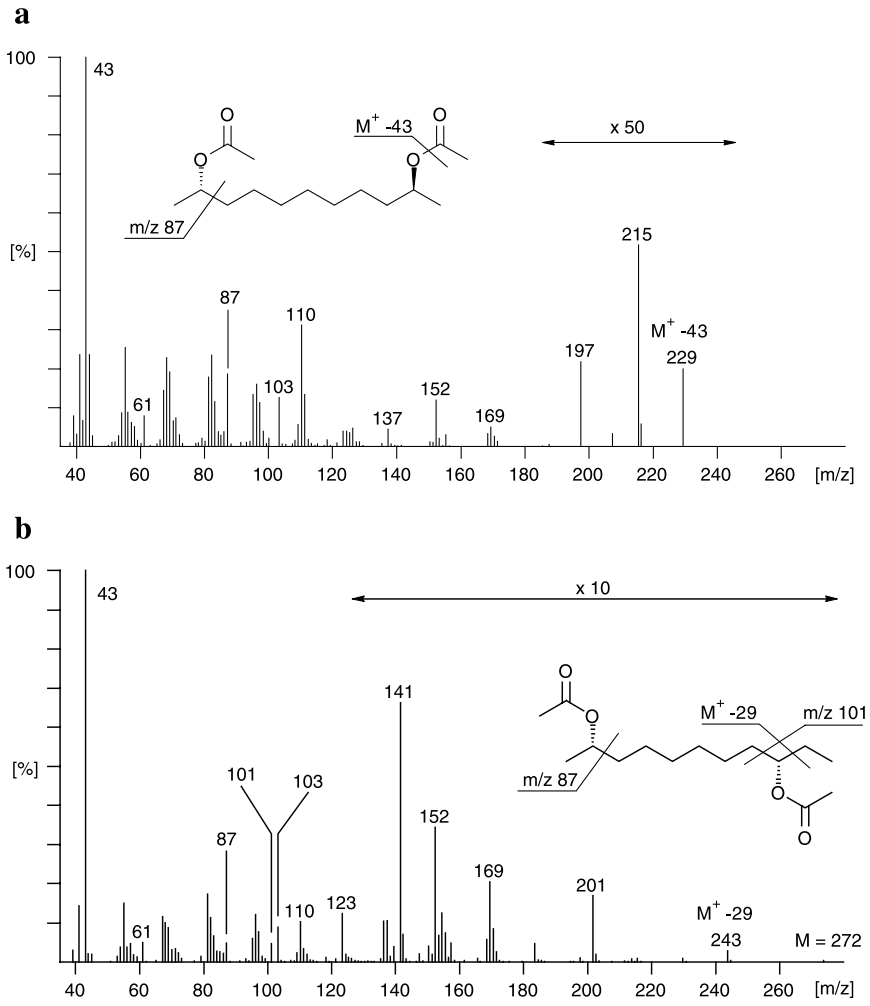

FIG. 3. Mass spectra (70 eV) and structures of (a) (2*S*,10*S*)-2,10-diacetoxyundecane and (b) (2*S*,9*S*)-2,9-diacetoxyundecane.

The corresponding diacetate was produced upon reaction with acetic anhydride. Following the same sequence but using racemic 2-methyloxirane furnished a mixture of the three stereoisomers in a ratio of 1:1:2. The two enantiomers of 2,10-diacetoxyundecane as well as the *meso*-form were well separated upon enantioselective gas chromatography. Hydrodex as a chiral stationary phase furnished an α value of tr(2*R*,10*R*)/tr(2*S*,10*S*) = 1.033 (36.36 min/35.21 min).

Structure assignment of stereoisomers was carried out by using (2*S*,10*S*)-2,10-diacetoxyundecane as a reference. Coinjection of the synthetic enantiomer and a *C. nasturtii* extract showed (2*S*,10*S*)-2,10-diacetoxyundecane to coelute with the natural product.

In analogy to the *C. pisi* pheromone, this result suggested the other EAD-active compound to be (2*S*,9*S*)-2,9-diacetoxyundecane. The synthesis of this target compound started from 7-bromo-1-heptene, which was chain elongated with (*S*)-methyloxirane via Grignard reaction (Figure 1). The resulting (*S*)-9-decen-2-ol was benzylated and reacted with AD-Mix-β to furnish (2*S*,9*S*)-9-benzyloxydecane-1,2-diol. After tosylation of the primary hydroxy group, chain elongation was carried out with methyl magnesium chloride in the presence of CuI. Debenzylation upon hydrogenation and acetylation completed the synthesis of (2*S*,9*S*)-2,9-diacetoxyundecane. Following a similar sequence but using racemic methyloxirane and nonstereoselective oxidation of a double bond furnished a mixture of the four stereoisomers, which were well separated upon enantioselective gas chromatography. Using Hydrodex as a stationary phase, the peak corresponding to (2*S*,9*S*)-2,9-diacetoxyundecane was assigned upon comparison of the retention time of the pure stereoisomer with those of the mixture

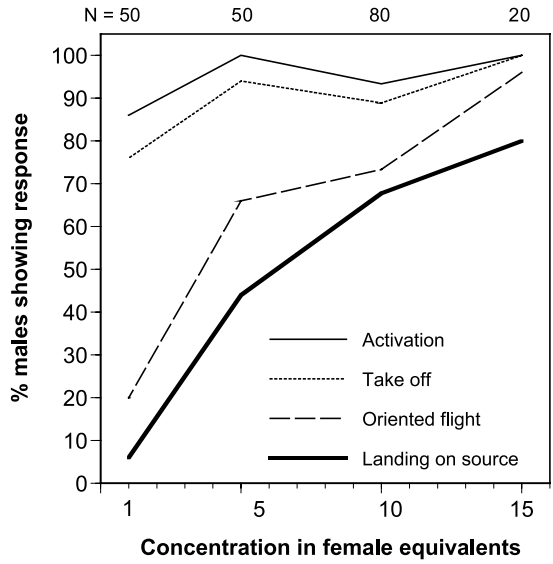

FIG. 4. Wind tunnel response of male *C. nasturtii* to extracts of calling females. The behaviors observed were activation (walking and wing fanning in glass tube), takeoff, oriented flight upwind towards the odor source, and landing on the odor source. *N* = number of males tested at each concentration.

of stereoisomers. On this column, synthetic (2*S*,9*S*)-2,9-diacetoxyundecane co-eluted with the natural product.

Because the structures of the two EAD-active compounds were closely related to the two main compounds of the pheromone system of *C. pisi*, we looked for the presence of another *C. pisi* analog, 2-acetoxyundecane. With a synthetic sample to check for retention times, and using single ion monitoring mass spectrometry (*m*/*z* 43, 87), we detected the target compound in the natural extract in extremely small amounts. Because of its low concentration, we were unable to determine the enantiomeric composition.

Wind Tunnel Assay. The number of males flying upwind and landing on the odor source increased with increasing doses of female extract on the filter paper (Figure 4). At the dose of 15 female equivalents, 92% of the males approached the odor source, and 80% landed on it. This result proved that the method was suitable for testing synthetic potential pheromone compounds.

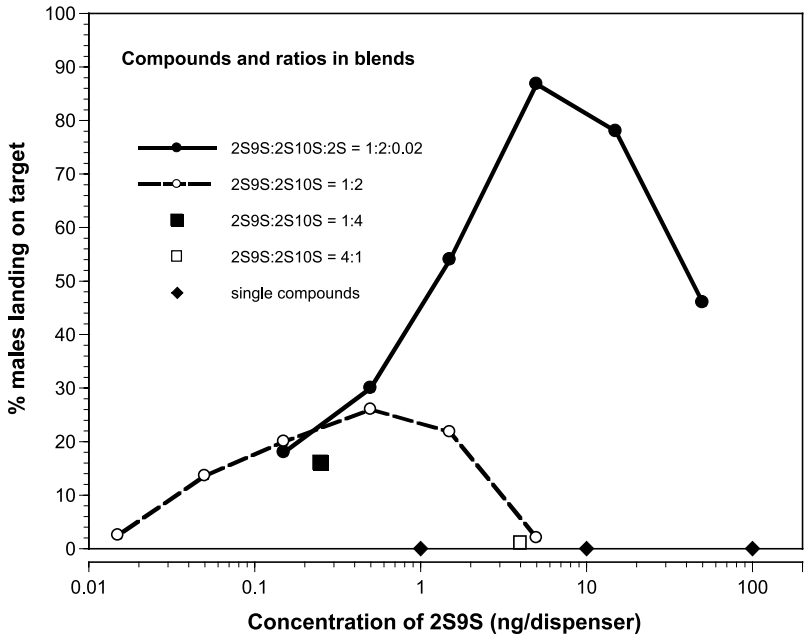

FIG. 5. *C. nasturtii* males (%) contacting the odor source in the wind tunnel when exposed to synthetic compounds. 2S9S = (2*S*,9*S*)-2,9-diacetoxyundecane, 2S10S = (2*S*,10*S*)-2,10-diacetoxyundecane, 2S = (*S*)-2-acetoxyundecane. Response to any one of the three single components at the doses indicated was always 0. $N \geq 50$ for all concentrations of blends tested; $N \geq 20$ for all concentrations of single components.

When tested singly, (2*S*,9*S*)-diacetoxyundecane and (2*S*,10*S*)-diacetoxyundecane did not elicit responses directed towards the odor source (Figure 5). Less than 15% of the tested males took off from the release point, and no males flew towards the odor source. A blend of the two components mixed in a ratio similar to that found in the pheromone gland extract at best stimulated 26% of the males to show the whole behavioral sequence from takeoff to landing on the odor source (Figure 5). Changing the ratio in the mixture more towards either (2*S*,9*S*)-diacetoxyundecane or (2*S*,10*S*)-diacetoxyundecane did not result in a higher proportion of males approaching the odor source (Figure 5). However, adding only 2% of (*S*)-2-acetoxyundecane to the blend gave a much more attractive blend with a landing rate of 87% for the most attractive dose, 5 ng (2*S*,9*S*)-diacetoxyundecane, 10 ng (2*S*,10*S*)-diacetoxyundecane, and 0.1 ng (*S*)-2-acetoxyundecane per dispenser (250 males tested, Figure 5). At this dose and ratio, the two-component blend made up of the two diacetates was unattractive to males, and the percentage landing did not differ significantly from 0 (Fisher's exact test; $P = 0.51$). The three-component blend was also significantly more attractive than the two-component blend at 1.5 ng/dispenser

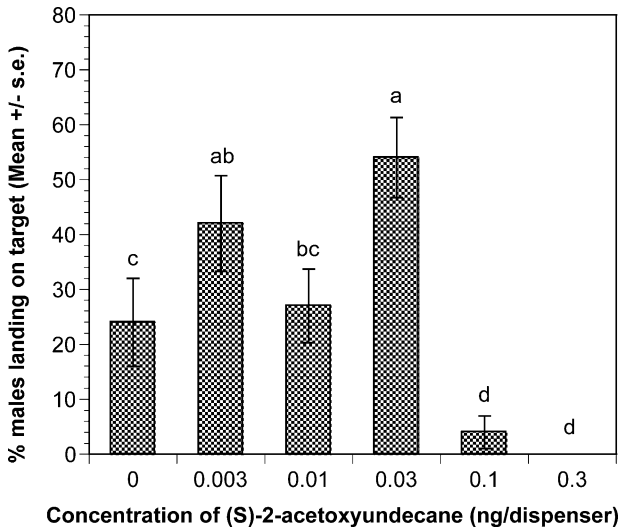

FIG. 6. *C. nasturtii* males (%) contacting the odor source in the wind tunnel in response to three-component blends with varying doses of (*S*)-2-acetoxyundecane. The dose of (2*S*,9*S*)-2,9-diacetoxyundecane and (2*S*,10*S*)-2,10-diacetoxyundecane was constant in all blends (1.5 and 3 ng/dispenser, respectively). Each dose of (*S*)-2-acetoxyundecane was tested with 45 (0.01 and 0.1 ng) or 50 (other doses) males. Different letters indicate values that are significantly different from each other in pairwise comparisons (Fisher's exact test; $P < 0.05$).

of (2*S*,9*S*)-diacetoxyundecane (Fisher's exact test; $P = 0.002$), whereas at the two lower doses tested, 0.15 and 0.5 ng/dispenser, there was no significant difference in attractiveness between the two- and the three-component blends (Fisher's exact test; $P > 0.05$).

To further investigate the influence of the relative ratio of (*S*)-2-acetoxyundecane, blends were tested with constant doses of (2*S*,9*S*)-diacetoxyundecane (1.5 ng/dispenser) and (2*S*,10*S*)-diacetoxyundecane (3.0 ng/dispenser) and varying doses of (*S*)-2-acetoxyundecane. Of the tested doses, 0.03 ng (*S*)-2-acetoxyundecane per dispenser was most attractive (Figure 6). At that dose and

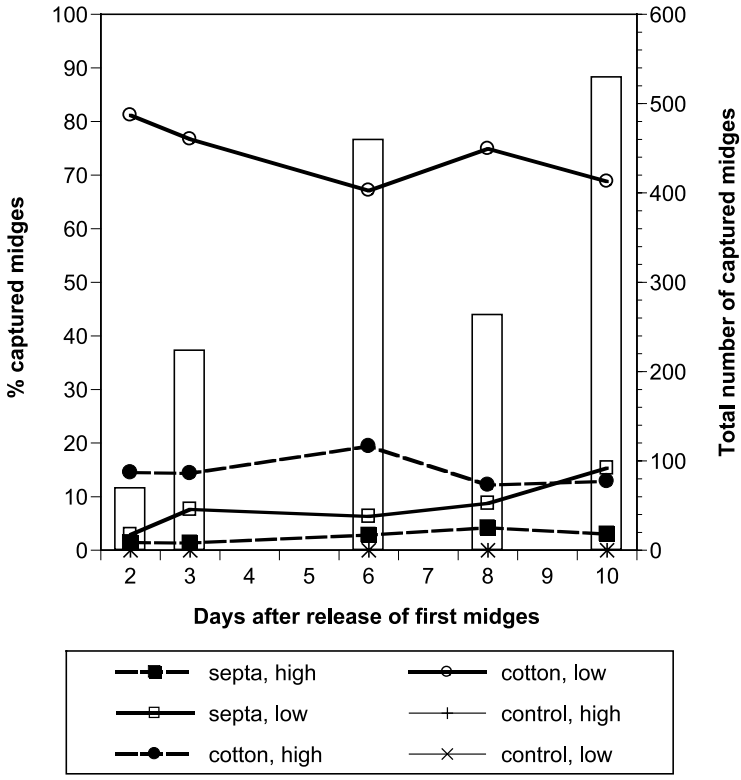

FIG. 7. Field assay with released midges and pheromone traps baited with a three-component pheromone blend [(2*S*,9*S*)-2,9-diacetoxyundecane/(2*S*,10*S*)-2,10-diacetoxyundecane/(*S*)-2-acetoxyundecane = 500:1000:10 ng] on either rubber septa or cotton rolls. Traps without dispensers were used as controls. For each treatment, traps were positioned at 20 cm (low) and 55 cm (high) above ground. Bars indicate the total number of midges in all traps on respective days. Lines indicate the distribution (%) of these midges among the treatments.

lower, at least as many males landed on the source as when exposed to the binary blend [Figure 6, dose (*S*)-2-acetoxyundecane = 0]. Higher relative ratios of (*S*)-2-acetoxyundecane significantly reduced the proportion of males landing (Figure 6). A ratio of components corresponding to the blend with 0.03 ng/dispenser in this experiment, i.e., (2*S*,9*S*)-diacetoxyundecane/(2*S*,10*S*)-diacetoxyundecane/(*S*)-2-acetoxyundecane = 1:2:0.02, was therefore used in the following field experiments.

Field Assay. An estimated total of 3000–5000 males were released in this experiment, and 1543 males were captured in the traps over 10 d. None of the control traps (without pheromone dispensers) ever caught a *C. nasturtii* male (Figure 7). During the whole assay, the traps in the lower position caught on average 81.9 ± 2.1% of the males (mean ± SE, $N = 5$ dates of counting midges). Traps with cotton roll dispensers caught 88.4 ± 2.4% of all midges captured (mean ± SE, $N = 5$ dates of counting midges), indicating that the pheromone blend released from the cotton rolls was more attractive than that released from the rubber septa.

DISCUSSION

Accumulating data on gall midge pheromones have revealed a high degree of conservatism with regard to molecular structure of pheromone components within the family Cecidomyiidae (identified compounds are listed in Choi et al., 2004). The behaviorally active three-component pheromone blend of *C. nasturtii* shows a striking similarity to that of *C. pisi*, consisting of (2*S*,11*S*)-diacetoxytridecane, (2*S*,10*S*)-diacetoxytridecane, and 2-acetoxytridecane (Hillbur et al., 1999). Except for the difference in chain length—11 carbons in *C. nasturtii* and 13 carbons in *C. pisi*—they seem identical, with regard to the number of compounds, their ratios in female gland extract, and the stereochemistry of the diacetates. In both species, however, the enantiomeric composition of the monoacetate is still unknown because the minute amount of the compounds present in gland extracts has prevented determination by GC-MS. Still, the clear behavioral activity of (*S*)-2-acetoxyundecane confirms that it is a pheromone component and that the (*S*)-enantiomer is likely to be identical with the female-produced compound. All presently identified pheromone components in gall midges are chiral compounds, and males have been shown to respond enantiospecifically, i.e., only to the female-produced stereoisomer (Harris and Foster, 1991; Gries et al., 2000, 2002; Hillbur et al., 2001; Choi et al., 2004).

At the optimal dose of the three-component blend that induces landing in almost 90% of the males, the two-component blend was unattractive. However, at low doses, the blends were equally attractive. This response pattern is partly inconsistent with the response specificity observed in several moth species using

multicomponent pheromone blends (reviewed by Linn and Roelofs, 1995). In these species, males need to perceive the natural blend of components to exhibit the complete behavioral response and will display their lowest response threshold to that blend (Linn and Roelofs, 1995). Further experiments will be needed to explain the observed behavior in *C. nasturtii*. The result of the GC-EAD analysis obtained with several pheromone gland extracts that showed no male antennal response at the retention time of (*S*)-2-acetoxyundecane suggests that the antennal sensitivity to this compound may be relatively low. To verify this, electroantennography (EAG) could be used to establish antennal dose response curves for all three pheromone components. The rather high release rate from filter paper, presumably higher for the monoacetate than for the diacetates, may distort the blend emitted. Thus, at low doses, the males may have been exposed to three-component blends with an amount of the monoacetate below the antennal detection threshold. Consequently, at the physiological level, the mixture of the two diacetates and the three-component blend may have seemed identical to the males.

In the experiment investigating the relative ratio of (*S*)-2-acetoxyundecane, 0.03 ng/dispenser [1% of the dose of (2*S*,10*S*)-diacetoxyundecane]—i.e., the same relative dose that was used in the preceding wind tunnel experiment (Figure 5)—elicited the highest behavioral response. Lower and higher relative ratios of (*S*)-2-acetoxyundecane were significantly less active (Figure 6), suggesting an optimal relative ratio of approximately 1%. An optimal relative ratio of the monoacetate was not conclusively demonstrated for *C. pisi* (Hillbur et al., 2000). In the wind tunnel, a blend with a relative proportion of 1% of the monoacetate was as attractive as pheromone gland extract of the same dose, but no other ratios were tested, and a three-component blend with a high ratio of the monoacetate (presumably 1:1:1) was not inhibitory in the field (Hillbur et al., 2000). With regard to the optimal relative ratio of (*S*)-2-acetoxyundecane in *C. nasturtii*, the inhibitory effect of higher ratios demonstrated in the wind tunnel ought to be considered if the pheromone is used for monitoring.

The field assay was conducted in a field that had had little *C. nasturtii* damage during recent years. Hence, the indigenous population was likely to have been low during the experiment. Therefore, a comparison between the estimated number of released midges and captured males indicates a high recapture rate of between 30 and 50%. This high recapture indicates that the trap is highly efficient within the tested range (3–5 m), despite the simultaneous presence of calling females. The majority of the males were caught down low in the crop (20 cm above ground), suggesting that *C. nasturtii* males, at least for short-range dispersal within the fields, fly close to the soil. The same behavior has been reported for *C. pisi* (Wall et al., 1991). Water traps at 0–30 cm above ground caught high numbers of *C. pisi* males during the morning hours, whereas traps positioned higher up caught almost exclusively females later in the day.

The result was explained by flight activity in the early morning being dominated by males in search of calling females, followed by flight of mated females to suitable oviposition sites during the afternoon (Wall et al., 1991). The *C. nasturtii* field bioassay also showed that dental cotton rolls were most effective as dispensers. Similar results were obtained previously in a field experiment with the *S. mosellana* pheromone (Celander and Hillbur, 2003) and in a release rate experiment in the laboratory with the *C. pisi* pheromone (Hillbur et al., 2000). Dental cotton rolls baited with small amounts of pheromone (1–100 μg) have also been found to stay attractive for several weeks in the field (Hillbur et al., 2001; Celander and Hillbur, 2003; Baur et al., unpublished). Cotton rolls may thus be a suitable and economic dispenser material for these types of pheromone compounds.

An important characteristic of pheromone-based monitoring systems is species specificity. To confirm the specificity of the *C. nasturtii* lure, a total of 100 male midges, retrieved from sticky papers of pheromone traps, were identified based on a molecular diagnosis method (Frey et al., unpublished). All analyzed specimens were *C. nasturtii*, confirming that attraction is species specific. To verify this finding, the pheromone blend will be tested throughout the world in regions with *C. nasturtii* populations, so that the species specificity of the catches can be assessed for the respective regions.

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RESPONSES OF FLEA BEETLE *Phyllotreta cruciferae* TO SYNTHETIC AGGREGATION PHEROMONE COMPONENTS AND HOST PLANT VOLATILES IN FIELD TRIALS

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Abstract—Male-specific compounds, previously identified from *Phyllotreta cruciferae* and synthesized or isolated from natural sources, attracted both sexes of the beetle in field trials and therefore function as components of a male-produced aggregation pheromone. Six field experiments of 7 to 10 d duration each were conducted over 2 yr using modified boll weevil traps and two doses of pheromone. Treatments containing two doses of allyl isothiocyanate (AITC), a breakdown product of glucosinolates in *Brassica napus* L., a host plant of the beetles, were included in the study. A dose response was observed for both the pheromone components and AITC, and combinations of the pheromone and AITC generally attracted greater numbers of flea beetles than did either component itself. This increased attraction to a combination of beetle-produced compounds and host odors has not been previously demonstrated in halticine beetles and could help explain patterns of movement by *P. cruciferae* into field crops.

Key Words—*Phyllotreta cruciferae*, crucifer-feeding flea beetle, aggregation pheromone, Chrysomelidae: Alticinae, field trials, kairomone.

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INTRODUCTION

The crucifer flea beetle *Phyllotreta cruciferae* (Goeze) (Coleoptera: Chrysomelidae) is a major pest of canola and crucifer crops in North America and is consistently found feeding in oilseed rape or canola fields (*Brassica rapa* L. or *B. napus* L.) on the Canadian prairies (Burgess, 1977; Lamb and Turnock, 1982; Lamb, 1989). Damage and control costs for this and a related species, *P. striolata* (F.), exceed $300 million annually (Knodel and Olson, 2002). Insecticides are the main means of control of flea beetles in canola crops, with more than 90% of the 5 million ha seeded to canola in North America treated with insecticides (Waite et al., 2001). Integrated methods of management of this chronic agricultural pest are urgently needed, including tools based on pheromones and host plant volatiles.

Males of *P. cruciferae* were previously reported to emit an aggregation pheromone that attracts both sexes based on field and laboratory experiments with live beetles (Peng and Weiss, 1992; Peng et al., 1999). Subsequently, the volatiles emitted by the beetles were analyzed, and six sesquiterpenes were identified (Figure 1). These were produced only by males, but at least one was readily sensed by the antennae of both sexes (Bartelt et al., 2001). Thus, these compounds or a subset of them was thought likely to constitute the pheromone. All six compounds are chiral, and only the enantiomers shown in Figure 1 are emitted by the beetles. Racemic forms (50:50 mixtures of the two enantiomers) of **1**, **3**, **5**, and **6** were synthesized (Bartelt et al., 2003). Subsequently, Muto et al. (2004) synthesized the individual enantiomers of compounds **1**, **3**, **5**, and **6**, using citronellal of known configuration as the chiral starting material. These studies confirmed the basic structures determined by Bartelt et al. (2001) and resolved uncertainty about the absolute configurations of these compounds.

The host range of *P. cruciferae* is confined to the order Capparales, principally to the family Brassicaceae (Feeny et al., 1970). All members of this family contain one or more anionic glucosinolate compounds. Glucosinolates and their metabolites are thought to act not only as deterrents for generalist insect feeders, but also as attractants and stimulants for specialist crucifer feeders such as crucifer-feeding flea beetles (*Phyllotreta* spp.) (Chew, 1988; Louda and Mole, 1991). The mustard oil allyl isothiocyanate (AITC), a glucosinolate breakdown product, is attractive to *P. cruciferae* in the field (Vincent and Stewart, 1984; Pivnick et al., 1992). Host plants also can affect the production of, and responses to, insect pheromones. In particular, male-produced pheromones of various bark beetles, weevils, nitidulid beetles, and other insects act synergistically with volatiles from the host plant (Landolt and Phillips, 1997). However, there is no specific information about such interactions in halticine flea beetles.

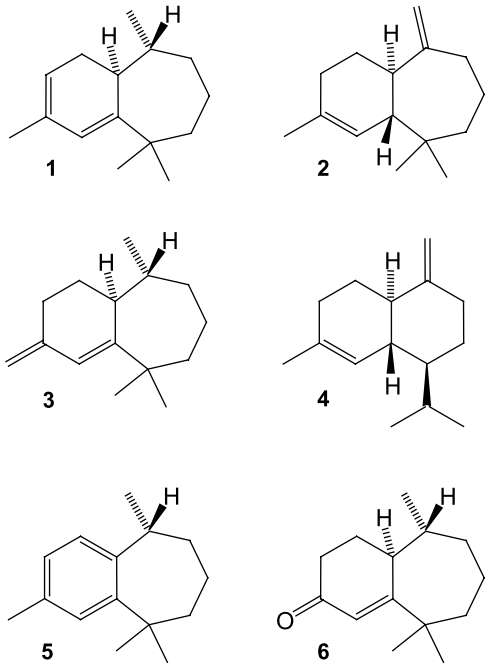

FIG. 1. Male-specific compounds previously identified from *P. cruciferae*. The compounds are numbered in the order of elution from a nonpolar GC column.

The primary objective of this research was to test whether the compounds identified from male *P. cruciferae* and subsequently synthesized were attractive to beetles in the field, relative to controls and to the host-derived attractant, AITC. A second objective was to explore beetle responses to the combination of male-specific compounds and the host plant volatile AITC.

METHODS AND MATERIALS

Male-Specific Compounds. Racemic compounds **1**, **3**, **5**, and **6** were synthesized as described previously (Bartelt et al., 2003). Compound **2** was not available for field tests. Compound **4**, γ-cadinene, was isolated from citronella essential oil (Vig et al., 1970) in a procedure described in the Appendix (available online at www.springerlink.com; search for DOI: 10.1007/s10886-005-5929-2; Electronic Supplementary Material can be found at the end of the article).

Pheromone Lures. Rubber septa (McDonough, 1991) were used as dispensers for the beetle-produced compounds in field experiments. Before loading the chemicals, all septa (20 × 11 mm diameter, Aldrich Chemical Co., Milwaukee, WI, USA) were cleaned by Soxhlet extraction for 8 hr with methylene chloride. Septa with the high dose were prepared such that the total weight of the natural enantiomers of **1**, **3**, **4**, **5**, and **6** was 500 μg and that the proportions of these enantiomers in the initial emissions from the septa were the same as the emissions from male *P. cruciferae* (Bartelt et al., 2001). The correct blend was found by an iterative approach because the proportions of the applied compounds differed from those emitted due to widely differing volatilities. Details are given in the Results section. Low-dose septa were prepared with 50 μg of the same blend used in the higher dose septa. After loading the chemicals, all septa were aired in a hood until the solvent had evaporated and then stored in tightly closed vials at −20°C until used for field tests.

The emissions from four high-dose septa were monitored in the laboratory for 2 wk to evaluate changes over time. Each septum was placed into a 50-ml flask through which air was passed at a rate of 100 ml/min. Volatile emissions were collected onto Super Q filters (Bartelt et al., 2001). The collection apparatus was kept in an incubator in the dark at 27°C. Volatiles were recovered every 1–3 d by rinsing the filters with hexane and quantified, relative to the internal standard nonadecane, by gas chromatography (GC) with a flame ionization detector. Emission per day was modeled for each component over time by linear regression.

Host Volatile Lures. Technical grade (95%) allyl isothiocyanate (AITC) (Sigma-Aldrich Chemicals, Oakville, ON, Canada) was placed in the traps in one of two configurations. In high-dose AITC lures, disposable glass culture vials (50 × 6 mm, 3.8 mm inside diameter, 1 ml capacity, Kimble Co., Nepean, ON, Canada) were filled with 0.2 ml AITC so that approximately 40 mm of the vial remained empty. At the end of the first trial, some of the vials contained one to several flea beetles in or near the surface of the AITC. Thus, in all subsequent trials the top of each vial was plugged with a small piece of foam rubber to prevent beetles from entering the vial. Evaporation rates of AITC were tested from capillary tubes with and without plugs by weighing before and after several days in the field. Plugs did not appear to hamper evaporation of the AITC.

For low-dose AITC lures, a 200-μl capillary tube (125 × 2.2 mm, 1.6 mm inside diameter, Goldseal Glassware, Becton Dickinson and Co, Parsippany, NJ, USA), was filled with approximately 10 μl AITC so that 70 mm of the inside length of the tube was not filled.

All vials and microcapillary tubes containing the AITC were weighed immediately before being placed in and after removal from the field traps to determine the average rate of AITC emission per day.

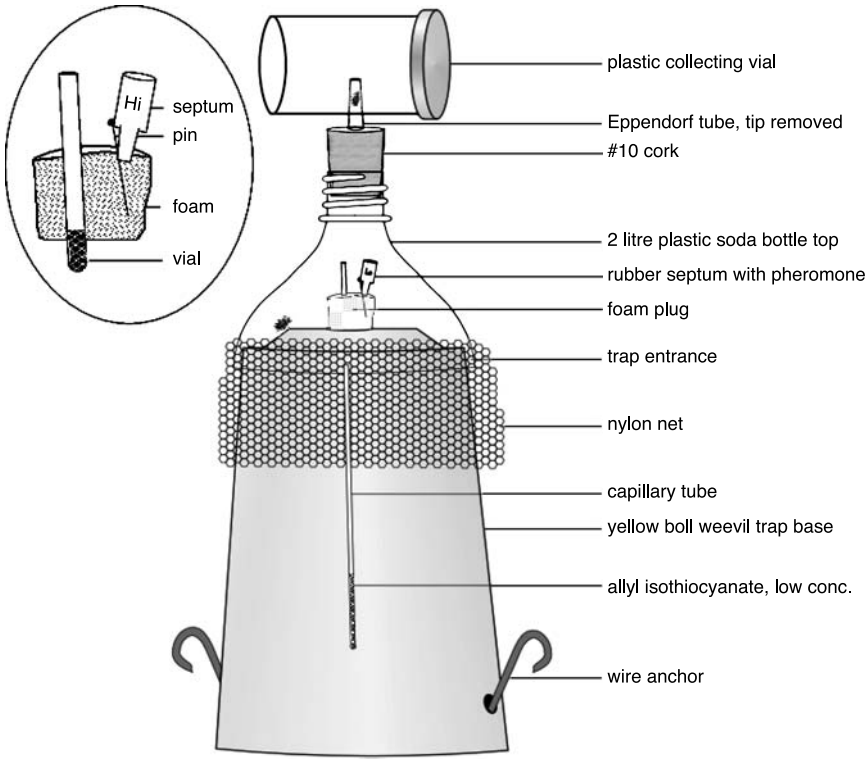

FIG. 2. Schematic diagram of trap used to test attractiveness of synthetic flea beetle compounds and/or AITC to crucifer-feeding flea beetles, *Phyllotreta* spp., in the field near Saskatoon, SK, 2001–2002. Trap shows Treatment 9, low concentration of AITC in a capillary tube, and low concentration of pheromone on a rubber septum. Enlargement shows Treatment 6, high concentrations of both allyl isothiocyanate and pheromone.

Traps. Yellow plastic boll weevil traps were used (Story Chemical Co., Starkville, MS, USA) modified as follows (Figure 2): the funnel-shaped plastic top of each trap was replaced by the upper third portion of a clear, 2-l plastic soft drink bottle and glued to the base of the trap. A No. 10 cork (30 × 20 mm diameter) was drilled with a No. 2 cork borer, and a Gilson Diamond D1000 plastic micropipette tip (Mandel Co., Guelph, ON, Canada) was placed through the cork with the tip up. Approximately 1 cm of the micropipette tip end was removed to form a hollow tube of the remainder, and the cork and modified pipette were inserted into the neck of the soft drink bottle. A #2 sized hole was bored into the side of a 100-ml plastic collecting vial, which was placed firmly on top of the pipette. A 30 × 20 mm diameter foam plug was placed in the hole

at the top of the yellow base through or onto which was inserted the appropriate treatment.

The three lure elements, that is, glass vials with AITC, capillary tubes with AITC, and rubber septa with beetle-produced compounds, were inserted onto the foam plug so that the top of each was at the same level above the top of the foam plug. In the first two trials, larger insects congested the traps. In Trials 3 to 6, a strip of nylon netting wide enough to hang one third of the way down the yellow base was taped to the soda bottle top to prevent access by large insects to the trap. Entry by flea beetles and other small insects into the traps was not impeded by the netting.

Field Sites and Experimental Design. Field trials were conducted at the Agriculture and Agri-Food Canada, Saskatoon Research Centre farm and at the Crop Development Centre of the University of Saskatchewan, near Saskatoon, SK, Canada, latitude 52°09′N, longitude 106°34′W, for 7 to 10 d duration on six occasions: early June, late June, and early September, 2001, and mid-June, late June–early July, and early September, 2002. The trials were set up in a randomized complete block design of six replicates and nine treatments per replicate, with randomization only at the beginning of each trial. For five of the trials, traps were set in a line along the edge of canola fields, with a minimum of 10 m between traps and with traps within 0.3 m of canola (*Brassica napus*) plants. In the trial conducted in September 2001, the traps were placed 5 m apart within replicates, with traps separated from canola by approximately 1.5 m. The first trials in both years were arranged in an east–west orientation, whereas all other trials were arranged in a north–south orientation. Prevailing winds in the area are from the northwest. The nine experimental treatments in each trial were the high and low pheromone doses; the high and low AITC doses, the four possible combinations of high and low doses of pheromone and AITC, and the control (foam plug alone).

The contents of the collecting vials were emptied daily (every second day in Trial 3) into plastic bags, returned to the laboratory, and the number of flea beetles counted. Flea beetles were identified to species level according to the characters described by Burgess (1977). In a subsample, the sex of 20 randomly selected flea beetles in each sample was determined by examining the ventral surface of the abdominal tip or by gently squashing it and examining the extruded genitalia. The tip of the male terminal abdominal segment is cup shaped; the male copulatory organ is a sclerotized, tobacco-colored, spoon-shaped aedeagus. Female abdomens are usually larger, with a smooth terminal abdominal segment and a clear spermatheca with a red line through the center, ending in cilia externally. The number of newly emerged or teneral adults from a 100-beetle subsample from each replicate on September 10, 2001, and from a 20-beetle subsample from each treatment and replicate of each day of Trial 6 in September 2002 also was determined. Teneral flea beetle adults, those that had emerged from

pupation less than 2 d from collection, had discernibly lighter elytra and other body parts than older flea beetles.

Statistical Analyses. For each of the six trials, the daily trap catches were summed over the days of the experiment for each of the 54 individual trap sites (9 treatments × 6 blocks). These sums were then transformed to $\log_{10}(x + 1)$ to stabilize variance, and analysis of variance was conducted using the mixed model procedure of SAS ("Proc Mixed") (SAS Institute, Inc., 2001) or Statistix software (Analytical Software, 2003). Treatments were considered as fixed effects and blocks were considered as random effects. If a significant F statistic resulted, comparisons of means were conducted. Initially, comparisons were made between the various treatments and the control using Dunnett's test (Steel and Torrie, 1980). Linear contrasts were then constructed, using means in the $\log_{10}(x + 1)$ scale, to compare the overall effects of pheromone or AITC at differing doses with the four pheromone/AITC interactions. For example, the contrast for effect of the high-pheromone dose was the mean over the three treatments that contained the high-pheromone dose (high pheromone, high pheromone plus high AITC, and high pheromone plus low AITC) minus the mean over three corresponding treatments that did not contain pheromone (control, high AITC, and low AITC). The other contrasts involved analogous sets of means. Probability values for the t statistics for these contrasts are presented with the results. The antilog of each contrast value represents the *factor* by which the subject effect increased the trap catch above the number found in the unbaited control. In analyses of beetle sex ratios, data were transformed by arcsine square root to stabilize variances. Analyses were conducted on data from individual trials, on data combined over June and September trials, and on data combined over all trials. When a significant F statistic resulted, Tukey's Studentized comparisons of means were determined.

RESULTS

Lure Emission Data. The preparation and emission properties for the high dose of male-specific compounds are summarized in Table 1. The proportions of emitted compounds differed slightly from those that were applied because of different volatilities. Compound **1** was the most volatile and became depleted most rapidly from the lures. However, the composition of the emitted blend was relatively stable over a 1-wk period.

The high-dose dispensers released 4300, 5500, 5200, 7200, 8200, and 4800 μg per day of AITC for Trials 1–6, respectively (means over 18 dispensers per experiment). The low-dose AITC dispensers had mean ($N = 18$) release rates of 620, 220, 340, 310, 590, and 280 μg per day for experiments

TABLE 1. EMISSION RATES OF SYNTHETIC *Phyllotreta cruciferae* PHEROMONE COMPONENTS FROM HIGH-DOSE RUBBER SEPTA

Compound	Weight of natural enantiomer per septum (μg)[a]	Relative amounts applied (**1** = 100)[b]	Natural relative emission rates (**1** = 100)[c]	Measured relative emission rates (**1** = 100; mean ± SE for others)[b]		Measured emission rates (μg/day) (mean ± SE)[a]	
				Initial	After 7 d	Initial	After 7 d
1	250	100	100	100	100	11.8 (±0.4)	8.2 (±0.3)
3	17	6.7	4.4	4.4 (±0.3)	5.4 (±0.2)	0.54 (±0.03)	0.41 (±0.02)
4	123	49.0	26.6	25.2 (±2.8)	39.0 (±1.6)	3.5 (±0.3)	2.9 (±0.2)
5	28	11.1	6.0	5.7 (±0.8)	9.2 (±0.5)	0.80 (±0.08)	0.67 (±0.04)
6	82	32.6	3.9	2.8 (±0.9)	7.7 (±0.5)	0.56 (±0.06)	0.54 (±0.03)

[a] Synthetic compounds **1**, **3**, **5**, and **6** were racemic, whereas compound **4** was the pure beetle-produced enantiomer. Thus, the actual gravimetric and achiral GC measurements for the amounts of **1**, **3**, **5**, and **6** were double the values shown. SE = standard error of fitted value from regression (see text).
[b] Ratios of compounds applied or measured are based on the amounts of natural enantiomers present. SE = standard error of fitted value from regression.
[c] Relative emission rates based on Bartelt et al. (2001).

1–6, respectively. Typical standard errors were in the range of 5–20% for both dosages. Release rates per day did not appear to vary appreciably over the short length of the experiments (7–10 d). For example, in the third trial, high-rate vials were removed after the second day and were found to have lost an average of 7700 μg AITC per trap per day, whereas over the entire 10 d of the test the average release rate was 5180 μg per trap per day. Flea beetle elytra were noted in some of the microcapillary tubes and may have contributed to the variability in the amount of AITC dispersed in this treatment by partially blocking air flow.

Field Experiments: General Observations. Large numbers of flea beetles were captured during the study, and over 99.5% of them were *P. cruciferae*. The totals for *P. cruciferae* captured in Trials 1 to 6, respectively, were 8,610; 10,151; 13,908; 12,940; 1,460; and 73,321. Other flea beetles captured included a total of 5 *P. striolata* and 94 *Psylliodes punctulata*, numbers too low to make inferences about treatment effects. Hymenoptera of various species also were captured in the experiments. Traps in Trials 1 and 2 frequently contained up to four *Banchus flavescens* Cresson, a large ichneumonid parasitoid of the bertha armyworm (*Mamestra configurata* Walker). Flea beetles may have been hindered in entering traps already containing *Banchus* because numbers of beetles tended to be lower in traps with the large parasitoids. After the netting was put on in Trials 3–6, larger insects were excluded from the traps. Of the micro-Hymenoptera collected, most were *Microctonus vittatus* L., a native parasitoid of *P. cruciferae*. Details of parasitoid responses to the various treatments will be the subject of a subsequent paper.

Weather conditions had a critical effect on the number of flea beetles captured, with highest numbers found after warm, sunny, and calm days, and low numbers found after cold, rainy, or windy days. Total numbers of flea beetles caught in the 54 traps in a 24-hr period varied from 1 flea beetle on June 6, 2001 (average daily temperature 12.9°C, 13 mm precipitation, north wind 10–20 kph), to 20,061 flea beetles on September 15, 2002 (average daily temperature 19.0°C, sunny, light winds). Flea beetles displayed little activity and were not attracted to any traps in very hot weather. For example, relatively few beetles were collected in Trial 5, during which maximum temperatures exceeded 35°C for several days.

Treatment Effects. Differences among treatment means were found in all six experiments (Table 2). The treatment containing high levels of both pheromone and AITC collected the greatest number of flea beetles, being more attractive than the control at $P \leq 0.001$ in all trials except the second one. All combined pheromone–AITC treatments had greater numbers of flea beetles than did the control. Furthermore, all later treatments with combinations of attractants had numerically greater numbers of flea beetles than single attractants (Table 2). In later trials, when modifications excluded large para-

TABLE 2. MEAN DAILY NUMBER OF *Phyllotreta cruciferae* PER TRAP IN FIELD TRIALS TESTING ATTRACTION OF SYNTHETIC MALE PHEROMONE OR/AND AITC

	Trial					
	1	2	3	4	5	6
Treatment	(Jun 5–Jun 12)	(Jun 22–Jun 29)	(Aug 31–Sep 10)	(Jun 13–Jun 21)	(Jun 25–Jul 2)	(Sep 11–Sep 18)
Control	6.99	17.3	16.3	0.13	0.16	75.0
Pheromone (high)	17.1	22.0	123***	1.90***	0.31	156
Pheromone (low)	9.23	13.6	58.1***	0.66	0.85	105
AITC (high)	28.9**	28.0	103***	16.0***	1.08	129
AITC (low)	8.07	13.4	50.2**	5.21***	0.60	171
Pheromone (high) + AITC (high)	46.3***	27.6	209***	88.1***	8.26***	420***
Pheromone (high) + AITC (low)	11.5	37.9	149***	33.4***	3.47***	277**
Pheromone (low) + AITC (high)	24.7*	37.9	143***	47.1***	5.00***	287**
Pheromone (low) + AITC (low)	27.3**	19.6	103***	29.9***	5.96***	223*
F statistic (8, 40 df)	6.39	3.83	16.2	37.0	10.2	4.79
P value	<0.001	0.002	<0.001	<0.001	<0.001	<0.001
Pooled % SE	21.9	21.5	22.1	32.1	34.2	22.8

Analysis was done on $\log_{10}(x + 1)$ transformed data. Means ($N = 6$) were returned to the numerical scale and then rescaled on a per-day basis for presentation. Pooled % standard errors were calculated by determining % standard errors over replicates and then averaged or pooled over treatments. Trials 1–3 were conducted during 2001, and 4–6 were conducted during 2002.
* $P = 0.05$, ** $P = 0.01$, *** $P = 0.001$; significant difference from control for each trial (Dunnett's test).

sitoids from the traps and flea beetles from the AITC vials, the means for combination treatments frequently exceeded the sum of the means for the individual treatments, indicating a synergistic response.

An examination of single-lure treatments found that the pheromone-only treatments had greater numbers of flea beetles than did the controls for the high dose in Trials 3 and 4 according to Dunnett's test (Table 2). Similar trends were seen with the AITC-only treatments, wherein high-dose AITC had a significant effect in experiments 1, 3, and 4.

The significant treatment effects indicated by the F tests were further elucidated by linear contrasts (Table 3). Overall, the high dose pheromone treatment significantly increased trap catches in all trials, and low dose pheromone traps failed to do so only once, in Trial 2. Similarly, the high dose of AITC always increased flea beetle capture, and the effect of low dose of AITC was significant in all but Trials 1 and 2. For both types of lures, the effects of the

TABLE 3. EFFECTS OF SYNTHETIC PHEROMONE AND AITC ON TRAP CATCH OF FLEA BEETLES, CALCULATED FROM LINEAR CONTRASTS

Contrast	Trial					
	1	2	3	4	5	6
Effects of	(Jun 5–Jun 12)	(Jun 22–Jun 29)	(Aug 31–Sep 10)	(Jun 13–Jun 21)	(Jun 25–Jul 2)	(Sep 11–Sep 18)
Pheromone, high dose	1.8×	1.5×	3.6×	6.5×	3.7×	2.2×
t Statistic	2.68*	2.56*	8.13***	6.89***	4.21***	3.95***
Pheromone, low dose	1.6×	1.2×	2.2×	3.7×	4.8×	1.6×
t Statistic	2.08*	0.88	4.93***	4.82***	5.09***	2.31*
AITC, high dose	3.1×	1.8×	3.0×	55×	7.2×	2.3×
t Statistic	5.30***	3.51**	6.97***	14.8***	6.40***	4.17***
AITC, low dose	1.3×	1.2×	1.9×	24×	4.9×	2.0×
t Statistic	1.29	1.32	4.03***	11.7***	5.14***	3.53**

Contrasts were calculated in the $\log_{10}(x + 1)$ scale. The antilog of the calculated contrast shown here represents the effects of the treatments, expressed as *factor increases* in numerical trap catch when the treatments are present vs. when they are not. The null hypothesis for the *t*-tests was that the value of the contrast is equal to 0, which is equivalent to saying that the factor of increase is equal to 1.00. * $P = 0.05$, ** $P = 0.01$, *** $P = 0.001$.

high doses were numerically higher than those for the low doses, except for pheromone treatments in Trial 5 (Table 3). Comparable trends can be seen in the treatment means in Table 2. These results indicate positive dose–response relationships for both types of attractants.

Over the entire study, the three treatments that contained the high dose of pheromone had mean trap catches that were 1.5–6.5 times higher than the corresponding treatments without the pheromone. For the low dose of pheromone, the factors ranged from 1.2 to 4.8; the 1.2 value, from Trial 2, was not significantly different from 1.0. All of the values for high-dose effects of AITC (1.8 to 55), were significant, as were four of the six values for low-dose effects of AITC (1.9 to 24). Only two values for the low dose of AITC, those for Trials 1 and 2, were not significantly different from 1.0 at the $P = 0.05$ level (Table 3).

Generations, Sex Ratios, and Teneral Adults. Newly enclosed adults responded similarly to trap lures (Trials 3, 6 in Table 2) as did overwintered adults (Trials 1, 2, 4, 5 in Table 2). However, absolute numbers of beetles caught in September were considerably higher than those caught in June. The pattern of attraction to treatments was different between the sexes, with greater numbers of females collected in traps with high and low levels of AITC alone than in unbaited control traps or in traps baited with high levels of pheromone alone (Table 4).

The proportion of teneral adults measured from the September 10, 2001, subsamples was 15.2% of the flea beetles caught on that date. A slightly greater

TABLE 4. SEX RATIO OF *P. cruciferae* CAUGHT IN TRAPS BAITED WITH AITC AND/OR PHEROMONE IN HIGH OR LOW DOSES

Treatment	% Females
Control	46.0 a
Pheromone (high)	44.6 a
Pheromone (low)	48.4 ab
AITC (high)	60.2 c
AITC (low)	57.5 bc
Pheromone (high) + AITC (high)	53.2 abc
Pheromone (high) + AITC (low)	51.1 abc
Pheromone (low) + AITC (high)	53.6 abc
Pheromone (low) + AITC (low)	52.1 abc
F statistic (13, 270 df)	4.56
P value	<0.001

Analysis of variance was conducted on arcsine (square root) transformed data. Means were returned to the numerical scale for presentation. Data were combined over trials and means followed by a common letter are not statistically different at $P<0.001$ Tukey's Studentized Range test.

proportion of teneral adults was noticed at the beginning of the trial. In contrast, in 2002 the number of teneral adults averaged 5.47% over the 7-d period ending September 18, with the greatest number of new adults collected on the last day of the trial. The proportion of teneral adults did not vary among trap treatments (data not presented).

DISCUSSION

The present results support the hypothesis that compounds **1**, **3**, **4**, **5**, and **6**, or a subset of these, function as a male-produced aggregation pheromone for *P. cruciferae*. Furthermore, the pheromone compounds act in conjunction with the host volatile AITC, in combination generally attracting greater numbers of beetles than each semiochemical source additively. In these ways, the chemical communication system in *P. cruciferae* is similar to those in various curculionid, scolytid, and nitidulid beetle species (Borden, 1985; Landolt and Phillips, 1997).

The dose responses of *P. cruciferae* to AITC alone were consistent with results reported earlier. Pivnick et al. (1992) found that in the field the lowest rate of AITC able to attract large numbers of flea beetles was 4000 μg/d, with minimum thresholds of about 400 μg/d, and with beetles responding to baits from less than 2 m away (Pivnick and Jarvis, 1991). It is likely that lower release rates would be attractive when insects are very close to the source or when flight occurs on calm days (Pivnick and Jarvis, 1991). Interestingly, the

responses to AITC and the male-specific compounds by themselves were generally similar in terms of trap catch, yet the emission rates for AITC were nearly 1000 times higher than for the male-specific compounds. The attraction of female *P. cruciferae* to AITC has been shown previously (Wylie, 1981; Vincent and Stewart, 1984). In our trial, one possible reason why female beetles appear to be less attracted to lures baited with pheromone may be the lack of AITC attractant in these treatments rather than the pheromone acting as a repellent. Curiously, the pheromone was still active in September, at a time of year when adults are preparing to enter overwintering diapause, rather than reproduce. This is undoubtedly a complex semiochemical system with as yet unknown biological and environmental factors affecting the way the attractants function and interact.

Several issues need to be resolved regarding the male-produced pheromone blend of *P. cruciferae*. First, it is unknown whether male-specific compound **2** elicits behavioral responses, either alone or as part of a blend. Compound **2** is a relatively minor constituent of the male-specific volatile emissions (7%) (Bartelt et al., 2001), but relative amount does not necessarily correlate with biological activity. Testing of compound **2** will be possible only when sufficient material becomes available through synthesis or isolation from a natural source. Second, racemic forms of **1**, **3**, **5**, and **6** were used in the present study, rather than the pure enantiomers that are emitted by the beetles. It is unknown whether the unnatural enantiomers in the racemic blends have any behavioral effect, either positive or negative (e.g., Mori, 1998). However, because beetles were attracted to the racemic compounds, the "unnatural" enantiomers cannot be strongly antagonistic. Third, it is unknown whether all of the compounds added to the rubber septa were in fact essential for attraction. There are examples known of male-specific compounds that are chemically related to the aggregation pheromones of nitidulid beetles, for example, that have no defined behavioral activity (Bartelt, 1999). Determining the minimum combination of chemicals required to attract flea beetles will be critically important for potential commercialization of the pheromone.

The pheromone system in *P. cruciferae* is one of only a few known examples of male-produced aggregation pheromones in leaf beetles of the Chrysomelidae, a large and important family whose pheromone biology and chemistry is poorly characterized. The first Chrysomelid pheromones to be characterized were from the *Diabrotica* spp. rootworm beetles. These are produced by females and attract only males (reviewed by Krysan et al., 1989). However, more recent identifications in other species have found male-produced pheromones that attract both sexes. These include the Colorado potato beetle (Dickens et al., 2002) and the cereal leaf beetle (Cossé et al., 2002; Rao et al., 2003). Crucifer-feeding flea beetles are an economically important insect group in the chrysomelid subfamily Alticinae. Continued exploration and elucidation of the pheromone systems of this beetle will aid in the understanding of

communications within the Alticinae and in the utilization of pheromones in practical insect management in canola.

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PRIMER AND SHORT-RANGE RELEASER PHEROMONE PROPERTIES OF PREMOLT FEMALE URINE FROM THE SHORE CRAB *Carcinus maenas*

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Abstract—The European shore crab *Carcinus maenas* is considered to rely on a female pheromone when mating. Evidence, however, is scarce on how the urine pheromone in itself affects males. We investigated male primer and releaser responses to female pheromones with methods that minimized effects from females, delivering female urine either as a pump-generated plume or deposited on a polyurethane sponge. We delivered the pheromone at different concentrations in far, near, and close/contact range to get a picture of how distance affects behavioral response. Our results show that substances in premolt female urine (PMU) function as primer and potent short-range releaser pheromones. Based on the olfactometer and sponge tests, we conclude that PMU stimulus in itself is sufficient to elicit increased search and mating-specific behaviors such as posing, posing search, cradle carrying, and stroking. Pheromone concentrations do not seem to be important for attenuating search and posing as long as the level is above a certain threshold concentration. Instead, pheromone levels seem to play a role in male acceptance of females, recruiting more males to respond, and generating better responses with increasing concentration.

Key Words—*Carcinus maenas*, Crustacea, shore crab, green crab, olfaction, chemical communication, sex, urine pheromone, primer, releaser, scaling, lek, hot spot.

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INTRODUCTION

During reproduction, matched event timing between the sexes is essential for individual fitness. The need for synchronous reproductive readiness is especially evident for mating by decapods because they can mate only during a limited time after the female has molted. Different signals (chemical, visual, and tactile) may be used by crustaceans to guarantee that a male and a female are in contact during this period.

In decapod crustaceans, mating is highly dependent on chemical cues. The presence of female sexual pheromones has been indicated in lobsters (Atema and Engstrom, 1971), crayfish (Stebbing et al., 2003), crabs (Ryan, 1966; Eales, 1974; Gleeson, 1980; Asai et al., 2000; Kamio et al., 2000), hermit crabs (Hazlett, 1996), and shrimp (Kamiguchi, 1972; Diaz and Thiel, 2004). There are also indications that tactile, visual, and hydrodynamic stimuli affect sequences of mating behavior (Bamber and Naylor, 1997; Sneddon et al., 2003). Attraction of a partner may occur on the individual level, i.e., a solitary female or a male will attract members of the opposite sex. It can also take place in a social context where groups of animals attract individuals of both sexes over a long range. This behavior is referred to as lekking and has been described in a number of animal groups (Davies, 1991), from arctiid moths (Willis and Birch, 1982) to black grouse (Hovi et al.,1997) and harbor seals (Hayes et al., 2004). According to van der Meeren (1994), lekking behavior is present in *Carcinus maenas*, but the mechanism of this long-range attraction is unknown. At near range, male cancrid and portunid crabs are able to locate a female conspecific and guard her for a period before, during, and after the molt (Hartnoll, 1969). This behavior, males locating and guarding females, is considered to be controlled by female sexual pheromones (Gleeson et al., 1984; Bamber and Naylor, 1996a). In both the blue crab *Callinectes sapidus* and the shore crab *C. maenas*, the main pheromone source in the females is believed to be the urine, although additional sources or release sites may be present (Bamber and Naylor, 1997; Bushmann, 1999). Differences in female molt state generate variation in male pheromone response (Christofferson, 1978; Bamber and Naylor, 1996b) and courtship behavior (Jivoff and Hines, 1998), reflecting changes in pheromone content or composition.

Animals showing more or less complex social hierarchies are known to use primer pheromones for regulating social and sexual behaviors. Priming pheromones from mammals such as mouse, rat, and goat (Iwata et al., 2000; Moffatt, 2003) and insects such as fire ants (Fletcher and Blum, 1981) and honeybees (Le Conte et al., 2001; Grozinger et al., 2003) are described, but evidence for primer pheromones in crustaceans is lacking. Aggregative social and dominance behaviors seem to play a role in shore crab mating (van der Meeren, 1994; Sneddon et al., 2003). Further, earlier studies on releaser

pheromones in portunid crabs have revealed temporal variability (Gleeson, 1991) and increase in precopula male responsiveness (Bamber and Naylor, 1996a). These facts have led us to believe that primer pheromones may be used in shore crab chemical communication. In decapods, physiological modification of behaviors through neurohormonal action is a well-known phenomenon (Kravitz, 1988; Wood and Derby, 1995; Doernberg et al., 2001; Tierney and Mangiamele, 2001), which supports the notion of primer pheromones modifying mating behaviors.

Crab mating systems are a complex web of chemical communication, and only basic parts are presently understood. To better understand reproductive signaling, the role of each stimulus in eliciting a part of the behavioral sequence needs to be isolated, and the responses to specific signals studied without influence from the other cues. The aim of this study was to investigate which primer and releaser effects are induced in male *C. maenas* by controlled delivery of female premolt urine. We chose female premolt urine as the stimulus for the investigation because no additional sources have been identified. To ascertain that no other uncontrolled female-released stimuli were present, premolt urine was delivered artificially by using either a stimulus pump or a treated sponge. Urine pheromone-induced priming effects were investigated at close/contact range, whereas releaser behaviors were investigated in three different spatial contexts: far range, near range, and close/contact range.

METHODS AND MATERIALS

Animals. Shore crabs *C. maenas* (L.) in the releaser experiments were caught during mating season, between July and September, with funnel nets off the Swedish west coast. Animals were discarded if they lacked a cheliped or more than one pereopod on each side. Crabs showing external signs of sacculinid infestation were also removed. Water for the tanks was pumped from outside the Gullmar fjord from a depth of 5 m. During the experimental period, water temperature was 16–22°C and salinity 27–32 ppt. Animals in holding tanks were kept at ambient lighting regimes and fed *ad libitum* amounts of blue mussels or white fish daily, the surplus food being removed 4–5 hr later. In these experiments, only males with green to orange-yellow carapace were used. This was because of the notable differences in both behavior and physiology that occur between males of long (red carapace color) and short (green carapace color) intermolt duration in this species (Abello et al., 1994; Reid et al., 1994, 1997).

Urine Collection. Female shore crabs were kept at high density ($100/m^2$) in communal tanks ranging from $1 \times 1 \times 0.5$ to $2 \times 2 \times 0.7$ m. One male crab for every 10 females was added to the tank to find females of the appropriate molt status, indicated by male precopulatory cradle carrying. Twice daily, tanks were

searched for precopula pairs, which were removed from the communal tanks and separated. After separation, males were transferred back to the communal tanks, while females were transferred to screening aquaria, each containing three males. Females in renewed precopula after 2 hr were transferred to individual tanks for urine sampling. Urine was collected from the opening of the antennal opercula with a micropipette (Bamber and Naylor, 1997). Collection from each female was made at a daily basis until 7 d after molt. The urine from each individual female was transferred to a microcentrifuge tube and immediately frozen at −20°C. Time of urine sampling and molting were noted for future reference. Two weeks after molt, each female was dissected and checked for internal signs of sacculinid infestation. Urine samples from parasitized females were not used because this parasite has a profound effect on crab physiology that interferes with molting processes and reproductive behavior (Hoeg, 1995). For sponge and olfactometer tests, urine samples from females between 2 d and 12 hr before molt were used.

Stimulus Preparation. Urine from *C. maenas* females between 2 d and 12 hr pre-molt was diluted according to the following plan and used as a stimulus source for all the experiments.

The primer experiment used frozen urine from 15 females, pooled, and diluted to 1:10 in distilled water to make a 4-ml stock. Seventy aliquots of 55 μl from the stock and 70 aliquots of 55 μl of the distilled water were used to dilute the samples and frozen for use as stimulus [premolt female urine (PMU)] and controls, respectively. Aliquots were stored at −20°C until used.

For the far-range experiment, a total of 5-ml urine from 123 females was pooled and diluted 1:40 in 200-ml filtered seawater. Two hundred milliliters of filtered seawater taken at the same time served as control.

The near range experiment used frozen urine samples from 19 females, pooled and diluted to 1:10 in distilled water to make a stock of 7 ml. Dilutions were made by sequentially taking 700 μl from the prior, tenfold more concentrated stock and diluting it to 7 ml in distilled water. Forty aliquots of 150 μl were taken from each of the 1:10, 1:100, 1:1000, and $1:10^4$ dilution stocks. These and 40 150-μl aliquots of distilled water serving as controls were frozen in microcentrifuge tubes and stored at −20°C until used. Immediately before each stimulus presentation, the 150-μl aliquot was thawed and diluted to 150 ml in filtered seawater, making final dilutions of 10^4, 10^5, 10^6, and 10^7 times.

For the close/contact experiment, frozen urine from 68 females was pooled to make a stock of 3.5 ml. Dilute stocks were made by sequentially taking 350 μl from the prior, tenfold more concentrated stock and diluting it to 3.5 ml in distilled water. Fifty four aliquots of 55 μl were taken from the undiluted pooled urine (1:1) and each of the 1:10, 1:100, and $1:10^4$ dilutions and frozen in microcentrifuge tubes. Fifty four aliquots of 55 μl of the distilled water were

used to dilute the samples, frozen, and used for controls. Aliquots were stored at −20°C until used.

Sponge Preparation. Dummy females used in the primer and close/contact experiment were made from 35 × 20 × 10 mm polyurethane (PU) sponges, prepared the following way. All sponges were washed in water, 95% alcohol, and water again to remove residual chemicals from manufacturing (crabs may eat them otherwise; Ekerholm, personal observations). They were then weighted with two 37-mm galvanized nails and immersed in a petri dish with Artificial Sea Water (ASW) (Aqua-medic reef salt®). Sponges were compressed to remove air and allow full absorption of ASW. For the primer and close/contact experiment, the sponge was placed with the long side protruding a few millimeters above the water surface to allow 50 μl of the stimulus or control (above) to be pipetted onto the front end of the sponge, immediately before serving it to the male. Each sponge was only used once.

Polyurethane sponges used for the far-range experiment were 70 × 20 × 25 mm. Before use, they were washed as above and allowed to dry. Each sponge was then saturated with liquid by absorption of 10 ml of the test stimulus (control or PMU 1:40) in a plastic bag. Sponges in bags were stored at −20°C until used. All sponges in the far-range experiment were used frozen to prolong the substance release time.

Behavioral Criterion. The following behaviors shown by males when searching for or contacting females were used. These were chosen on the grounds that they represent true sexual behaviors and could not easily be confounded with eating or agonistic behaviors.

(1) Posing: Males exposed to females or their pheromones stand up high on pereopods 2, 3, and 4 with the propodus of the chelipeds pointing forward at an angle of 90° to the front of the carapace. Pereopods 5 are held almost horizontally at or above carapace level. Bamber and Naylor (1996b) described this as a reliable criterion for sexual arousal in *C. maenas*.
(2) Posing search: Males sufficiently stimulated start a slow high-stance walk using only pereopods 2, 3, and 4 for most of the time, while keeping chelipeds and pereopods 5 as in (1).
(3) Cradle carrying: Keeping a fairly upright stance, the male grabs and holds the female with pereopods 2 or 3, not using the chelipeds. In pre- and post-copulatory behavior, the female carapace faces the male sternum, while in-copula pairs have the sterna facing each other (Bamber and Naylor, 1996b).
(4) Stroking: While cradle carrying, the male uses the medial side of the propodus and dactylus of the chelipeds for stroking against the front of the female.
(5) Pleopod insertion: The male unfolds the abdomen and inserts his pleopods in the female vulvae.

In addition to the mating-specific behaviors, number of search bouts and time spent per search bout in response were recorded in the olfactometer to see if PMU altered these. A search bout was defined as an activity period mainly directed towards the stimulus source. A search bout ended when the crab entered the shelter zone (Figure 1) to stay there for more than 90 sec.

Dummy Female Assay. For the primer experiment, and close/contact experiment, we used a modified version of the dummy female assay (Kamio et al., 2000) to examine pheromone elicited sexual behavior of male crabs towards a pheromone-treated target sponge. Male crabs were exposed to stimulus- and control-treated sponges the following way. Each sponge was submersed in the test tank approximately 10 cm from the crab and slowly moved toward the male, making sure the sponge was below the upper carapace level of the test crab. At a distance of 1–5 mm from the antennular exopodites, the sponge was held still for 20 sec, waiting for the crab to respond. When the crab grabbed the sponge with claws or second pereopods, the sponge was released. If the crab did not grab the sponge after 20 sec, the sponge was released onto the bottom. Recording of the behavior began as soon as the sponge entered the water and

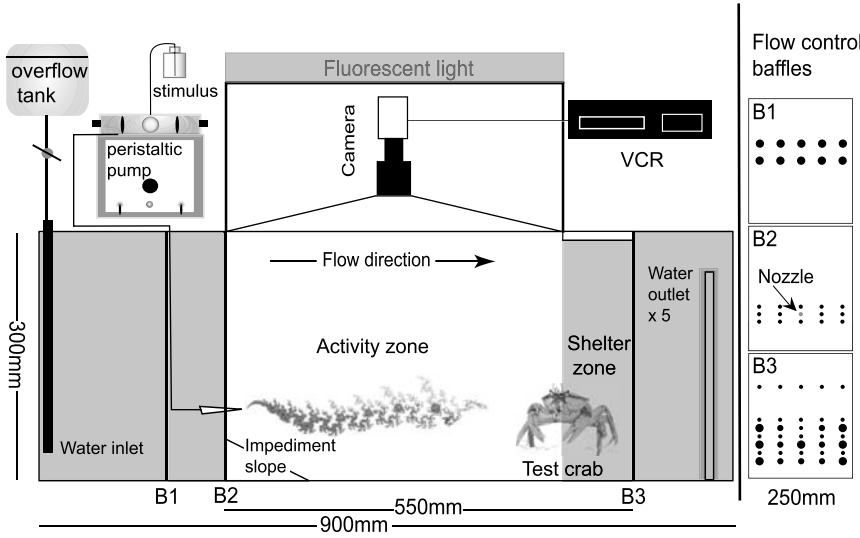


FIG. 1. Olfactometer setup for recording PMU-related activity changes in male shore crabs. The activity chamber consisted of a lighted activity zone and a dark shelter zone. PMU and control stimuli were delivered using a peristaltic pump. Activity patterns were recorded by an overhead video camera and were later analyzed manually. Baffles for controlling the stimulus flow through the activity chamber (B1, B2, and B3) are depicted to the right. Black circles represent holes mediating flow through the baffle.

continued until 90 sec had passed from the release of the sponge. Behaviors (1) and (3)–(5) (above) were registered directly into a score card, but were also videotaped for reference and later recording of cradle-carrying times. The small size of the test aquaria prevented us from reliably registering behavior (2).

Primer Experiments. To investigate if PMU has any priming effects on male behavioral response, 16 males caught off mating season (first week of April) were exposed to the following treatments. Three days before experimentation day, they were brought up to room temperature (18–21°C) and summer season lighting regimes (14:10 light/dark period) in individual 150 × 150 × 110 mm glass aquaria oxygenated by an airstone. The next day, animals were each fed with a shrimp. Water was changed daily during the whole experiment, on experimental days immediately before testing. After 4 d, the animals were considered fully acclimatized, and experimentation began. Before each test sponge, a control sponge was served to detect unselective sexual responses. None were detected in this experiment.

On the first treatment day, animals were divided into two groups (N = 8 each) and subjected to the dummy female assay (above) using either a PMU or a control sponge (above). After each test, sponges were squeezed with the forceps three times to release PMU or control water. The released substances served to prime the crabs, which were left in the treated water until the next day. By subjecting one group to control the first day, differences due to acclimatization time were controlled. On days 2, 3, and 4, both groups were tested with PMU sponges, and were left in PMU water until the next day. In this experiment, only cradle carrying and stroking behavior were analyzed. One crab in each group was excluded from the analysis because of internal sacculinid infestation or death.

Far-Range Releaser Experiments. Foldable square crab traps (700 × 500 × 250 mm) were baited with PMU-treated sponges in a setup using the pheromone as a cue for long-range search and capture. Each replicate con-

TABLE 1. RESULTING CATCHES OF SHORE CRABS *Carcinus maenas*, FROM FIELD-TRAPPING EXPERIMENTS, USING A PAIRED CONTROL-FEMALE PREMOLT URINE TREATMENT SETUP WITH 20 REPLICATES

	Statistics			
	Treatment mean ± SE			
Parameter	Control	PMU	Test statistics	P
Total catches	1.25 ± 0.36	1.85 ± 0.43	Wilcoxon Z = 1.17	ns
Male catches	0.70 ± 0.23	1.00 ± 0.20	Wilcoxon Z = 1.10	ns
Female catches	0.55 ± 0.21	0.85 ± 0.37	Wilcoxon Z = 0.45	ns

sisted of two crab traps baited with control and PMU sponges ("Stimulus Preparation," above), respectively. Pairs of traps were connected by a 6-m line. Each trap was weighted to minimize wave actions. From each trap, a rope led up to a surface marker.

Twenty pairs of traps were laid out between the 15th and 20th of August at between 1900 and 2000 hr and were taken up at between 0600 and 0700 hr the following morning. Traps were only laid out in areas where overnight food-baited trapping usually catches approximately 10 crabs. Care was taken to ensure matched environments (nearby rocks and vegetation, distance to shore and reefs) for both traps in a pair. Immediately after taking up the cages, a subset of the sponges (eight) was tested for residual pheromone activity using the dummy female assay (above). Cradle carrying was evoked in response to 63% of these, showing activity at the end of the experiment.

Near-Range Releaser Experiments. Males used for experiments were held in large communal tanks without females for between 1–2 wk at natural photoperiod. Three days before the experiment, males were put in small tanks together with two other males and an intermolt female. This equalized environments for the experimental males and avoided increased aggressiveness due to isolation effect. During these days, they were fed *ad libitum* amounts twice daily.

Search activity of 37 males was investigated with a controlled flow olfactometer. It consisted of a four-chambered flow-through tank (900 × 260 × 300 mm) with three baffles for guiding the flow (Figure 1). The activity chamber was located between baffles 2 and 3, which served to direct the stimulus flow. Inlet of ambient temperature seawater from the Gullmar fjord (see above) was regulated via a constant level overflow tank and a tap. Flow rates were 1 cm/sec, based on fluorescein dye visualization 10 cm above the activity chamber floor. Five standpipes provided controlled outlet. Stimuli were delivered through a micropipette tip in the center of baffle 2, 10 cm above the olfactometer floor. Ninety-second stimulus pulses, followed by pauses of the same duration, were generated by a Harvard apparatus 55-1762 peristaltic pump with Tygon® Pharmed® (3.2/6.4 mm inner/outer diam) tubing. Pulsed stimuli were used because continuous stimulus delivery resulted in decreasing male activity (Ekerholm, personal observation). Flow rates of the stimulus jet were adjusted to obtain a filamentous plume, shedding periodical vortices at 2- to 3-cm intervals. This corresponded to a flow rate of 150 μl/sec. An impediment slope prevented the crab from passively locating itself below the nozzle (Bamber and Naylor, 1996a).

Prior to an experiment, each crab was put into the activity chamber of the olfactometer and acclimatized for at least 4 hr without any chemical stimulus. The crab was then familiarized with the hydrodynamic stimulus from the pump by pumping filtered seawater through the experimental chamber for 30 min. During experimentation, crabs were exposed to odorants for 30 min in

order of successively higher PMU concentrations. Presentations of PMU were separated by a 15-min interval in which seawater was pumped through the experimental chamber. Seawater control and dilutions of $1{:}10^7$, $1{:}10^6$, $1{:}10^5$, and $1{:}10^4$ were used. The nonrandom approach was used to minimize effects of contamination and residual activity from previous treatments. Between trials, the tank was thoroughly washed, and the activity chamber coral gravel bedding was changed. Activities of the subject crabs were recorded using a Sony DCR-TRV730E digital-8 camera connected to a Panasonic S-VHS recorder. Number of search bouts, search time in the activity zone, and posing search were registered from video recordings. The tapes were analyzed in random order to minimize biasing.

Close/Contact-Range Releaser Experiments. Behavioral responses to PMU dilutions from $1{:}10^4$ to 1:1 were performed using the dummy female assay (above). Before testing, crabs were taken from communal holding tanks and allowed to acclimatize 2–4 hr in individual test aquaria (glass, 200 × 150 × 170 mm) with 28 ppt ASW. Temperatures were between 16 and 19°C. Fifty male crabs were subjected to a sequence of test sponges treated with increasing concentrations of PMU. Control and dilutions of $1{:}10^4$, 1:100, 1:10, and 1:1 (see "Stimulus Preparation" above) were used. After each treatment, the animal was moved to a new aquarium containing fresh ASW and given 20 min to acclimatize. Cardboard box covers minimized interference from the surroundings.

Statistics. Statistical analysis for all experiments was performed using SPSS 12.01 (SPSS Inc.) for Windows XP. For the primer experiment, presence or absence of cradle carrying and stroking was entered as dichotomous data for each exposure time and individual and analyzed using Cochran's *Q*-test for overall comparison between groups of different exposure time. Two McNemar tests were used for planned comparisons between groups of 0- and 1-d and of 1- and 2-d exposure time. Far-range trap catches were analyzed using Wilcoxon's signed ranks test. Results for each individual behavior in the near-range and close/contact-range experiments were analyzed with a global repeated measures test for all concentrations and controls followed by planned comparisons between control and individual dilutions and between pairs of sequential dilutions. Data on time per search bout (near-range experiment) was log-transformed to meet the assumptions of the following repeated-measures ANOVA. Planned comparisons used a LSD test. In the case where the transformed values (number of search bouts: near-range experiment; cradle-carrying time: close/contact range experiment) did not meet the ANOVA assumptions, Friedman's test was used together with multiple Wilcoxon's signed ranks tests. Presence/absence of posing, posing search, cradle carrying, and stroking were entered as dichotomous data and analyzed using Cochran's *Q*-test for overall comparison and multiple McNemar tests for the planned comparisons. Tests between individual treatments in the nonparametric analyses were corrected for repeated testing

using the sequential Bonferroni method (Holm, 1979) for a total experimentwise error rate of 0.05.

RESULTS

Primer Experiment. No unprimed or control primed animals responded to the PMU sponges, irrespective of groups. In both groups, three of seven animals cradle-carried the PMU sponges after 1 d of PMU exposure. After one additional day of PMU priming, six of seven animals responded to the PMU sponge by cradle carrying it. Control sponges did not yield cradle carrying or stroking responses. Because both groups showed equal trends irrespective of the 1-d adaptation length difference, statistical analysis was based on data pooled over pheromone exposure duration. Only the first three tests with PMU sponges (i.e., unprimed or control primed, 1-d pheromone priming and 2-d pheromone priming) were analyzed. The proportion of cradle-carrying and claw-stroking males differed between priming treatments (Cochran $Q_2 = 18.00$, $P < 0.001$ and Cochran $Q_2 = 13.00$, $P = 0.001$, respectively). The number of crabs showing cradle-carrying behavior differed between control and 1 d PMU-treated animals (McNemar, $P = 0.031$) and also between 1 d and 2 d PMU-treated animals (McNemar, $P = 0.031$) (Figure 2A). The number of crabs showing claw stroking differed between control and 1 d PMU-treated animals (Figure 2B) (McNemar, $P = 0.031$). No difference in claw stroking between 1 d and 2 d PMU-treated animals was found (McNemar, $P > 0.05$).

Far-Range Releaser Experiment. There were no significant differences in total, male or female trap catches (Table 1).

Near-Range Releaser Experiment. Male search activity increased in response to higher PMU concentrations in the olfactometer, indicated by a higher number of search bouts (Figure 3A) (Friedman $\chi^2_4 = 20.70$, $P < 0.001$) and longer search bouts (Figure 3B) (rmANOVA: $F_{4,144} = 8.47$, $P = 0.001$). More males responded with a posing search to higher PMU concentrations (Figure 3C) (Cochran $Q_4 = 19.82$, $P < 0.001$), verifying the sexual nature of the activity increase. Comparisons between controls and individual PMU treatments and between all pairs of sequential PMU concentrations revealed a similar pattern for the three parameters investigated. More and longer search bouts were evoked, and more males displayed a posing search at a threshold dilution of 10^6 (LSD test, $P < 0.05$; Wilcoxon, $P < 0.05$; McNemar, $P < 0.05$), but responses did not increase more with a further increase in concentration (LSD test, $P > 0.05$; Wilcoxon, $P > 0.05$; McNemar, $P > 0.05$).

Close/Contact-Range Releaser Experiment. The dummy female assay successfully elicited posing, cradle carrying, and, at higher concentrations,

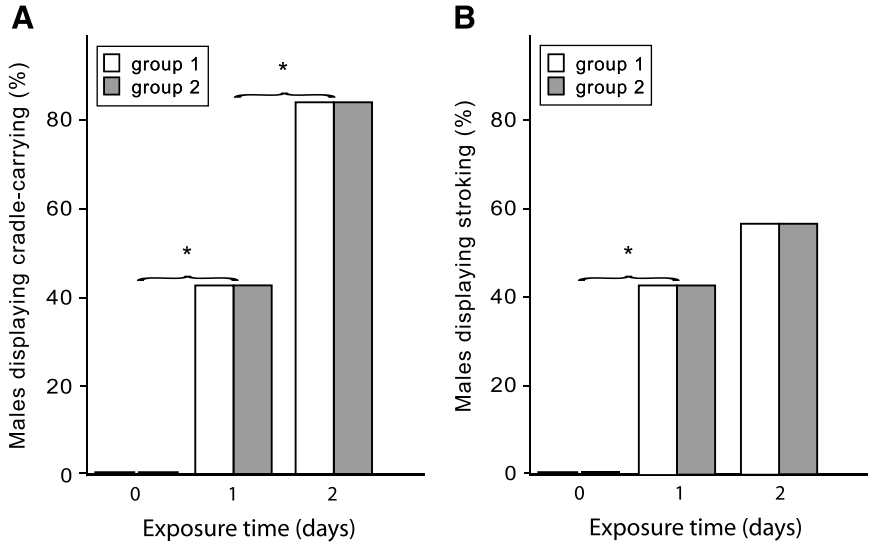
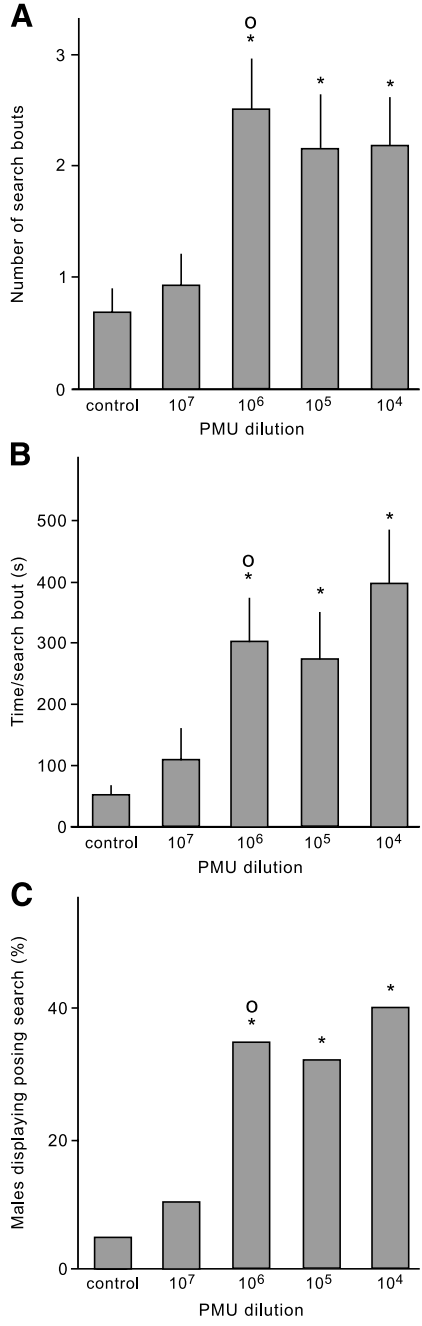


FIG. 2. Proportion of the off-mating season male *Carcinus maenas* responding to a sponge treated with 50 µl PMU diluted 1:10. (A) The percentage of males displaying cradle carrying at each exposure time. (B) The percentage of males displaying claw stroking at each exposure time. On the first experiment day, group 1 males (white bars) received a control sponge, whereas males in group 2 (gray bars) received a PMU-treated sponge serving to test and prime the animal. This generated a 1-d difference between groups in exposure time in relation to experiment days. On experiment d 2–4, both groups received PMU treatments. Crabs having no previous exposure of PMU did not cradle-carry or stroke the sponge irrespective of experiment day. After 1 d of PMU exposure, significantly more males displayed cradle carrying and stroking than in the control (both groups pooled). After 2 d of exposure, more males displayed cradle carrying than after 1 d, whereas no differences in displayed claw stroking were found. *indicates statistically significant ($P < 0.05$) differences between groups exposed to PMU for different durations.

stroking behaviors. No pleopod insertion response was observed in response to any of the dilutions. Males displaying posing differed between treatments (Cochran $Q_4 = 29.00$, $P < 0.001$). Male-characteristic pose response to PMU-treated sponges occurred at dilutions of 10^4, but significantly more poses than in the control only occurred with PMU dilutions 100 or 10 (McNemar, $P < 0.05$) but not 1 (McNemar, $P > 0.05$) (Figure 4). Male display of cradle carrying and stroking increased with concentration (Cochran $Q_4 = 91.33$ and 46.18, respectively, $P < 0.001$). Males showed cradle carrying when subjected to PMU diluted 100 times or less. The number of responding males increased with


A
Number of search bouts
0
1
2
3
control
10^7
10^6
10^5
10^4
PMU dilution
B
Time/search bout (s)
0
100
200
300
400
500
control
10^7
10^6
10^5
10^4
PMU dilution
C
Males displaying posing search (%)
0
20
40
control
10^7
10^6
10^5
10^4
PMU dilution

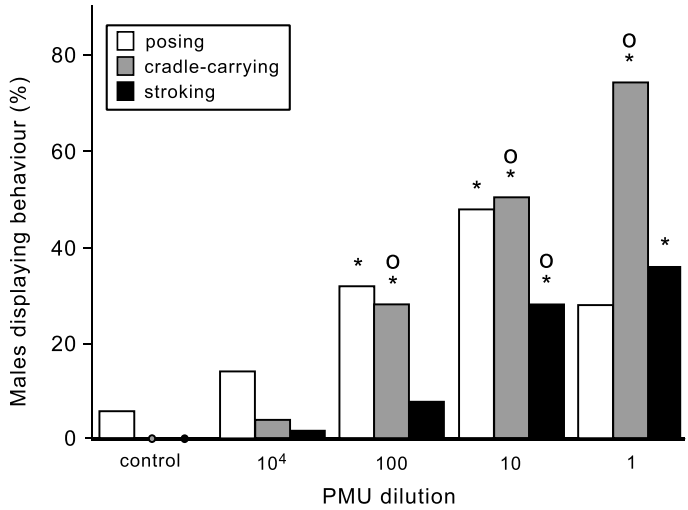


FIG. 4. Proportion of male *C. maenas* showing mating-specific posing, cradle-carrying, and stroking behaviors, when exposed to a sponge treated with increasing amounts of PMU. *indicates significant differences from control (McNemar test, $P < 0.05$). O indicates significant difference from previous, more dilute, treatment (McNemar test, $P < 0.05$).

each concentration increase (Figure 4) (McNemar, $P < 0.05$). Male cheliped stroking of the female dummies differed from the control only at PMU dilutions 10 or 1. The two active treatments did not differ from each other (McNemar, $P > 0.05$) (Figure 4). Cradle-carrying times increased with concentration

FIG. 3 Mean responses of *C. maenas* males exposed to a control plume and increasing concentrations of PMU. (A) Mean number of search bouts differed among treatments (Friedman $\chi^2_4 = 20.70$, $P < 0.001$). Urine diluted 10^6 or less evoked more search bouts than control; urine diluted 10^6 also differed from the previous, more dilute treatment ($P < 0.05$, multiple Wilcoxon's signed ranks test, sequential Bonferroni correction at $\alpha = 0.05$). (B) Time per search bout differed among treatments (rmANOVA: $F_{4,144} = 8.47$, $P = 0.001$). Urine diluted 10^6 or less evoked longer search bouts than control; urine diluted 10^6 also evoked longer search times than the previous, more dilute treatment (LSD test, $P < 0.05$). (C) Male *C. maenas* showing a posing search differed among treatments (Cochran $Q_4 = 19.82$, $P < 0.001$). Urine diluted 10^6 or less evoked less posing search than control; urine diluted 10^6 evoked more posing search than the previous, more dilute treatment ($P < 0.05$, multiple McNemar tests, sequential Bonferroni correction at $\alpha = 0.05$). Error bars represent SEM. *indicates significant differences from control. O indicates significant difference from previous, more dilute, treatment.

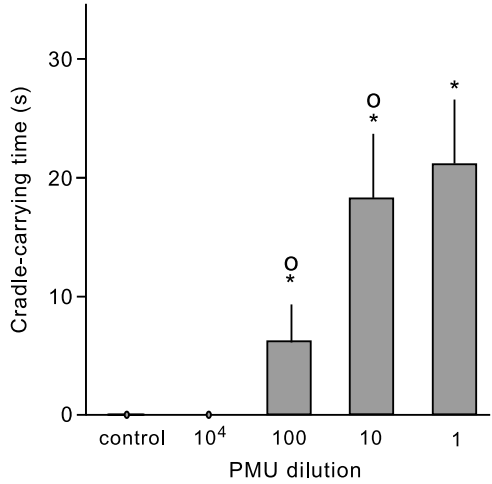


FIG. 5. Mean cradle-carrying time for male *C. maenas* exposed to a sponge treated with increasing amounts of PMU. Error bars represent SEM. *indicates significant differences from control (Wilcoxon test, $P < 0.05$). O indicates significant difference from previous, more dilute, treatment (Wilcoxon test, $P < 0.05$).

(Friedman $\chi^2_4 = 67.52$, $P < 0.001$). Dilutions 100, 10, or 1 differed from the control. Longer cradle-carrying times were observed at the dilution 10 compared with 100 (Wilcoxon, $P < 0.05$). There were no differences between dilutions of 1 and 10 (Wilcoxon, $P > 0.05$) (Figure 5).

DISCUSSION

Our investigation shows that in *C. maenas*, female premolt urine has both primer and short-range releaser functions. The female PMU is sufficient to elicit a chain of precopulatory behaviors in male crabs. These behaviors are elicited according to a response hierarchy where lower concentrations ($1{:}10^6$ dilution) evoke increased search activity and posing search. Increasing PMU concentrations to a 1:100 dilution evokes cradle carrying, and finally a 1:10 dilution evokes cheliped stroking.

Novel Primer Pheromone Function. The priming function of PMU, inducing male cradle-carrying responsiveness to PMU, represents a previously unknown pheromone quality of the female premolt urine. Because a direct behavioral reaction to the PMU was not present on the first exposure, the effect

fits the description of a primer pheromone, functioning to induce or increase male receptivity to the component(s) in PMU responsible for generating cradle carrying. When using stroking behavior as a response quality indicator, it is notable that primed off-season males responded at least as well as males caught in mating season (Figures 2 and 4). It will be impossible to determine whether the priming effect is caused by the same chemical eliciting cradle carrying and stroking until the substance(s) have been identified.

Reproductive pheromones possessing both primer and releaser properties are known in other aquatic species, such as goldfish and salmonids (Vermeirssen et al., 1997; Sorensen et al., 1998; Yambe et al., 1999; Stacey, 2003). The best understood of these are preovulatory steroids and postovulatory prostaglandins in goldfish (Stacey, 2003). As in crabs, these pheromones are released in urine, but may also be released across the gills. Regardless, the PMU-pheromone synthesis is probably not dependent on gonad maturity in *C. maenas* because gonad maturation occurs at a considerable time after the molt (Hartnoll, 1969). A more probable candidate would be a secondary metabolite or hormone indicative of molt status and female sex. Bamber and Naylor (1997) speculated that the pheromone may be a low molecular weight substance, associated with early ovarian development, carried in the hemolymph. Hardege et al. (2002) have made further progress towards identifying the pheromone, a low molecular weight substance, less than 1 kDa in size.

Because little is known about the identity of the primer pheromone, and how it exerts its effects, one may only speculate about physiological actions. In the blue crab *C. sapidus*, neuromodulatory action working in courtship display has been described by Wood et al. (1995). They showed that the peptide proctolin and the amines dopamine and octopamine influence courtship display postures. Application of dopamine modulates proctolin-evoked courtship display by lowering response thresholds, whereas application of octopamine extinguishes the display (Wood, 1995). A possible role of the primer pheromone may be to regulate activities affected by neuromodulators, which is consistent with previous observations suggesting that at least some reproductive behaviors are under neuromodulatory control (Wood, 1995).

Our investigation shows that off-season males respond better to PMU-treated sponges when previously exposed to PMU. None of the male crabs caught off mating season responded to the PMU-treated sponges when previously unexposed to the PMU. This suggests a seasonal decrease in male receptivity to the pheromone, which can be reversed with PMU exposure.

Multiple Releaser Effects. In the long-distance attraction experiment, no differences in male, female, or total trap catches were detected between control and treatment sponges. The question is why these fairly high amounts of pheromones fail to attract and capture them. The traps were baited with a much higher concentration than required to elicit posing search (2.5×10^4 times), had

a much larger volume (500 times) than needed for eliciting cradle carrying, and were placed where food-baited traps earlier caught approximately 10 males each. We believe that males do not use these pheromones to locate females over longer distances. But, we do not know the properties of the flow environments for each cage, or how well the sponge system mimics the female urine release pattern, so it is possible that hydrodynamic factors account for the lack of male attraction and capture. However, long-distance attraction of males may not be necessary in shore crab mating because premolt females seek out hot spots where mates are readily available (van der Meeren, 1994).

Search responses and posing search behaviors were elicited to PMU diluted 10^6 times or lower in the olfactometer. Number of search bouts and time spent per search bout increased by three to six times compared to the controls. Males also started displaying posing search behavior at the same PMU dilution. Increased male activity in the presence of premolt females or female-treated water has been reported in several investigations (Seifert, 1982; Bamber and Naylor, 1996b; Hardege et al., 2002). Our results further show that there is a threshold stimulus concentration, where a shift to mating-related search occurs. Higher PMU concentrations did not increase search activity. Although not indicated here, this concentration information could be important for locating females.

Posing, cradle carrying, and stroking associated with mating were evoked in response to the dummy females. Posing was present in males exposed to female dummies at PMU diluted 10^2. Most poses were displayed in response to PMU 10^1, whereas undiluted PMU did not differ from control. Cradle carrying was evoked in a concentration-dependent manner. Some males displayed cradle carrying, starting at PMU diluted 1:100, and increasing proportions at 1:10 or 1:1. Cradle-carrying time varied in the same manner indicating that increases result from both a higher proportion of males responding and stronger responses from individual males. Stroking behavior is also indicative of response quality. This only differed from the control in 10^1 or 10^0 diluted PMU treatments. The PMU-evoked cradle carrying concurred with a previous study, where premolt female treated water caused a pairing stance similar to cradle carrying (Hardege et al., 2002). Also, the threshold level of PMU-evoked cradle carrying agrees well with data for postmolt urine in *Telmessus cheiragonus* (Kamio et al., 2000). Hence, no female input apart from urine odor (and a target) is required for establishing the first steps towards mating.

Roles for PMU in Chemical Communication During Mating. Male *C. maenas* congregate at hot spots weeks before mating season (van der Meeren, 1994). During mating season, premolt females enter the hot-spot area, where they are intercepted and cradle-carried by one or more males until molt. The flow environment of the shore crabs may limit the ability of males to locate individual females over long distances. Our results suggest that PMU functions

as a short-range, signal-stimulating increased local search activity by males, who initiate search and posing behavior upon perception of the pheromone(s) in female urine. This posing may serve as a visual cue for the female, signaling male readiness to mate. Females actively seek out males (Bamber and Naylor, 1997; Sneddon et al., 2003). When the male and female get closer to each other, the male perceives more concentrated female pheromones. At a certain distance, the optimum male response is to stop posing and to seize and cradle-carry the female. According to our experiments, this shift happens between dilutions of 1:10 and 1:1, corresponding to distances of a few centimeters. At contact distance, a higher pheromone concentration may instead indicate a female closer to molt, given that pheromone concentrations increase throughout the premolt period. From a male perspective, pheromone concentration would then be correlated to the time and, therefore, the cost involved in guarding the female until molt and mating. A cost-based male choice fits well with our results, which clearly show that a higher proportion of the males cradle-carry female dummies at higher PMU concentrations. In addition, increased stroking behavior and cradle-carrying times of the individual males verifies increased attractiveness to increased PMU concentrations, which further supports the notion of PMU concentration as a cost/benefit indicator for the male.

The priming quality of the female urine represents a possible way for the female to increase male receptivity and responsiveness at the mating site. The primer pheromone could also strengthen the bond to the male presently carrying her, thereby securing her mating and survival past molt.

In summary, our results show that substances in premolt female urine function as primer and potent short-range releaser pheromones. Based on the olfactometer and dummy female test, we conclude that PMU stimulus in itself is sufficient to elicit search behaviors and mating-specific behaviors such as posing, posing search, cradle carrying, and stroking. Search and posing are elicited at threshold concentrations but do not increase with greater concentrations. Cradle carrying and stroking behavior, however, increase in a dose-dependent manner. From this, we conclude that pheromone levels are not important for attenuating search and posing as long as the level is above a certain threshold. Pheromone levels seem to play a role in male acceptance of females, recruiting more males to respond, and generating better responses with increasing concentration. The chemical communication system in *C. maenas* reflects a higher degree of complexity than previously thought. Semiochemical dependency, stereotypical behavior, and lekking in this species offer possibilities for answering many ecological and physiological questions involved in marine chemical communication.

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CHEMICAL SIGNAL MEDIATED PREMATING REPRODUCTIVE ISOLATION IN A MARINE POLYCHAETE, *Neanthes acuminata* (*arenaceodentata*)

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Abstract—*Neanthes acuminata* Ehlers (1868) is a monogamous coastal polychaete with male parental care and a high level of sexual selection. We measured the level of prezygotic isolation among allopatric populations of *N. acuminata*; from the East and West Coast of the USA, a population from Hawaii, and a laboratory culture originating from Los Angeles, CA. All populations were found to preferably mate with members of their own population. Individuals from populations from Atlantic vs. Pacific Ocean failed to pair and to mate, either during the 10 min or 48 hr experiments. Instead, individuals showed high levels of aggressive behavior. Experiments measuring the levels of interpopulation aggression, established that individuals can recognize and discriminate among different populations of *N. acuminata* on the basis of olfactory cues. Aggressive behavior was induced by exposure of animals to seawater "conditioned" by individuals from the other populations, thus demonstrating the role of olfaction in the detection of "home" populations. The aggressive display was stronger upon exposure to seawater conditioned with "unrelated" populations and especially between Pacific and Atlantic populations.

Key Words—*Neanthes acuminata*, premating isolation, sex pheromones, population variations in chemical cues.

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INTRODUCTION

The reproductive behavior of nereidid polychaetes has been studied for many years, and most species are known to spawn as heteronereids following a metamorphosis into this sexually mature form. In the broadcast spawning species, reproduction takes place in the free seawater column when performing a typical swimming behavior, the "nuptial dance," which culminates in the release of gametes by the sexual partners (Hardege, 1999). The reproduction of an individual is timed via environmental triggers including temperature, day length, lunar cycle, and additional cues such as daytime and weather conditions (Hardege et al., 1990, 1994; Bentley and Pacey, 1992) to coincide with the majority of worms in the same population. For monotelic species that die after reproduction, such coordination is essential to ensure successful fertilization (Denny and Shibata, 1989). The spawning process itself is controlled by sex pheromones released from sexually mature individuals of the opposite sex (Boilly-Marer and Lassalle, 1980), a number of which have been identified (Zeeck et al., 1996, 1998; Hardege, 1999).

Nereis (*Neanthes*) *arenaceodentata* (Moore, 1903), also known under the synonyms *Nereis* (*Neanthes*) *caudata* (Delle Chiaje, 1841) and *Neanthes* (*Nereis*) *acuminata* (Ehlers, 1868), is widely distributed along coastlines of North America, Europe, Africa, and the Indo-West Pacific (Pettibone, 1963; Day, 1973). This population complex is characterized by numerous paragnaths on both rings and hooked falcigerous seta on the proboscis. The "original" species was described as *Neanthes caudata* from European waters. Pettibone (1963) corrected this and named it *Neanthes arenaceodentata* based on the material from New England. Day (1973) renamed a North Carolina population as *Neanthes acuminata*. Reish established a laboratory population in 1964 from six specimens collected at Los Angeles Harbor, and referred to it as *N. arenaceodentata*. Weinberg et al. (1990) studied the chromosome number of different populations in this species complex. *N. acuminata* from New England has $2N = 22$, *N. arenaceodentata* from Reish's laboratory culture has $2N = 18$; collections from the mouth of San Gabriel river and Newport Bay has $2N = 18$, but the centromere of one chromosome is in a different location, and a Hawaii population has $2N = 28$. We are using the name *N. acuminata* to designate the New England populations and use *N. arenaceodentata* for California populations with specific site designation.

For over 40 yr, the species has been used as a laboratory experimental animal for ecotoxicology studies because of the ease of culture and its well-studied reproductive behavior and early development (Reish and Stevens, 1969; Reish, 1985). Among the nereidids, it is an unusual species because of its premating pairing behavior, male parental care, and the direct development of the young (Reish, 1957; Reish and Alosi, 1968). Typical behavior in encounters

between individuals, especially between those of the same sex, often involves aggressive displays, and fights that can cause cannibalism and mortality (Starczak, 1984). In contrast, intersexual encounters of ripe individuals show little degree of aggression (Reish, 1957), with the partners passing each other, and secreting mucus to form a burrow/tube inside which they remain for 1–3 wk. During reproduction, the male will increase movement (rhythmically beating of tail) within the tube and release a small amount of a white, mucus-like cloud that resembles the gamete release behavior of broadcast spawning nereidids (Weinberg and Starczak, personal communication). This is followed by the release of eggs by the female that are fertilized by the male, releasing a whitish cloud of gametes. Once the female has laid her eggs into the mucus-lined tube, she dies outside of the tube within a few hours. The male then incubates the young; the exact nature of his care is unknown but it was observed that males rhythmically beat the length of the body while positioned in the mucus tube adjacent to the egg mass. The larvae leave the parental tube after a period of approximately 21–25 d (under laboratory conditions) and construct their own individual tubes. The adult male may then find a second mate and may continue to mate. In total, the life cycle takes approximately 12–16 wk to complete in the laboratory (Reish, 1985).

When attempting to pair individuals from populations of *Neanthes acuminata* collected from various sites off the Atlantic and the Pacific coast of the US, Weinberg et al. (1990) found a significant level of interpopulation aggression that in some cases prevented the worms from pairing. While two populations from the Los Angeles area (San Gabriel River and Newport Beach) showed little aggression toward each other, there was some initial aggression between two Atlantic coast populations (Falmouth, MA, and Stonybridge, CT), but eventually these animals did pair during an observational period of 36 hr. In contrast, all attempts to pair Atlantic populations with Pacific populations failed, and the worms showed high levels of aggression and a substantial number of casualties because of the intense fighting. Interestingly, Weinberg et al. (1992) found that both Pacific populations failed to pair and mate with individuals taken from the above-mentioned laboratory-cultured collected at Los Angeles Harbor by Reish in 1964. Although these three sampling sites are separated by less than 25 miles, no successful offspring could be reared, and aggression levels were high. These results suggested evidence for a rapid species isolation following a founder event (Weinberg et al., 1992), a theory that was since rejected by Rodriguez-Trelles et al. (1996). Based on allozyme electrophoretic studies, the authors found differences between these populations that were as large as those to the Atlantic populations, and concluded that at the time when the Los Angeles Harbor (laboratory culture) population was collected it was already genetically distinct from the San Gabriel and Newport populations.

None of these studies provided an explanation of the possible causes of interpopulation aggression that prevent mating and may function as mechanisms in reproductive isolation. Weinberg et al. (1990), discussing the interpopulation aggression, suggested that one of the mechanisms by which individuals may be able to recognize kin could be the use of population-dependent chemical signals, which he described as "differences in their sex pheromones." Sex pheromones have been described in a number of nereidid polychaetes (see Hardege, 1999 for review), but little is known about pheromone variability among populations in marine invertebrates. "Kin recognition" and "detection of self" via chemical signals has been described in a number of species (see Cardé, 1986 for review) including lizards (Cooper et al., 1999; Bull et al., 2000), bumblebees (Ayasse et al., 1999), and salamanders (Rollmann et al., 2000). In the aquatic environment, little is known about the chemistry involved, and few studies have addressed this topic. Stanhope et al. (1992) describes behavioral assay-based evidence for a habitat-modified, race-specific sex pheromone in amphipods (*Eogammarus confervicolus*) based on genetic differences in the algal diet of the gammarids. Here, we demonstrate that premating isolation in *N. acuminata* populations is based on chemical cues rather than visual or tactile ones. "Conditioned seawater" taken from the various populations induces "fighting behavior" indicating that the species is capable of chemically based kin (population) recognition.

METHODS AND MATERIALS

Experimental Animals. Experimental animals represent cultures originating from collections by Dr. J. Weinberg from the intertidal sands at the mouth of the San Gabriel River, Long Beach, CA, USA, from the upper bay at Newport Beach, CA, from Alwife Cove, New London, CT, USA, West Falmouth Harbor, MA, USA, Hawaii, and the laboratory culture of Dr. D. Reish. The latter culture ("Reish culture") was established in 1964 from six animals collected at Los Angeles Harbor, allowed to grow into a population of thousands of individuals, and is maintained until today at California State University, Long Beach. A subculture of the "Reish culture" was established at Woods Hole, MA, by Weinberg in 1986. Additional cultures were established by Weinberg from field collections at West Falmouth Harbor, MA, Hawaii, San Gabriel River, Long Beach, CA, from the upper bay at Newport Beach, CA, and from Alwife Cove, New London, CT in the early 1990s. For the present study, approximately 300 individuals per population were obtained from Weinberg and cultured in the laboratory starting in 1995. Collection of worms in the field was undertaken in June between 1997 and 2002 at West Falmouth Harbor, MA, and Alwife Cove (New London), CT.

Laboratory Animal Culture. Worms were kept in natural seawater at a salinity of 32‰ and a water temperature of 20°C. Seawater was changed on a weekly basis, and the temperature and salinity were checked twice a week. The nereids were initially fed four times a week with fish food flakes (Tetra Min™) as the primary source of food, and later with low protein rabbit food and freeze-dried or frozen algae (*Enteromorpha* ssp.). These were soaked in seawater before feeding. The small particles were pipetted and dispersed through the water. The term "sexually mature" is used as a loose definition to distinguish "adult" nereids from the immature individuals. It is not a direct indication of the specimen's level of sexual receptiveness, although this is intrinsically linked. Females were identified as being mature on the basis of visible presence of eggs in the coelom. Occasionally, males could be distinguished by the whitening of coelom, due to the presence of sperm. An increase in size from the juvenile stages was the most obvious indication of maturity. Behaviorally, the mature males were also easily recognized, as they would often attempt to pair and mate. Only those worms that had been previously paired with an individual from their own population were used in behavioral experiments. This procedure was chosen to guarantee that all worms were capable of pairing given contact with the "right" partner. "Conditioned seawater" samples were produced through incubating 100 ml sterile filtered seawater (0.2 μm) with five mature males for 24 hr.

Behavioral Assays. For prezygotic isolation experiments (intra- and interpopulation aggression), individuals were taken from culture and were defined as sexually mature on the basis of physical morphology as described above, and then isolated in small glass beakers (50 ml) for 24 hr. Two individuals (one of either sex) were placed in a Petri dish with seawater and left for a 10-min period to determine if they would pair. Pairing was defined as "both individuals laying alongside one another with no signs of aggression toward or movement away from the other individual" as stated by Weinberg et al. (1990). The pairing as defined above indicates a pair bond between two individuals, which would eventually lead to mating. All experiments were carried out over a 6-wk period to minimize any differences in aggression through seasonal effects, and all measurements were taken between 12:00 and 17:00 hr to reduce diurnal variations in aggression that may occur. A Petri dish (diam 6.6 cm) was filled three quarters with seawater, and two individuals were placed into the dish, one immediately after the other. Timing was initiated at the first point of contact (proximity of less than 0.5 cm, usually followed by behavioral change such as extending jaws within seconds, but not always followed by physical contact) between the two specimens. "Conditioned water" samples used to investigate whether the observed kin recognition behavior is based on chemical cues were obtained by placing 25 individuals of a given population in 50 ml sterile filtered (0.2 μm) seawater for 2 hr. Samples were

applied to individual worms with glass pipettes (0.1 ml) at 1 cm distance in front of the individuals, and this was defined as "contact" to "odor samples." "Point Sampling" (action at a particular point in time) was used at 30-sec intervals to compare the action of the nereids with the aggression score. Sampling continued for 10 min, and all scores were tallied. Scoring of aggression was modified slightly from Reish and Alosi (1968, modifications are shown in italics): Score 0—no aggressive behavior; Score 1—defensive or avoids contact with other specimen; palpi may be flared; *or aggressive displays may be shown while the two nereids are distanced within 1 cm from one another*; Score 2—fighting position assumed; palpi flared and jaws may be extended; Score 3—aggressive, with violent attacks upon other specimen; jaws extended and biting, *following a level 3 aggression further aggressive displays may be shown while the two nereids are distanced no more than 1 cm from one another and worms actively avoid close contact*.

Statistical analysis of variance within and differences among samples was undertaken using a median test and a Kruskal–Wallis test (ANOVA). A rigorous Wald–Walfowitz test (ANOVA) was used for testing significant differences between "exposure to conditioned seawater" and direct encounters.

RESULTS

Pairing of sexually mature individuals from the five populations show significant levels of premating aggression among all specimens, except when paired with individuals from their "own" population (Figure 1a, e.g., San Gabriel males vs. other populations: $\chi^2 = 24.996$, $FG = 4$, $P < 0.001$ median test, $P < 0.001$). Specimens used for the experiment were taken from tanks with populations kept in the laboratory for at least 3 mo and in some cases ("Reish culture") nearly 40 yr. Although San Gabriel River and Newport Beach populations are separated by less than 15 miles, significant levels of premating isolation ($\chi^2 = 15.7$, $P = 0.013$ median test, $P = 0.011$) were observed with aggressive encounters reaching behavioral score 3.

Exposure of mature specimens to seawater "conditioned" by individuals from other populations also induced a behavioral response in the worms, which in some cases resulted in attacks on the glass pipette used. Similarly to worm–worm encounters, aggression levels were higher between populations than within the populations (e.g., San Gabriel male conditioned seawater: $\chi^2 = 26.499$, $FG = 4$, $P < 0.001$, median test, $P < 0.001$; Figure 1b). The chemically induced (population odor) premating isolation levels were generally lower than during worm–worm direct contact encounters, but not often statistically significant. For example, in the San Gabriel worms "conditioned seawater" was only significantly less effective than direct contact using samples from the

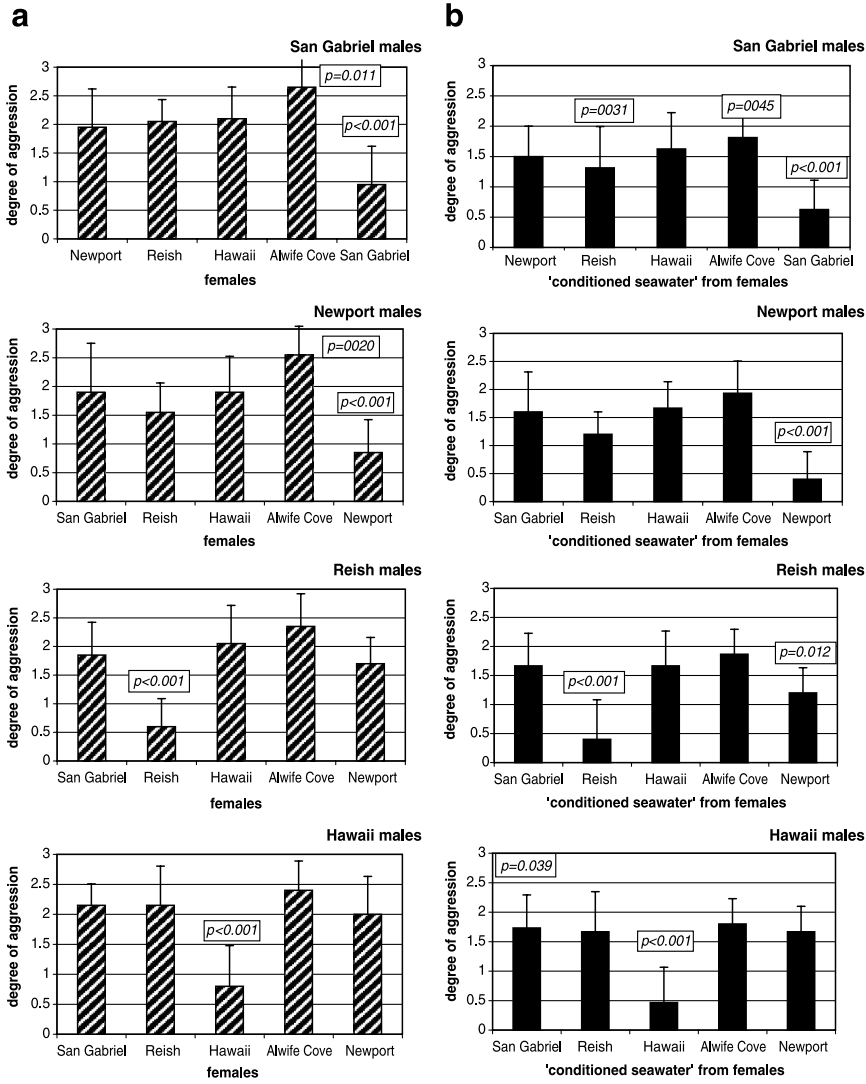

FIG. 1. Behavioral assay on pairing behavior towards various populations using: (a) exposure of individual mature males to females and (b) to "conditioned seawater" representing the (female) odor of the various populations. The *P* values, presented in boxed text, show significant differences using Wald–Walfowitz tests (ANOVA) for testing significant differences between "exposure to conditioned seawater" and direct encounters (b vs. a), and a Kruskal–Wallis test (ANOVA) to examine differences within an experiment. Number of pairing experiment repeats vary between different combinations ($N = 19-22$ repeats) due to availability of worms, error bars represent mean/SD.

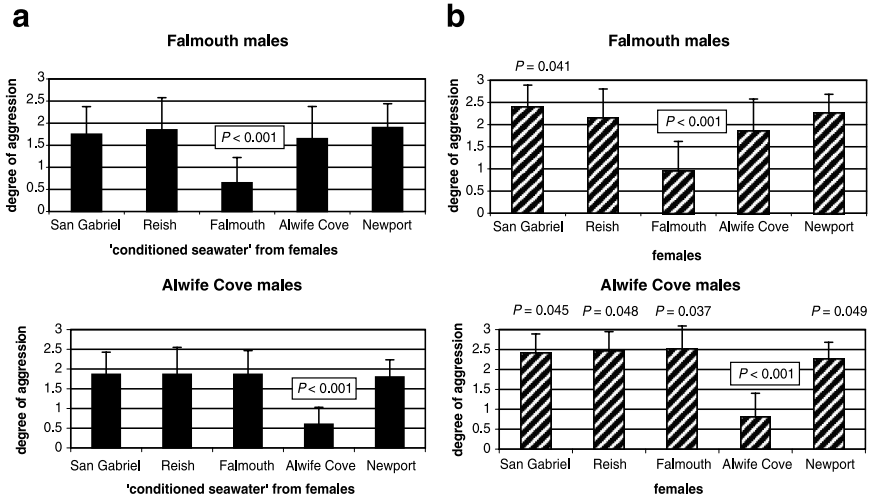

FIG. 2. Behavioral assay on pairing behavior of mature male individuals from Alwife Cove (Connecticut) and Falmouth harbor (Massachusetts) collected in the field 2 wk prior to the assays towards various populations using: (a) exposure to males, and (b) exposure to "conditioned seawater" representing the (male) odor of the various populations. The *P* values, presented in boxed text, show significant differences using Wald–Walfowitz tests (ANOVA) for testing significant differences between "exposure to conditioned seawater" and direct encounters, and a Kruskal–Wallis test (ANOVA) to examine differences within an experiment. Number of pairing experiments: $N = 21$ per data set, error bars represent mean/SD.

Alwife Cove and Reish culture samples (Reish vs. Reish H_2O: $P = 0.03$, Alwife Cove vs. Alwife Cove H_2O: $P = 0.045$; Figure 1b).

Figure 2 shows data obtained from specimens collected in the field (Falmouth, Alwife Cove) and assayed within less than 2 wk after sampling. As with all assays with culture-reared worms, the field collected population from Falmouth and from Alwife Cove showed aggressive displays toward all other populations expect to individuals of their own population ($\chi^2 = 22.467$, $FG = 4$, $P < 0.001$ median test, $P < 0.001$; Figure 2b), and the aggressive display was also inducible using "conditioned seawater" (Figure 2a).

DISCUSSION

Extreme levels of inbreeding are found in a number of species, particularly in species that are of "domestic use" and as such artificially selected and reared for consistency. In rodents, such inbreeding is thought to compromise an

individual's ability to discriminate among individuals (Nevison et al., 2000). Individual recognition and kinship play an important role in social organisms and influence competition and mate choice. In mice, as with the majority of animals, mate choice is influenced by chemical signals, pheromones. Hurst et al. (2001) demonstrated that wild house mice (*Mus domesticus*) use mouse urinary proteins (MUPs) that bind and release small volatile pheromones to mediate individual recognition. Wild mice show a large degree of diversity in the expression of MUPs that might be as great for the major histocompatibility complex (MHC) that has been suggested as the main source of the immense odor complexity in rodents, fish, and possibly in humans (Yamazaki et al., 1999). Consequently, "odor individuality" in mice and other organisms that use urine marking may be based on specific release rates of such volatile compounds rather than differences in MHC. Currently, we do not have a well-characterized aquatic invertebrate system to study this question, but the observed population differences in *Neanthes acuminata* may enable us to address this in the future. This will require studies of the chemistry involved as well as the biodiversity of MHC complexes.

The use of chemical signals in discrimination between self-produced, own population, and those produced by conspecifics has been studied in gregarious lizards, *Cordylus cordylus* (Cooper et al., 1999) and *Egerinia stockesii* (Bull et al., 2000), a number of insects (Gemeno et al., 2001; Evenden et al., 2002), and rodents (Hurst et al., 2001). In the aquatic environment, a number of studies have shown that closely related species can distinguish other species by the use of odor (e.g., stomatopods, *Gonodactylus zacae* and *G. bahiahondensis*; Caldwell, 1982), but little is known about the chemical basis of such phenomena. Kin recognition studies have focused on amphipods, *Eogammarus confevicolus*, where race-specific sex pheromones were postulated by Stanhope et al. (1992), and copepods, *Tigriopus californicus* (Palmer and Edmands, 2000).

Our experiments using *Neanthes acuminata* confirm that marine invertebrates can use odor for kin recognition. Aggression levels within a population were lower than between populations (Figure 1a), and the high degree of fighting behavior suggests that this could lead to premating reproductive isolation among the different populations. Exposure of male individuals to "conditioned seawater"—meaning incubation water in which females from various other populations were kept (see Methods and materials)—induces "aggressive display" demonstrating the importance of "chemical cues" in this behavior (Figure 1b). Odors induced aggression levels between worms from the East and West Coast of the USA are significant and confirm the allozyme electrophoretic studies by Rodriguez-Trelles et al. (1996), and the chromosome complements undertaken by Pesch et al. (1988), suggesting that it is unlikely that successful offspring can occur. Nevertheless, in all "conditioned seawater" experiments, the aggression levels are lower than in direct confrontation be-

tween worms. Although these differences are only in some cases statistically significant (see Figures 1b and 2), the ubiquitously lower level of chemical odor induced aggression (many P values between 0.06 and 0.14) indicates that the level of such interactions may not be solely chemical. Behavioral responses, such as avoidance, fleeing, and aggression display, cause additional stress, which increases the level of fights between "unfamiliar" individuals while exerting little to no effect upon "familiar, own population" pairs. This is known from crayfish (Breithaupt and Eger, 2002) and American lobster, *Homarus americanus* (Bushmann and Atema, 2000; Breithaupt and Atema, 2000), both of which use chemical signals for dominance fights to establish social hierarchies. Specimens collected in the field (Falmouth, Alwife Cove) and assayed within less than 3 wk after sampling (Figure 2b) showed aggression levels not significantly different from those obtained from the cultured worms (Figure 1a, b), indicating that culture in the laboratory did not influence the behavioral responses.

Aggression levels among individuals from the three Los Angeles area populations are significantly higher than within these populations, suggesting that all three southern Californian populations can discriminate each other, are independent, and have different odor profiles. Further genetic evidence is required to test whether these odor differences have led to the existence of at least three races within the L.A. area or even "represent a case for rapid speciation" in the laboratory, as hypothesized by Weinberg et al. (1992), but disputed by Rodriguez-Trelles et al. (1996). In addition to population genetics studies, future research will focus on high-performance liquid chromatography (HPLC) analysis of odor profiles from the various populations by using "conditioned seawater." Today, only a few examples exist where odor profiles of aquatic organisms have been studied. These includes lobster, where urine derived proteins may function in individual recognition (Karavanich and Atema, 1998; McLaughlin et al., 1999), and salmon, where odor profiles are implicated in "homing" (Solomon, 1973; Brannon and Quinn, 1990).

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Arabidopsis thaliana: A NEW TEST SPECIES FOR PHYTOTOXIC BIOASSAYS

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Abstract—Lettuce seeds (*Lactuca sativa* L.) and other crop species are often used in phytotoxic bioassays that are designed to detect allelochemicals. The seeds of these species are considered ideal because they are readily available, germinate rapidly and uniformly, and are routinely used in laboratories around the world. Despite their common use, however, the seeds of these species are often not as sensitive or responsive to some phytotoxic chemicals as are the seeds of other species. While searching for a more sensitive test species for phytotoxic bioassays, the Columbia ecotype of *Arabidopsis thaliana* exhibited greater sensitivity to seven potent allelochemicals than did lettuce seeds, which, in some cases, did not respond at all to those substances. Sensitivity satisfies one of the criteria for selecting a test species for bioassays. We now report on the results of our study and offer additional reasons for using *A. thaliana* seeds.

Key Words—Allelopathy, allelochemicals, phytotoxicity, phytotoxic bioassays, *Arabidopsis thaliana*, *Lactuca sativa*.

INTRODUCTION

Despite being first mentioned approximately 2000 years ago, allelopathy is a relatively new field of research (Rice, 1995) and refers to the interactions of secondary metabolites between neighboring plants as well as interactions between plants and other organisms such as bacteria, fungi, and algae (Rice, 1995). Secondary compounds, or allelochemicals, can induce both inhibitory

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and stimulatory effects on organisms and may play roles in shaping plant and microbial communities.

In the past, plants and their seeds have served as useful test organisms for screening allelochemicals that may affect seed germination, root growth, shoot elongation, membrane function, and photosynthetic activity (Hoagland and Williams, 2004). Where phytotoxic bioassays that specifically focus on detecting allelochemicals are concerned, lettuce seeds (*Lactuca sativa* L.) and other crop species have routinely served as the test species of choice. There is concern that these species do not reflect what is actually occurring in natural ecosystems (Inderjit and Dakshini, 1999). Jefferson and Pennacchio (2002) recently reported that lettuce seeds alone would not have detected, for example, that extracts from Australian chenopod species promote germination and growth in some of their own species instead of inhibiting it. Despite such concerns, bioassays of this type offer researchers a starting point from which to learn more about phytotoxicity and allelochemicals. Before seeds are selected for use in phytotoxic bioassays, however, they must first satisfy a number of selection criteria: be readily available; be affordable; germinate quickly, completely, and uniformly; and produce repeatable and reliable results. They must also be sensitive enough to respond to a variety of chemicals with different biochemical effects. This is especially critical where crude extracts are concerned (Hoagland and Williams, 2004). Another consideration is that the test species need to offer researchers a means by which to help identify the mechanisms of action of active compounds.

We evaluated the potential use of *Arabidopsis thaliana* (L.) Heynh (Brassiaceae) seed for use as part of a set of standard target species (STS) in phytotoxic bioassays. In our study, we tested seven potent allelochemicals and compared their effects on the seeds of Columbia (Col-3) ecotype of *A. thaliana* with those of the more commonly used lettuce seeds.

METHODS AND MATERIALS

Phytotoxicity Study. Commercially available lettuce (*L. sativa*) seed were used in this study, along with the Col-3 ecotype of *A. thaliana* seeds. This ecotype was selected after a factorial study involving 20 commonly used ecotypes. Temperature and pH studies revealed that it germinated rapidly and at a variety of temperatures and pH ranges. Furthermore, it could be tested under the same laboratory conditions as lettuce seed, which were previously established by Macias et al. (2000).

A total of 25 lettuce seeds were evenly spaced onto sterile Petri dishes (90 mm diam) lined with Whatman filter paper No. 1. Since it is known that filter

paper may influence seed germination in *A. thaliana* seeds (Rehwaldt, 1968), paper from the same batches was used in each trial. In addition, because of their smaller size, 50 *A. thaliana* seeds were used in each replicate. There were four replicates for each control and treatment group. Each Petri dish received either 3 ml of distilled water (control) or one of three different concentrations of allelochemicals. All Petri dishes were sealed with parafilm (Pechiney Plastic Packaging, Neenah, WI, USA) and incubated at 25.0°C and at a pH of 6.0. Lettuce seeds were also tested at a pH of 6.0 and at a temperature of 25.0°C as recommended by Macias et al. (2000).

All seeds were monitored for germination for a period of 7 d. Only those seeds with an emerging root length of 1 mm or greater were considered germinated. Attempt was made to minimize contamination by fungi and bacteria, but no fungicides or other antimicrobial agents were used. The study was designed to evaluate specifically the effect of allelochemicals on seed germination, and no attempt was made to determine the effects on root and shoot length. However, measuring the lengths of the emerging roots and shoots is useful in phytotoxicity studies since both may be affected regardless of whether or not germination is affected.

Allelochemicals. Seven known and commercially available compounds were used as test solutions. The allelochemicals tested were tannic acid (Sigma Chemicals), juglone (5-hydroxy-1,4-naphthoquinone, $C_{10}H_6O_3$; Acros Organics), cinnamic acid (Acros Organics), caffeic acid (MP Biomedicals), coumarin (Acros Organics), salicyclic acid (Acros Organics) and 1,8-cineole (1,8-epoxy-*p*-menthane, 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane, $C_{10}H_{18}O$; Aldrich Chemicals). These were tested at the following concentrations: control: distilled water; 1,8-cineole (0.65, 6.5, and 65 mM); coumarin (0.01, 0.10, and 1.00 μM); juglone (1, 10, and 100 μM); tannic acid (0.1, 1.0, and 10.0 mM); cinnamic acid (0.01, 0.1, and 1.0 mM); caffeic acid (0.001, 0.01, and 0.1 M; salicyclic acid (0.01, 0.1, and 1 mM).

Concentration ranges were selected based on the results of previous studies and reports, e.g., cineole concentrations for lettuce seed by Flematti et al. (2001) and coumarin concentrations by Vyvyan (2002). Those that were not reported in the literature were determined during a preliminary concentration–response study (data not shown). Because of their poor water solubility, coumarin and 1,8-cineole were initially dissolved in acetone (Sigma Chemicals), which was allowed to evaporate completely from the filter papers lining the Petri dishes. The volumes used for both acetone and water depended on the final concentration for each trial. Juglone and caffeic acid were gently heated in distilled water to dissolve these chemicals. Each of the compounds was distributed evenly across the filter paper.

Data Analysis. Germination counts were performed daily for a period of 7 d. Final germination percentage (FG%), rate of germination (RG), and mean

TABLE 1. THE EFFECTS OF KNOWN ALLELOCHEMICALS ON THE FG%, RG, AND MPFG OF *A. thaliana* (COL-3 ECOTYPE) AND *L. Sativa* SEEDS

Allelochemical	Concentration	FG%		RG (d)		MPFG (d)	
		At	Ls	At	Ls	At	Ls
1,8-Cineole (mM)	Control	100.0 ± 0.0 a	100.0 ± 0.0 a	27.8 ± 4.4 a	24.1 ± 0.4 a	2.0 ± 0.2 a	1.1 ± 0.1 a
	0.65	96.0 ± 2.2 b	100.0 ± 0.0 a	15.5 ± 0.4 b	17.6 ± 0.9 a	3.2 ± 0.0 b	1.8 ± 0.1 a
	6.50	12.5 ± 7.2 c	60.0 ± 5.9 b	1.0 ± 0.6 c	4.5 ± 0.3 b	3.2 ± 1.9 b	3.6 ± 0.1 b
	65.00	0.0 ± 0.0 c	24.0 ± 7.1 c	0.0 ± 0.0 d	2.0 ± 0.8 c	0.0 ± 0.0 c	3.6 ± 0.8 b
P value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Coumarin (μM)	Control	100.0 ± 0.0 a	100.0 ± 0.0 a	27.8 ± 4.4 a	24.1 ± 0.4 a	2.0 ± 0.2 a	1.1 ± 0.1 a
	0.01	59.5 ± 2.1 b	78.0 ± 15.7 ab	9.1 ± 0.8 b	4.6 ± 1.0 c	3.7 ± 0.3 b	4.7 ± 0.3 b
	0.10	37.5 ± 5.7 c	70.0 ± 10.9 ab	5.9 ± 0.9 b	10.8 ± 2.1 ab	3.5 ± 0.1 b	2.0 ± 0.1 c
	1.00	0.0 ± 0.0 d	54.0 ± 9.6 b	0.0 ± 0.0 c	9.1 ± 1.8 b	0.0 ± 0.0 c	1.7 ± 0.1 c
P value		<0.001	0.015	<0.001	<0.001	<0.001	<0.001
Juglone (μM)	Control	100.0 ± 0.0 a	100.0 ± 0.0 a	24.6 ± 0.2 a	24.9 ± 0.1 a	2.1 ± 0.0 a	1.0 ± 0.0 a
	1.00	100.0 ± 0.0 a	100.0 ± 0.0 a	19.5 ± 0.6 a	24.8 ± 0.1 a	2.7 ± 0.1 b	1.0 ± 0.0 a
	10.00	99.5 ± 0.5 a	100.0 ± 0.0 a	19.3 ± 3.1 a	24.9 ± 0.1 a	3.2 ± 0.1 c	1.0 ± 0.0 a
	100.00	57.0 ± 5.3 b	100.0 ± 0.0 a	4.8 ± 0.5 b	19.6 ± 0.6 b	6.1 ± 0.1 d	1.5 ± 0.1 b
P value		<0.001	–	0.104	<0.001	<0.001	<0.001
Tannic acid (mM)	Control	100.0 ± 0.0 a	100.0 ± 0.0 a	24.6 ± 0.2 a	24.9 ± 0.1 a	2.0 ± 0.0 a	1.0 ± 0.0 a
	0.10	100.0 ± 0.0 a	100.0 ± 0.0 a	17.4 ± 0.4 a	24.9 ± 0.1 a	3.0 ± 0.1 a	1.0 ± 0.0 a
	1.00	4.0 ± 1.4 b	100.0 ± 0.0 a	0.3 ± 0.1 b	25.0 ± 0.0 a	6.0 ± 0.6 a	1.0 ± 0.0 a
	10.00	0.0 ± 0.0 c	99.0 ± 1.0 a	0.0 ± 0.0 c	10.6 ± 0.3 a	0.0 ± 0.0 b	0.9 ± 0.1 a
P value		<0.001	–	<0.001	0.588	<0.001	0.588

Cinnamic acid (mM)	Control	100.0 ± 0.0 a	100.0 ± 0.0 a	24.6 ± 0.2 a	24.9 ± 0.1 a	2.1 ± 0.0 a	1.0 ± 0.0 a
	0.01	72.0 ± 7.8 b	61.0 ± 13.0 b	10.3 ± 1.0 b	9.9 ± 2.8 b	3.7 ± 0.1 b	1.8 ± 0.2 b
	0.10	52.0 ± 13.3 bc	55.0 ± 4.1 b	6.0 ± 1.6 b	10.0 ± 1.5 ab	4.6 ± 0.3 c	1.8 ± 0.2 b
	1.00	0.0 ± 0.0 c	38.0 ± 5.8 b	0.0 ± 0.0 c	4.3 ± 1.3 b	0.0 ± 0.0 d	3.4 ± 0.4 c
P value		<0.001	<0.001	0.002	0.001	<0.001	<0.001
Caffeic acid (M)	Control	100.0 ± 0.0 a	100.0 ± 0.0 a	24.6 ± 0.2 a	24.9 ± 0.1 a	2.1 ± 0.0 a	1.0 ± 0.0 a
	0.001	98.5 ± 0.9 ab	99.0 ± 1.0 a	23.1 ± 0.5 a	24.5 ± 0.2 a	2.2 ± 0.1 ab	1.0 ± 0.0 a
	0.01	94.5 ± 2.2 b	99.0 ± 1.0 a	20.3 ± 0.9 b	23.4 ± 0.4 a	2.5 ± 0.2 b	1.1 ± 0.0 a
	0.10	0.0 ± 0.0 c	98.0 ± 2.0 a	0.0 ± 0.0 c	23.5 ± 0.7 a	0.0 ± 0.0 c	1.1 ± 0.1 a
P value		<0.001	0.782	0.006	0.095	0.017	0.164
Salicyclic acid (mM)	Control	100.0 ± 0.0 a	100.0 ± 0.0 a	24.6 ± 0.2 a	24.9 ± 0.1 a	2.1 ± 0.0 a	1.0 ± 0.0 a
	0.01	98.0 ± 0.8 b	100.0 ± 0.0 a	14.1 ± 0.6 b	23.9 ± 0.4 a	3.6 ± 0.1 a	1.1 ± 0.0 a
	0.10	0.0 ± 0.0 c	59.0 ± 3.8 b	0.0 ± 0.0 c	4.6 ± 0.3 b	0.0 ± 0.0 b	3.3 ± 0.1 b
	1.00	0.0 ± 0.0 c	6.0 ± 3.5 c	0.0 ± 0.0 c	0.3 ± 0.2 c	0.0 ± 0.0 b	2.6 ± 1.5 c
P value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Different letters indicate significant differences between extract concentrations, using Tukey's compromise test. FG% = final germination percentage; RG = rate of germination (d); MPFG = mean period to final germination (d); At = *A. thaliana*; Ls = *L. sativa*. See Methods and materials for calculation of RG and MPFG.

period to final germination (MPFG) were calculated to determine the percentage of seed that had germinated at the conclusion of the study. They also revealed the rate at which germination had occurred. These three germination parameters, previously reported by Saxena et al. (1996), were calculated as follows: (1) FG%: This is the maximum average percentage of seeds that germinated during the experiment; (2) MPFG = $\sum N_i D_i$/FG; (3) RG = $\sum N_i / D_i$; where N is the daily increase in seedling number, D is the number of days from seed placement, and $\sum$ is the sum of the others.

Final germination percentages were arcsine transformed before statistical analyses according to Ott (1998). Both RG and MPFG data were $\log_{10}$ transformed. All data were analyzed by using the statistical package Systat (v. 10.2). A one-way analysis of variance (ANOVA) was performed on all transformed data to reveal differences in means. Probabilities of less than 0.05 were considered significant. Differences between means were determined by using Tukey's compromise test. Means ± SE are included in the results.

RESULTS

Sensitivity to Allelochemicals. The Col-3 ecotype of *A. thaliana* seeds was significantly more sensitive to all seven allelochemicals than were the lettuce seeds (Table 1). The FG%, RG, and MPFG were all affected in a concentration-dependent manner (Table 1). Lettuce germination was not inhibited by any concentration of tannic acid, juglone, or caffeic acid (Table 1). The RG and MPFG of lettuce were, however, affected by the highest concentration of juglone ($P < 0.001$ for both). In contrast, tannic acid inhibited *A. thaliana* FG% ($P < 0.001$) and RG ($P < 0.001$) at a concentration of 1.0 mM and prolonged MPFG at a concentration of 10.0 mM (Table 1). Juglone significantly inhibited *A. thaliana* seed germination (i.e., FG%, RG, and MPFG) at 100 μM, whereas a concentration of 0.01 M caffeic acid was sufficient to significantly affect all aspects of germination (Table 1).

Caffeic acid's chemical analog, cinnamic acid, significantly inhibited germination in both seed types (Table 1). The FG%, RG, and MPFG of both species were inhibited at a concentration of 0.01 mM ($P < 0.001$ for all). Coumarin, in contrast, produced mixed results. For example, the FG% of lettuce seed was inhibited, whereas MPFG was prolonged starting at a concentration of 0.01 μM (Table 1). The RG decreased initially at 0.01 μM (4.6 ± 1.0), but then increased at 0.1 (10.8 ± 2.1) and 1.0 μM (9.1 ± 1.8). The Col-3 ecotype was more consistent. It was inhibited ($P < 0.001$ for all three germination parameters) at a concentration of 0.01 μM (Table 1).

Finally, salicyclic acid inhibited the Col-3 ecotype at a concentration of 0.10 mM. *L. sativa* seed germination (all three measured parameters), in con-

trast, was inhibited at a higher concentration of 0.1 mM (Table 1). Eucalyptol (1,8-cineole) inhibited ($P < 0.001$) the Col-3 ecotype seed germination (i.e., FG%, RG, and MPFG) at the lowest concentration of 0.65 mM, whereas *L. sativa* seed germination was inhibited at a higher concentration of 6.5 mM (Table 1).

DISCUSSION

Other researchers, such as Leather and Einhellig (1986), Inderjit and Dakshini (1995), and Inderjit and Nilsen (2003), have discussed the shortcomings of using bioassays in laboratory-based phytotoxicity studies. However, despite some of the problems associated with them, they are useful because they focus on important aspects of allelopathy, e.g., detecting the presence of allelochemicals, mapping the fate of compounds in soil, and determining the effects on microbes in an ecosystem (Inderjit and Nilsen, 2003).

One test species, which is ideal for use as part of a set of STS in bioassays, is the cruciferous weed, *A. thaliana*. This species is sensitive to a variety of potent allelochemicals and satisfies all of the selection criteria for target species. In addition, it has been researched extensively in laboratories worldwide for a broad spectrum of molecular and other purposes. Many have already increased our understanding of plant growth and development, plant–pathogen interactions, plant pattern formation, plant physiology, and plant biochemistry (Bowman, 1994). Furthermore, the entire genome of this species has recently been mapped (The Arabidopsis Genome Initiative, 2000). As a result, there are approximately 10,000 references to this species alone in the literature and in various databases.

Also of significance are the more than 200 qualitative mutations that have been linked with the five chromosomes of *A. thaliana* (Griffing and Scholl, 1991). Some of the more interesting mutants include herbicide resistance, which may make it an attractive model for determining the mechanisms of resistance to toxic substances (Mitterbauer and Adam, 2002). This mutant and others could potentially help with identifying the mechanisms of action of certain allelochemicals.

In addition, proteomic studies will reveal the biochemical pathways affected by allelochemicals during seed germination and growth. Using a reverse proteomics approach, Gallardo et al. (2002) highlighted the importance of methionine biosynthesis in *Arabidopsis* seed germination. Tomita-Yokotani et al. (2003) reported that *A. thaliana* seed exudates promoted their own growth while inhibiting that of the fungus, *Neurospora crassa*.

With such an extensive resource available to researchers, along with the sensitivity to potent allelochemicals exhibited by the Col-3 ecotype of

A. thaliana seed and seedlings, we believe that it is should be included as part of a set of STS in allelopathy studies. A number of different ecotypes and mutants exist, many of which can be used in comparative studies, as well as for templates for the discovery of new phytotoxic substances.

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HYDROXAMIC ACID CONTENT AND TOXICITY OF RYE AT SELECTED GROWTH STAGES

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Abstract—Rye (*Secale cereale* L.) is an important cover crop that provides many benefits to cropping systems including weed and pest suppression resulting from allelopathic substances. Hydroxamic acids have been identified as allelopathic compounds in rye. This research was conducted to improve the methodology for quantifying hydroxamic acids and to determine the relationship between hydroxamic acid content and phytotoxicity of extracts of rye root and shoot tissue harvested at selected growth stages. Detection limits for an LC/MS-MS method for analysis of hydroxamic acids from crude aqueous extracts were better than have been reported previously. (2*R*)-2-*β*-D-Glucopyranosyloxy-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA-G), 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA), benzoxazolin-2(*3H*)-one (BOA), and the methoxy-substituted form of these compounds, (2*R*)-2-*β*-D-glucopyranosyloxy-4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA glucose), 2,4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA), and 6-methoxy-benzoxazolin-2(3*H*)-one (MBOA), were all detected in rye tissue. DIBOA and BOA were prevalent in shoot tissue, whereas the methoxy-substituted compounds, DIMBOA glucose and MBOA, were prevalent in root tissue. Total hydroxamic acid concentration in rye tissue generally declined with age. Aqueous crude extracts of rye shoot tissue were more toxic than extracts of root tissue to lettuce (*Lactuca sativa* L.) and tomato (*Lycopersicon esculentum* Mill.) root length. Extracts of rye seedlings (Feekes growth stage 2) were most phytotoxic, but there was no pattern to the phytotoxicity of extracts of rye sampled at growth stages 4 to 10.5.4, and no correlation of hydroxamic acid content and phytotoxicity (I_{50} values). Analysis of dose–response model slope coefficients indicated a lack

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of parallelism among models for rye extracts from different growth stages, suggesting that phytotoxicity may be attributed to compounds with different modes of action at different stages. Hydroxamic acids may account for the phytoxicity of extracts derived from rye at early growth stages, but other compounds are probably responsible in later growth stages.

Key Words—DIBOA glucose, (2*R*)-2-*β*-D-glucopyranosyloxy-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one, DIBOA, 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one, BOA, benzoxazolin-2(*3H*)-one, DIMBOA glucose, (2*R*)-2-*β*-D-glucopyranosyloxy-4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one, DIMBOA, 2,4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one, MBOA, 6-methoxy-benzoxazolin-2(3*H*)-one.

INTRODUCTION

Rye (*Secale cereale* L.) is a popular winter annual cover crop in conventional and conservation tillage production systems of major vegetable and field crops including sweet corn (*Zea mays* L.) (Burgos and Talbert, 1994), soybean (*Glycine max* L.) (Liebel et al., 1992), and tomatoes (*Lycopersicon esculentum* Mill.) (Masiunas et al., 1995). Rye has many desirable attributes that make it a suitable winter cover crop for cool, northern climates: seeds germinate quickly, seedlings establish in cool weather, young plants produce deep roots that reduce soil erosion and recycle nutrients, and mature plants yield 7–8 t/ha of biomass that can enhance soil tilth. Rye residues exhibit numerous physical and chemical attributes that can influence subsequent crops and their environment. Physical attributes of rye on the surface of soils can affect the radiation, thermal, and hydrological environment that in turn can influence emerging crops, weeds, and pests (Teasdale and Mohler, 1993; Teasdale et al., 2004). Chemical release of toxins and production of phytotoxic microbial products from rye residues has been widely documented (Putnam, 1985; Niemeyer, 1988; Weston, 1996).

Allelopathy in rye is attributed to two major compounds: 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) and its breakdown product, benzoxazolin-2(*3H*)-one (BOA) (Barnes et al., 1987). Both DIBOA and BOA are abundant in rye. They arise sequentially as DIBOA glucose (DIBOA-G) is enzymatically degraded to DIBOA. BOA is formed by the natural breakdown of the DIBOA (Niemeyer, 1988). Additional allelopathic compounds have also been identified in rye (Shilling et al., 1986), but they are considered to have lower activity and are seldom considered when chemical-specific allelopathy studies on rye are carried out (Barnes et al., 1987; Niemeyer, 1988).

The concentrations of DIBOA and BOA in rye may be affected by many variables. Burgos et al. (1999) reported over a tenfold difference in the concentration of DIBOA among eight cultivars. Mwaja et al. (1995) showed that DIBOA and BOA concentrations and allelopathic activity varied with plant

fertility, being higher when rye was grown at low to moderate fertility than at high fertility. Reberg-Horton et al. (2005) found major concentration differences of DIBOA within several rye cultivars based on plant age and stage of development. Several studies have compared differences in phytotoxicity of rye root *versus* shoot tissue with conflicting results (Barnes and Putnam, 1986; Chase et al., 1991; Hoffman et al., 1996). Research is needed to determine changes in allelopathic compounds in rye tissue as a function of plant development.

When assessing the main allelopathic chemicals in rye, many researchers have attributed the major activity to DIBOA (Barnes et al., 1987; Burgos and Talbert, 2000). In the intact rye plant, the major form of DIBOA is the glycone form, DIBOA glucose (DIBOA-G), which rapidly loses the glucose moiety through enzymatic cleavage when the plant tissues are damaged (Hietala and Virtanen, 1960). Subsequent breakdown of DIBOA to BOA is enhanced through heating and mechanical action of the macerated plant extracts (Tang et al., 1975). These three DIBOA-related compounds make up the hydroxamic acid content in rye as described by Burgos et al. (1999). However, the methoxy-substituted family of hydroxamic acids has also been detected in rye, e.g., DIMBOA glucose, DIMBOA (Hofman and Hofmanová, 1969) and MBOA (Tang et al., 1975). Thus, to assess the total hydroxamic content of rye, one needs to measure both the DIBOA family, referred to here as the hydroxamic acid (HA) content, and the DIMBOA family, referred to as methoxy-substituted (MHA). Note that HA also has been classified as "BX" by Reberg-Horton et al. (2005) in reference to the fact that "B" and "X" are unique letters in the chemical name common to all of these compounds, "benzoxaz...one." Assessment of the total hydroxamic acid content of rye (HA + MHA) was a major goal of this study.

There are few analytical methods that have adequately accounted for all members of the hydroxamic acid (HA + MHA) family of compounds from rye. Early research focused on methods utilizing colorimetry and spectrophotometry by measuring the absorbance of a blue complex with $FeCl_3$ (Baker and Smith, 1977). This approach was laborious and limited by the fact that benzoxazolinones (BOA and MBOA) do not react with $FeCl_3$ (Lyons et al., 1988). At present, identification and quantification of HA and MHA are typically done by high performance liquid chromatography-ultraviolet (HPLC-UV) (Gutierrez et al., 1982; Lyons et al., 1988; Burgos et al., 1999) or gas chromatography (GC) (Tang et al., 1975; Woodward et al., 1979a,b). However, none of these methods is capable of detecting low (<1 μg/g dry wt) concentrations in rye. Gas chromatographic (GC) methods, both GC/flame ionization (FID) (Tang et al., 1975; Woodward et al., 1979a) and GC/mass spectrometry (MS) (Woodward et al., 1979b), provide sensitivities similar to HPLC-UV methods, and the MS method provides additional qualitative assurance of compound identity. However, current GC methods require lengthy derivatization steps that make the techniques

more complicated than LC. Only two studies report the presence of MHA components in rye, Hofman and Hofmanová (1969) and Tang et al. (1975). Most investigators do not look for the MHA compounds, even though Tang et al. (1975) reported fairly high levels in their study, i.e., 120 μg/g fresh wt. leaves of rye and 310 μg/g fresh wt. in rye roots using gas chromatographic methods. They reported 710 μg/g fresh wt. of BOA in these same extracts.

With the advent of liquid chromatography coupled to triple quadrupole mass spectrometry using electrospray ionization methods (LC/ESI/MS-MS), and the numerous improvements to simultaneous qualitative and quantitative characterization of liquid chromatographically separable materials that it offers, we decided to use these methods to develop a new analytical method for determining the analysis of benzoxazinone derivatives in rye extracts. Other investigators (Cambier et al., 2000; Bonnington et al., 2003a) explored variations of these methods for analysis of benzoxazinones in wheat and corn. The analytical goals were to develop an LC/ESI/MS-MS method that would work directly on crude aqueous extracts and one that would provide lower method detection limit values than currently available. Consequently, this study was undertaken to: (1) develop an LC/ESI/MS-MS method to work directly on crude aqueous extracts and lower detection limits; (2) evaluate phytotoxic activity in aerial and root tissues at various stages of plant growth from seedling to maturity; and (3) determine potential correlations between hydroxamic acid concentration and phytotoxicity at these different growth stages.

METHODS AND MATERIALS

Plant and Tissue Preparation. The experiments were conducted at the Beltsville Agricultural Research Center, Maryland, USA. Common "Abruzzi" rye was seeded in mid-September, 2002, using a no-tillage John Deere grain drill (Model No. 450, Westminster, MD, USA). Seeding rate was 90 kg/ha in rows 19 cm apart. No herbicide, fertilizer, or irrigation was applied until the last sample was taken on 17 June 2003. Rye aerial and root tissue was sampled at five dates from March through June of 2003, covering growth stages 4 to 10.5.4 on Feekes growth scale (Large, 1954), and again in November of 2002 corresponding with growth stage 2. Only aerial tissue was taken on the June sampling date due to the difficulty of recovering senescing root tissues.

Whole plants with intact roots were carefully dug, and the soil was washed gently from the roots. Weeds were separated and discarded. Aerial tissues were separated from roots, and the tissues were washed several times with distilled water and blotted on paper. They were then cut into 5-cm pieces, dried for 3 d in a forced-air oven at 65°C, ground in a Wiley mill (Model No. A75-A, Arthur Thomas Co., Philadelphia, PA, USA) to pass through a 40-mesh screen, and

stored in airtight bags at 4°C until use. Aqueous crude extracts from aerial and root ground tissues were prepared following established procedures (Barnes and Putnam, 1986; Burgos et al., 1999; Burgos and Talbert, 2000) with slight modification. Ground tissues (12 g) were incubated in 180 ml deionized water (1:15 w/v) in 500-ml Erlenmeyer flasks. The flasks were sealed with a parafilm layer, placed on a shaker (Gyrotory G-2; Edison, NJ, USA), and incubated for 24 hr in the dark at 4°C with constant shaking at 100 rpm. The slurry was filtered through six layers of cheesecloth and centrifuged for 10 min at 3,046 $\times$ *g* in a refrigerated centrifuge. The supernatant solution (134 $\pm$ 2 ml), which represented the crude aqueous extract, was held in ice until used in bioassays. Electrical conductivity of the crude extract was determined using a conductivity meter. Portions of the supernatant were filtered by using a 0.7-μm Whatman glass microfiber filter, and aqueous filtrate solutions were injected directly onto an LC column connected to the LC/ESI/MS-MS as will be described later.

Analytical Procedures. Chemicals used were derived from the following sources: benzoxazolin-2(3*H*)-one (BOA) (98%) and 6-methoxy-benzoxazolin-2(3*H*)-one (MBOA) (97%) were bought commercially from Sigma-Aldrich (St. Louis, MO, USA); 2,4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA) and (2*R*)-2-*β*-D-glucopyranosyloxy-4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA-G; estimated >90%) were isolated from corn according to the methods described by Klun et al. (1967); and 2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) and (2*R*)-2-*β*-D-glucopyranosyloxy-4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA-G) were synthesized following the methods of Sicker et al. (1989) for DIBOA (estimated >95%) and Kluge et al. (1997) for DIBOA-G (estimated >95%).

Analytical method development was aimed at improving the detection limits for the hydroxamic acids expected to be present in the various extracts. Initial full-scan analyses were carried out to determine what types of hydroxamic acid-related compounds could be found. The initial data produced spectra that could potentially arise from the hydroxamic acid family of compounds. Therefore, standards were obtained or synthesized to quantify these compounds as well as β-hydroxybutyric acid and β-phenylacetic acid.

Spike recovery experiments were carried out on leaf extracts from rye sampled at Feekes stage 2. These extracts produced the greatest amount of co-extracted materials, i.e., pigments and suspended cell materials that tended to clog the 1-μm pore size syringe filters used to clarify extracts for LC injection. Because of this clogging, only about 1 ml of material could be obtained before clogging occurred.

Instrumentation. The LC instrument was a Waters 2690 XE Separation module (Waters Corp., Milford, MA, USA). The LC column was a Waters X-Terra MS C-18 column, (5 μm 2.1 $\times$ 150 mm). The separation method was

adapted from Lyons et al. (1988). These conditions were set using known standards of the analytes. The liquid chromatographic protocol involved an isocratic elution program after a 10-μl injection of the sample. The mobile phase consisted of 75% solvent A, 30:70 (v/v) mixture of methanol and 1% formic acid and 25% solvent B, distilled water. A flow rate of 0.3 ml/min was maintained while maintaining the column temperature at 45°C.

Electrospray ionization mass spectrometry (positive and negative ion; ES+/−, MS/MS) was performed on a Quattro LC benchtop triple quadrupole mass spectrometer (Micromass Ltd., Manchester, UK) and analyzed using the multiple reaction monitoring mode (MRM). Exact identification and quantitation was aided by the specificity of the MS that monitored characteristic daughter ions from parent masses of BOA, DIBOA, DIBOA-G, MBOA, DIMBOA, and DIMBOA-G, and the selectivity of the LC separation producing matching peaks at the predetermined retention times for the standards. Nonspecific mass spectrometer settings were set as follows: capillary voltage was set at 3.0 kV; source and desolvation temperatures were set at 140 and 400°C; liquid nitrogen was used to supply the nebulizer and desolvation gas (flow rates were approximately 70 and 600 l/h, respectively). Argon was used as collision-induced decomposition gas to fragment the parent ions, typical pressure 2.9×10^{-5} mbar. MRM was chosen because it allows high sensitivity and selectivity by setting both quadrupoles to transmit selected ions only; the goal of analysts is typically to set the first quadrupole to select the quasi-molecular ($[M\text{-}H]^+$ or $[M\text{-}H]^-$) molecular weight ions (the exceptions here were DIBOA and DIMBOA-G; Table 1) that are allowed to react in the collision cell, and the second quadrupole is set to transmit only specific daughter ions. The compound-specific settings for each parent and daughter ion used for compound identification and quantification are listed in Table 1, along with the optimized cone voltages and collision energies used. Resulting retention times for the specific column conditions are also shown.

TABLE 1. COMPOUND-SPECIFIC MASS SPECTROMETER SETTINGS AND LC RETENTION TIMES

Compound	Parent ion (m/z)	Daughter ion (m/z)	Ionization mode	Retention time (min)	Cone (V)	Collision (eV)	Molecular weight
DIMBOA-G	380	218	ES+	5.8	36	20	373
DIMBOA	212	166	ES+	7.7	12	9	211
MBOA	166	110	ES+	11.4	35	20	165
DIBOA-G	342	134	ES−	5.3	15	11	343
DIBOA	164	80	ES+	7.0	15	20	181
BOA	136	108	ES+	10.2	35	16	135

Quantification was carried out by external standard method using a 5-point standard curve that included 0 with standards ranging from 0.05 to 5 ppm. Peak integration and quantification were performed automatically using the MassLynx 4.0 software (Micromass Ltd.) Instrumental Limit of Detection (LOD) and Limit of Quantification (LOQ) were estimated in terms of the baseline noise. LOD values were calculated using the Eurochem guidelines, where the standard deviation of the lowest visible standards was multiplied by 3.3 to provide method detection limit values (Standards Council of Canada, 2003). Quantification limits were based on the lowest standard that produced a signal to noise ratio greater than 3:1 (Table 2).

Bioassay Methods. Crude aqueous extracts were prepared in proportions of 1.0, 0.5, 0.25, and 0.125 for use in Petri dish bioassays to determine the suppressive activity of rye aerial and root tissue on germination and growth of "Great Lakes" lettuce and "Rutgers" tomato seeds. Lettuce and tomato seeds (50 per dish) were germinated in 100 × 15 mm Petri dishes over a Whatman No. 1 filter paper using 2.5 ml of extract per dish. Dishes were sealed with parafilm, placed on trays, and held in the dark in a growth chamber at a constant 26°C. Trays were positioned at a 45° angle to encourage geotropic growth and facilitate hypocotyl and root measurements (Burgos and Talbert, 2000). Percent germination and hypocotyl and root lengths were measured after 3 d for lettuce and after 5 d for tomatoes. There were five replications for each sampling date, extract, and proportion, and all extractions were repeated once. Data were expressed as the ratio of the treatment value divided by the control in which seeds were germinated in deionized water.

At the May 27 sampling, rye plants reached a growth stage of 10.3 on the Feekes scale. By that time, flowering was complete and the uppermost leaves were still green, whereas the lower leaves were senesced. Plants were harvested, and the aerial tissues were separated into upper green leaves, lower senesced

TABLE 2. ANALYTICAL METHOD PERFORMANCE

Compound	Limits of quantification (μg/g dry wt.)	Limits of detection (μg/g dry wt.)	Spike recoveries (%)	Precision for spike tests [(RSD[a,b] (replicates)]
DIMBOA-G	0.75	0.4	60	0.18 (N = 6)
DIMBOA	0.84	0.1	104	0.12 (N = 9)
MBOA	0.36	0.7	102	0.07 (N = 9)
DIBOA-G	0.75	0.6	60	0.10 (N = 9)
DIBOA	0.84	0.3	125	0.04 (N = 9)
BOA	0.36	0.5	102	0.03 (N = 9)

[a] RSD = relative standard deviation of the means.
[b] Spike concentrations varied from an average of 39–120 μg/g dry wt.

leaves, and leafless stems. The tissues were dried and ground as described earlier. Crude extracts from these tissues were used in bioassays to determine suppressive activity.

Statistical Analysis. All analyses were conducted using SAS Version 8.2 (SAS Inc., Cary, NC). Analysis of variance and mean separations were conducted using PROC MIXED. The nonlinear dose–response function $Y = 100/(1 + (X/c)^{**}b)$, where Y is the assay species response, X is the extract proportion, and c and b are coefficients, was determined using PROC NLIN (Seefeldt et al., 1995). The I_{50} value was determined by the c coefficient. Correlations among hydroxamic acid concentrations and I_{50} values were determined using PROC CORR.

RESULTS AND DISCUSSION

Analytical Observations. DIBOA and BOA (HA group) were the major compounds measured in shoot extracts, whereas DIMBOA-G and MBOA (MHA group) were the dominant constituents of root extracts (Table 3). The relative proportions of BOA and DIBOA agree with data from rye reported by other investigators (Barnes et al., 1987; Mwaja et al., 1995; Burgos et al., 1999; Reberg-Horton et al., 2005). The proportions depend on how completely the enzyme has reacted to remove the glucose precursor, and this may vary depending on extract preparation methods. Furthermore, many researchers do not monitor all of the precursor species or the methoxy-substituted forms. Our rediscovery of MBOA in rye may have significance as an important chemical responsible for toxicity.

Precision checks during routine processing resulted in duplicate analyses, one leaf and one root sample both from rye sampled at Feekes stage 2. For DIBOA in leaves, the relative standard deviation (RSD) was 0.08 [relative percent difference (RPD) of 12%], for BOA in the leaves the RSD was 0.22 (RPD 30%), and for root it was 0.47 (RPD 67%), and for MBOA in the root sample the RSD value was 0.19 (RPD 27%). Based on these routine tests, the overall precision of the analyses during processing of the samples was considered acceptable, e.g., <0.25, except for BOA in roots, which may have been caused by the relatively low concentration of this compound in these samples. Precision of the general method was also tested by using spike recovery experiments, where triplicate sample sets were analyzed on high (average of 120× background), low (average of 40× background), and nonspiked materials (concentrations ranged from 0.8 to 500 μg/g dry wt). There did not appear to be any effect of concentration on the precision of these results, which allowed all replicates to be averaged. Thus, the respective

TABLE 3. TOTAL HYDROXAMIC ACID (HA + MHA) CONCENTRATION (μg/g dw) IN RYE PARTS HARVESTED ON VARIOUS DATES

	HA			MHA					
Sampling date	DIBOA glucose	DIBOA	BOA	DIMBOA glucose	DIMBOA	MBOA	Total HA	Total MHA	Total All
Rye shoot tissue									
10 Nov.	2.8	287	114	–	–	2.7	404	2.7	407
24 Mar.	1.4	108	96.0	–	–	2.2	205	2.2	208
15 Apr.	–	12.8	60.9	–	–	2.7	73.7	2.7	76.4
6 May	17.2	15.0	22.8	–	–	0.7	55.0	0.7	55.7
27 May	7.7	4.0	3.7	–	–	<0.36 (0.32)	15.4	0.3	15.7
17 Jun.	–	2.3	2.3	–	–	–	4.6	–	4.6
Rye root tissue									
10 Nov.	2.2	6.6	22.5	–	1.0	155	31.3	156	187
24 Mar.	–	0.9	13.8	–	–	36.0	14.7	36.0	51.7
15 Apr.	lost	lost	lost	lost	lost	lost			
6 May	7.6	–	11.4	43.4	1.1	11.9	19.1	56.3	75.3
27 May	10.5	–	4.0	38.8	–	7.2	14.5	46.1	61.6

average RSD values for the analytes measured by this method were as follows: 0.18, 0.12, 0.07, 0.10, 0.04, and 0.03 for DIMBOA glucose, DIMBOA, MBOA, DIBOA glucose, DIBOA, and BOA, respectively (Table 2).

Spike recovery tests with Feekes stage 2 material revealed that all compounds were recovered at or greater than 60% (Table 2). The glucose-substituted forms of the hydroxamic acids were consistently low (60% ± 2.1 to 3.6% standard error, respectively, for DIBOA-G or DIMBOA-G compared to 102–125% for the other nonglucose-substituted compounds). Because of the low and consistent recovery of the two glucose-substituted forms, there could be a small amount of matrix suppression occurring here. This type of interaction is not uncommon when using the electrospray interface (Reemtsma, 2001). These matrix effects need to be assessed, especially when working with more contaminated extracts, such as were encountered in these analyses. Methods to correct for these effects include standard additions or passing the crude extracts through solid-phase cleanup cartridges, e.g., C-18 cartridges (Bonnington et al., 2003a; Schmitz-Afonso et al., 2003). The ideal method to compensate for possible matrix effects with these methods is to use isotope dilution methods utilizing labeled standards that behave exactly as the unlabeled materials. Even though they coelute with the natural material, each can be separately identified and distinguished because of unique molecular weights. Unfortunately, such isotopes are sparingly available and often require costly and time-consuming custom synthesis.

Whereas most current LC/UV methods require several steps to isolate the hydroxamic acid and related products, i.e., aqueous-phase extractions followed by organic solvent extractions of the aqueous phase and final solvent exchanges and filtration (Gutierrez et al., 1982; Lyons et al., 1988; Yenish et al., 1995; Melanson et al., 1997), our method requires a simple aqueous phase extraction, filtration, and analysis. Niemeyer et al. (1989) successfully employed a similar preparation procedure using LC/UV detection (263 nm) to analyze DIBOA and DIMBOA in wheat and rye tissues. Aside from some graphical data on spike performance, recoveries near 100%, and what appears to be low standard deviation of their replicated spikes, there was little additional detail on method performance. The authors made no mention of detecting any benxozalonones (BOA and MBOA) in their extracts.

Our studies had one drawback, namely, that crude extracts have limited shelf lives and must be analyzed within 1 d of preparation or the HA and MHA compounds appear to degrade. If longer holding times are necessary, then organic solvent extractions could be employed or additional studies might be needed. There appeared to be some minor matrix effects with the most contaminated extracts; however, cleanup of these extracts did not appear to be necessary because accurate quantitative and qualitative determinations were possible.

Detection limits were better than have been reported by others (Table 2), with the exception of the Bonnington et al. (2003a), who also employed LC/ESI/MS-MS methods. Their limit of detection values (μg/g dry wt) varied from 0.1 to 1.1, whereas our values ranged from 0.1 to 0.7 for the same analytes. Lyons et al. (1988) reported detection limits with their method, HPLC with UV detection (λ = 280 nm), in terms of lowest standard injected for DIMBOA-G and DIMBOA of 0.2 nmol (2.2 ppm DIMBOA and 3.8 ppm DIMBOA-G). Comparing this to our lowest standard of 0.056 ppm that was injected in 10 μl, our detection range was >39 times and >140 times lower for DIMBOA and DIMBOA-G, respectively. Using the LOD values in Table 2, our detection limits were >314 times lower than those of Lyons et al. (1988). Using their method for calculating limits of detection, our values computed to range from 0.7 to 2.8 pmol. Woodward et al. (1979a) used a GC/flame ionization detection (FID) method for DIMBOA and DIBOA and reported detection limits for 10 μl injections of 0.02 and 0.05 nmol for DIBOA and DIMBOA, respectively. Their analytical variation was expressed as a standard error of 2% of the mean, and their recoveries were 106% and 78% for DIMBOA-G and DIMBOA, respectively. Their standard error values for precision on five replicates were 434 ± 13 and 1,066 ± 19 for DIMBOA-G and DIMBOA, respectively, in corn that would correspond to RSD values of 0.067 and 0.040, respectively, for DIMBOA-G and DIMBOA. Gutierrez et al. (1982) also used HPLC-UV for quantification of DIMBOA and MBOA in corn extracts and reported recoveries of 104 ± 2% for DIMBOA and 106 ± 3% for MBOA. The variability values were not clearly defined and they did not provide detection limit data.

Qualitative identification of the different analytes is vastly improved using LC/MS methods *versus* non-MS methods discussed above, e.g., LC/UV and GC/FID. Cambier et al. (2000) utilized LC/MS for qualitative and semi-quantative analysis of the glucosylated forms of DIMBOA in corn tissues. They chose to use atmospheric pressure chemical ionization methods (APCI) rather than the electrospray ionization methods utilized here. Their investigation did demonstrate the utility of LC/MS for better characterizing the family of compounds comprising the benzoxazinone group. Bonnington et al. (2003a) conducted a more detailed study using LC/MS. Their LC/ESI/MS-MS methods differed from ours in that they conducted their acquisition in negative ionization mode *versus* the positive electrospray ionization method used here. Under negative ionization methods, the analytes produce different characteristic ions than in positive mode. For their method, more fragment ions were produced and quasi-molecular ions near the molecular weights were seldom detected. This apparently limited the choices of ion transition pairs that Bonnington et al. (2003a) selected for their quantitation parameters. Thus, they used identical daughter ions for three analyte pairs and, in two cases, the parent-to-daughter transition ion pairs were the same for two different compounds (DIMBOA and

MBOA). All of this limited the selectivity of their method, especially when compared to ours where none of the transition pairs were the same and in most cases (4 out of 6) the parent ion was the quasi-molecular $[M\text{-}H]^+$or $[M\text{-}H]^-$ form for the compound's molecular weight (Table 1). DIBOA was one of the compounds whose quasi-molecular parent ion was different, e.g., 18 mass units lower than its molecular weight; this suggests an initial loss of water from the molecule prior to formation of the MH^+ parent. The daughter mass for this pair was 80 *m*/*z*, which was also identified by Bonnington et al. (2003b). These authors also described many of the other transition ions observed here, excluding the glucosylated compounds. The DIMBOA glucose parent ion had a mass 7 units higher than its molecular weight. A possible explanation for this would be the formation of a sodium adduct of the glycosylated DIMBOA after loss of oxygen. While most sodium adduct molecules are stable to further breakdown, it has been observed that some sodium adducts of glucose-substituted molecules are susceptible to loss of the aglycones under collision induced fragmentation (Tolonen, 2003). Loss of the aglycone (162 *m*/*z*) seems the best explanation for the 282 *m*/*z* daughter fragment observed here, Table 1.

Two additional allelopathic compounds of lower activity, β-phenylactic acid and β-hydroxybutyric acid (Shilling et al., 1986), were also searched for in the rye extracts. However, consistent levels were not detected and the data are not presented. The absence of these compounds may have been caused by degradation during the 24-hr period of incubation of the crude extracts where myrosinase activity was allowed to proceed in order to convert the glucosides to their respective aglycones.

The levels of various hydroxamic acid (HA) and methoxy hydroxamic acid (MHA) compounds (Table 3) are similar to those reported by other researchers for rye. Niemeyer et al. (1989) reported fresh weight data that, if expressed on a dry weight basis, would have ranged from approximately 7,000 to 550 ppm. Several cultivars of rye were tested by Burgos et al. (1999) in which levels of BOA ranged from 18 to 229 μg/g dry wt and those of DIBOA ranged from 127 to 1,469 μg/g dry wt. The highest levels measured in our study were 287 μg/g DIBOA, 155 μg/g MBOA, and 114 μg/g BOA (Table 3). MBOA was highest in root tissues and DIBOA and BOA were higher in above ground tissues. Few researchers have reported concentrations for MBOA in rye. Hofman and Hofmanová (1969) were perhaps the first to mention that this methoxy-substituted form was present in rye. Their statement was that it was in "minute amounts." In our study, the nonglucose-substituted forms predominated when the tissues were younger, but by May, the glucose-substituted forms became more predominant, particularly in root tissue (Table 3). Appearance of these glucose-substituted forms could indicate that the enzymatic cleavage of the glucose moiety was not as efficient in these sample

preparations as it was in extracts of younger tissue. Paired comparisons of extraction results using our aqueous extraction methods *versus* use of organic solvents as has been utilized by other researchers (Lyons et al., 1988; Burgos et al., 1999) might have permitted more HA and MHA compounds to be recovered; however, measuring the levels in the aqueous extracts, as we have done here, was more relevant to the bioassay exposures that was the primary intent of the method.

Some of the important advantages in using our method include: the ease and speed at producing extracts for analysis, e.g., aqueous extraction, followed by filtration and the low detection limits possible, e.g., at least an order of magnitude lower than conventional detection methods, and the certainty of compound identification, since molecular mass identification is part of the detection method. While the detection levels are much lower than may be currently needed to associate concentration with allelopathic activities, following the fate of these compounds in the environment and in their biogenesis in plant tissues clearly becomes easier with these lower detection limits.

Bioassay Observations. Lettuce root growth was the most sensitive assay for phytotoxicity studies. Averaged over all factors, lettuce root length was inhibited by 49% by rye extracts, tomato root length was inhibited by 19%, and none of the other assays using hypocotyl length or germination showed inhibition greater than 10%. Averaged over all factors for both species, hypocotyl length was inhibited by 9%, and germination was the least sensitive assay of all, being inhibited by 4%. These results confirm previous results showing that lettuce root growth is a sensitive indicator of phytotoxic activity (Barnes and Putnam, 1986; Burgos and Talbert, 2000). Our results also confirm previous research showing that root growth is more sensitive than hypocotyl growth (Burgos and Talbert, 2000). Subsequent discussion of our results will focus on the lettuce and tomato root length assays. Similar results were obtained using other assays, but were less pronounced because of the relative insensitivity of these assays.

Across all proportions, extracts from rye shoot tissue were more phytotoxic than extracts from rye root tissue using either the lettuce or tomato root length assays (Figure 1). Barnes and Putnam (1986) also showed that rye shoots were more phytotoxic than rye roots. Lettuce was more sensitive than tomato to both rye shoot and root extracts. Tomato responses to extracts were insufficient to adequately fit the dose–response model and determine an I_{50} value; consequently, subsequent analyses of the influence of sampling date will be presented for the lettuce assay only.

Extracts of rye root and shoot tissue that were sampled at selected dates in the spring and fall differed in their phytotoxicity to lettuce root length (Figure 2). The dose–response model c parameter represents the proportion of

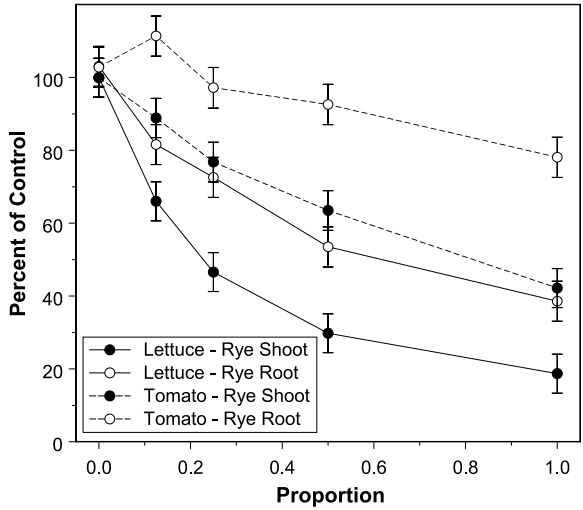

FIG. 1. Lettuce and tomato root length in response to proportions of full-strength extracts of rye shoot or root tissue averaged over spring sampling dates. Error bars represent the standard error (SE) of the mean.

full-strength extract required to inhibit lettuce root length by 50% (I_{50}). The I_{50} values were lowest (indicating the highest level of phytotoxicity) for both rye shoot and root tissue harvested in November, followed by that harvested in April (Table 4). There was not a clear decline in phytotoxicity with age of tissue

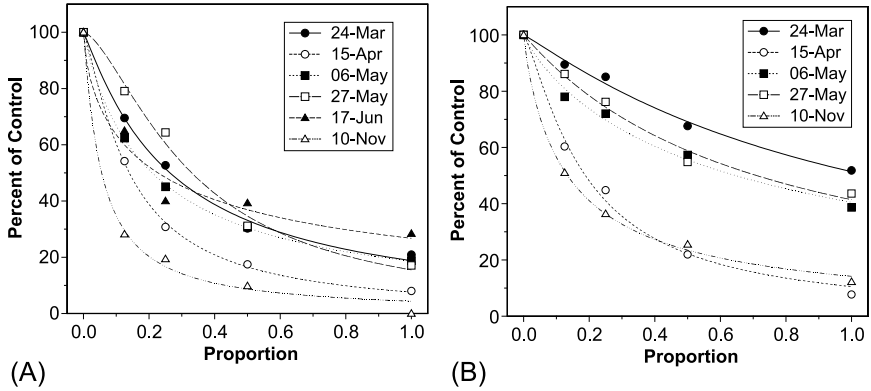

FIG. 2. Lettuce root length in response to proportions of full-strength extracts of rye shoot tissue (A) and rye root tissue (B) harvested at various sampling dates. Model parameters are presented in Table 2.

TABLE 4. DOSE–RESPONSE MODELS[a] OF LETTUCE ROOT LENGTH IN RESPONSE TO RYE SHOOT AND ROOT EXTRACTS

Rye tissue	Date	c (I_{50})	b	R^2
Shoot	10 Nov.	0.055 d	1.068 abc	0.78
	24 Mar.	0.264 b	1.100 b	0.82
	15 Apr.	0.140 c	1.267 ab	0.91
	6 May	0.210 b	0.944 bc	0.81
	27 May	0.330 a	1.531 a	0.90
	17 Jun.	0.227 b	0.679 c	0.58
Root	10 Nov.	0.134 d	0.895 b	0.88
	24 Mar.	1.053 a	1.084 ab	0.59
	15 Apr.	0.188 c	1.280 a	0.91
	6 May	0.645 b	0.880 b	0.82
	27 May	0.710 ab	1.017 ab	0.86
	17 Jun.	–	–	–

[a] Parameters c and b are from the model $Y = 100/(1 + (X/c)^b)$, where Y is percent of control and X is proportion of extract. Parameter c represents the proportion of the full-strength extract required to inhibit lettuce root length by 50 percent (I_{50}). All regression models were significant ($P < 0.0001$). Parameters within columns and rye tissue followed by the same letter were not significantly different according to 95% confidence limits.

during the spring months because shoot tissue sampled on March 24, May 6, and June 17 had similar I_{50} values, and root tissue sampled on May 27 had a similar I_{50} value to that sampled on March 24 and May 6.

The model b parameter defines the steepness of slope around the c parameter. A comparison was conducted between the model fitted with a common b parameter for all dates *versus* a model with separate b parameters for each date (Seefeldt et al., 1995). F values for these comparisons were significant for rye shoot extracts ($F = 8.31$, $P < 0.01$) and for rye root extracts ($F = 2.77$, $P < 0.05$), indicating that separate b parameters were required for each date. Comparison of 95% confidence intervals also indicated that b parameters differed between sampling dates (Table 2). The lack of equivalence among these steepness parameters indicates a lack of parallelism among models that suggests different modes of action may have occurred among extracts derived from different aged rye tissue (Streibig et al., 1993). The models also indicate that the b parameters did not follow any clear pattern relative to the age of tissue (Table 2). This result suggests that different compounds were probably responsible for the toxicity of extracts from different aged tissue.

Extracts of rye upper leaf tissue harvested on May 27 were more phytotoxic to lettuce and tomato root length than extracts of lower leaf or stem tissue at extract proportions of 0.5–1.0 (Figure 3). Extracts of lower leaf tissue, which was senescing at the time of harvest, varied in toxicity, exhibiting activity

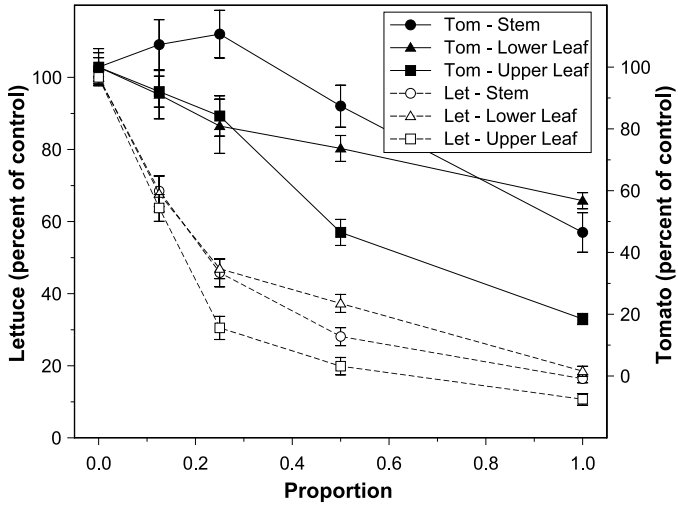

FIG. 3. Lettuce (Let) and tomato (Tom) root length response to extracts of stem, lower leaf, and upper leaf tissue of rye harvested on May 27, 2003. Error bars represent the standard error (SE) of the mean.

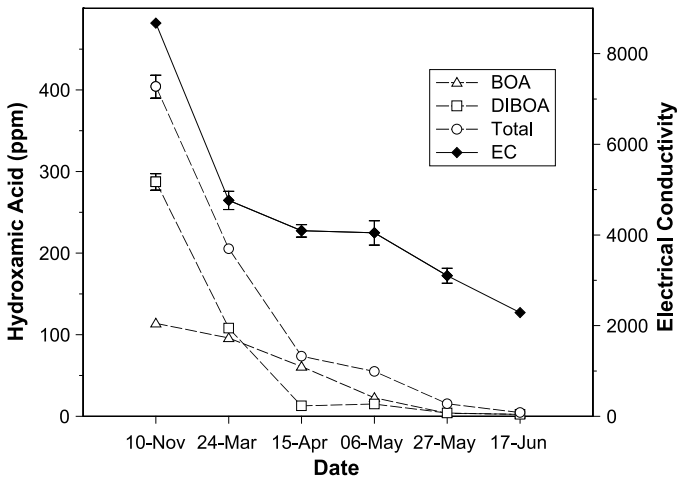

FIG. 4. Total hydroxamic acid, BOA, and DIBOA concentration and electrical conductivity (EC) of rye shoot tissue harvested at various dates. Error bars represent the standard deviation (SD).

greater than that of stem tissue in some cases, but not in others. These data suggest that more actively growing tissue tended to have the more phytotoxic extracts.

Total hydroxamic acid, DIBOA, and BOA as well as electrical conductivity were highest in rye shoot tissue at the earliest growth stage in November and declined as tissue aged through the spring (Figure 4). All of these measurements were highly correlated. Correlation coefficients between BOA and DIBOA were 0.84; between BOA and DIBOA with total hydroxamic acids they were 0.91 and 0.99, respectively; and between BOA, DIBOA, and total hydroxamic acids with electrical conductivity they were 0.86, 0.96, and 0.97, respectively. These high correlations suggest that it would be difficult to determine which hydroxamic acids may have contributed to the phytoxicity of these extracts, or whether other ionic compounds that contributed to electrical conductivity may also have been involved. The phytotoxicity of extracts did not follow a similar steady decline over time (Table 2) as that of the compounds in Figure 4. Correlations between phytoxicity as determined by I_{50} values and either the prominent hydroxamic acids, their totals, or electrical conductivity were not significant for rye shoot or root tissue. Burgos et al. (1999) also reported a low correlation between phytoxicity and HA content. These results suggest that compounds other than hydroxamic acids contributed to phytoxicity. DIBOA has been shown to be more phytotoxic than BOA (Barnes et al., 1987; Burgos and Talbert, 2000) and to be capable of inhibiting lettuce root growth at the concentrations observed in our November and March extracts (Burgos and Talbert, 2000). Thus, hydroxamic acids probably contributed to the phytoxicity of tissue sampled at early growth stages, but had minimal contribution at later harvest dates when tissue concentrations dropped to low levels.

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RELATIONSHIPS BETWEEN PHENOLIC ACID CONCENTRATIONS, TRANSPIRATION, WATER UTILIZATION, LEAF AREA EXPANSION, AND UPTAKE OF PHENOLIC ACIDS: NUTRIENT CULTURE STUDIES

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Abstract—Phenolic acid treatments of cucumber seedlings (*Cucumis sativus* cv "Early Green Cluster") inhibited transpiration, water utilization, leaf area, and absolute and relative rates of leaf expansion. The cinnamic acids, ferulic and *p*-coumaric acids, were two to five times more inhibitory than the benzoic acids, *p*-hydroxybenzoic acid and vanillic acid. When phenolic acid concentrations were maintained at inhibitory concentrations through multiple successive treatments, percent inhibition of water utilization remained relatively constant for a given concentration and phenolic acid, percent inhibition of leaf area initially increased and then leveled off to a constant percent, and percent inhibition of transpiration and rates of leaf area expansion declined over time. Subsequently, *p*-coumaric acid was chosen as the model compound for further study. When *p*-coumaric acid was inhibitory, percent inhibition of transpiration, water utilization, and rates of leaf area expansion of actively growing leaves rapidly declined (i.e., was lost) as *p*-coumaric acid concentrations surrounding roots decreased. Absolute and relative rates of leaf expansion, for example, declined approximately 12 and 14%, respectively, for every 0.1 mM decline in *p*-coumaric acid concentration. Uptake of *p*-coumaric acid by cucumber seedling roots was continuous over the 24- or 36-hr periods monitored, but was not consistently related to the initial *p*-coumaric acid treatment concentrations. However, declining *p*-coumaric acid concentrations monitored at 6- or 12-hr intervals over the 24- or 36-hr periods continued to be highly correlated to the initial *p*-coumaric acid treatment concentrations. A 25% depletion by 13-d-old cucumber seedlings took 8.5, 12, 19.5, 25, and 29.5 hr for 0.125-, 0.25-, 0.5-, 0.75-,

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and 1-mM treatments, respectively. Uptake during periods when phenolic acid concentrations and root uptake (depletion from solution) were related appeared to represent periods dominated by apoplastic movement into the intercellular spaces of roots. Uptake during periods without this relationship likely represented periods dominated by symplastic movement. The ability of cucumber seedlings to modify active phenolic acid concentrations surrounding their roots suggests that cucumber seedling can directly influence the magnitude of primary and secondary effects of phenolic acids through feedback regulation.

Key Words—Absolute and relative rates of leaf expansion, feedback regulation, inhibition, phenolic acids, phenolic acid depletion and uptake, recovery, transpiration, water utilization.

INTRODUCTION

Root membranes are a primary site of action for phenolic acids. Contact of phenolic acids with root cell membrane leads to depolarization, an efflux of ions, and a reduction of hydraulic conductivity and net nutrient uptake (Glass, 1973, 1974; Einhellig, 1986, 1995; Lyu and Blum, 1990; Bergmark et al., 1992; Booker et al., 1992; Baziramakenga et al., 1995; Lehman and Blum, 1999). Subsequent resulting changes in plant water relations and mineral nutrition lead to a cascade of secondary, tertiary, etc. effects, frequently referred to as secondary effects. Included among these are reductions in photosynthesis (Einhellig et al., 1970; Patterson, 1981), carbon allocation to roots (Blum and Rebbeck, 1989), leaf expansion (Blum and Dalton, 1985), transpiration (Blum et al., 1985a), increases in abscisic acid (Hollapa and Blum, 1991), and closure of stomata (Einhellig and Kuan, 1971; Patterson, 1981). The intensity and duration of primary and secondary effects are dependent on species sensitivity and the continued presence of active phenolic acid concentrations surrounding roots (Einhellig and Kuan, 1971; Glass and Dunlop, 1974; Blum and Dalton, 1985; Blum et al., 1985a, b; Blum and Rebbeck, 1989; Lehman and Blum, 1999). Soil solution concentrations of active phenolic acids are a function of the inputs of phenolic acids (e.g., leaching of plant materials, microbial activity, root secretions and exudations, and root cell autolysis; see Rice, 1984), losses of phenolic acids (e.g., sorption by soil particles, microbial utilization, uptake by roots; see Siqueira et al., 1991; Blum et al., 1999a, b), state of protonation (see Blum et al., 1985b, 1999b), and soil water content. Because seedlings can regulate transpiration rates and thus soil moisture, take up and detoxify phenolic acids, regulate phenolic acid utilizing microbes in the rhizosphere, and increase or decrease protonation of phenolic acids (pK_a approximately 4.5) through adjustments of rhizopshere and solution pH (Shann and Blum, 1987a, b; Blum et al., 1985b, 1999b; Blum, 2004), seedlings should also have the capability to

self-regulate the degree of expression of both primary and secondary effects of phenolic acids. In summary, phenolic acids affect seedling processes of sensitive species. Affected and unaffected seedling processes in turn reduce the active concentrations of phenolic acids surrounding their roots, and the resulting changes in active concentrations of phenolic acids surrounding roots lead to feedback regulation of or a decline in the effects. Feedback regulation is defined as a response in which the product of one of the final steps in a chain of events affects one of the first steps in this chain; fluctuations in rate or concentration are minimized with negative feedback or amplified with positive feedback (Lambers et al., 1998).

To explore the relationships between phenolic acid concentrations surrounding roots and transpiration, water utilization, phenolic acid uptake, and leaf area expansion, and as a first step towards understanding the role of seedling-induced feedback regulation, we experimentally addressed the following questions by using a cucumber seedling–nutrient culture system:

- How do phenolic acids affect transpiration, water utilization, and leaf area expansion?
- How do phenolic acid concentrations influence phenolic acid depletion from solutions surrounding roots?
- How does depletion of phenolic acid from solution surrounding roots influence transpiration, water uptake, and leaf area expansion?

METHODS AND MATERIALS

Experimental Designs. Experiment 1: Four phenolic acids (ferulic, *p*-coumaric, *p*-hydroxybenzoic, and vanillic acids); five concentrations (0, 0.25, 0.5, 0.75, and 1 mM); 8 AM phenolic acid treatment on 3 d (d 6, 8, and 10); four replicates; data collection: 8 AM and/or 5 PM on d 8–12; and final harvest on d 12. Experiment 2a: 1 Phenolic acid (*p*-coumaric acid); five concentrations (0.125, 0.25, 0.5, 0.75, and 1 mM); 8 AM phenolic acid treatment on d 13; four replicates; data collection: 8 AM, 2 PM, 8 PM, and/or 8 AM; and final harvest on d 14. Experiment 2b: 1 Phenolic acid (*p*-coumaric acid); five concentrations (0.125, 0.25, 0.5, 0.75, and 1 mM); 8 PM Phenolic acid treatment on d 14; three replicates; data collection: 8 PM, 8 AM, 2 PM, and/or 8 PM; and final harvest on d 15. Experiment 3: 1 Phenolic acid (*p*-coumaric acid); four concentrations (0, 0.125, 0.25, and 0.5 mM); 8 AM phenolic acid treatment on d 12; two solution changes (no change or every 4 hr over 36 hr); four replicates; data collection: 8 AM, 8 PM, 8 AM, and/or 8 PM; and final harvest on d 13. Experiment 4: 1 Phenolic acid (*p*-coumaric acid); two concentrations (0 and 0.5 mM); 8 AM phenolic acid treatment on d 12; three nutrient strengths (0.5, 1,

and 1.5); two aeration (plus or minus); four replicates; data collection: 8 AM, 8 PM, 8 AM, and/or 8 PM; and final harvest on d 13.

Nutrient Culture System. Cucumber seeds (*Cucumis sativus* cv. "Early Green Clusters," Wyatt Quarles Seed Company, Raleigh, NC, USA) were germinated for 2 d in the dark at 30°C in glass trays containing moist vermiculite. The resulting exposed hypocotyls were placed under a fluorescent light bank [cool white, 12-hr photoperiod (8 AM to 8 PM), 140 $\mu E/m^2/sec$] for 2 d to allow for the development of autotrophic seedlings. Resulting seedlings were transplanted into glass jars containing 110 ml Hoagland's solution (Hoagland and Arnon, 1950). The pH of all solutions was adjusted to 5.0 with sodium hydroxide. Seedlings were suspended by foam collars inserted through holes in the lids of the jar. Jars were inserted through holes in a wooden panel located under a light bank to protect the solutions from light. With the exception of treatment periods for experiments 3 and 4, solutions were completely changed every other day. Distilled water was added to jars daily at 8 AM as needed to compensate for water utilization for experiment 1 and for pretreatment seedling development of all other experiments. Water was not added during the 24- or 36-hr treatment periods for experiments 2–4. Light banks were located on laboratory benches. For additional details, see Blum and Dalton (1985).

Dry Weight, Leaf Area, and Absolute and Relative Rates of Leaf Expansion. Dry weights of tops and roots were based on oven-dried samples (100°C). Leaf areas were determined from leaf length (L) and width (W) measurements and the following equation: leaf area in $cm^2 = -1.457 + 0.008 (L \times W)$, $P < 0.001$, $r^2 = 0.98$, where L and W are in mm (Blum and Dalton, 1985). Surface areas for cotyledons were determined by measuring cotyledon length (L) and width (W) and the following equation: area of cotyledon in $cm^2 = 1.293 + 0.006 (L \times W)$, $P < 0.001$, $r^2 = 0.76$, where L and W are in mm. The absolute rates of leaf expansion (cm^2/seedling/unit time) for the cotyledons and/or true leaves were determined as follows: leaf area at $time_{x+1}$ − leaf area at $time_x$. The relative rates of leaf expansion (cm^2/cm^2/unit time) were determined as follows: ln (leaf area at $time_{x+1}$) − ln (leaf area at $time_x$). For a discussion on calculating growth rates, see Radford (1967).

Transpiration and Water Utilization. Transpiration (ml/cm^2 leaf area/hr) and water utilization (ml/seedling/hr) were determined by solution depletion over various time intervals. Volumes of solution in the jars at a given time were determined by the following regression: ml in jar = −10.549 + (1.523 × solution level), $P < 0.001$, $r^2 = 0.99$, where solution level in jar is in mm.

Depletion of Phenolic Acid from Nutrient Culture. One-milliliter subsamples were taken at various times from the treatment jars; the subsamples were filter sterilized with 2-μm filters (Supor −200, Gelman Sciences, Ann Arbor, MI, USA) before being injected into a high-performance liquid chromatograph (HPLC) to determine the amounts of phenolic acid in the sub-

samples (Blum et al., 1994). Total phenolic acid in jars and depletion of phenolic acid over time from jars were based on the HPLC data and the solution levels in the jar. Depletion of phenolic acid from jars was assumed to represent total uptake of phenolic acid by the cucumber seedling roots. Depletion associated with microbial utilization was not determined; however, HPLC-detectable breakdown products of *p*-coumaric acid were determined for experiment 4. Individual breakdown products were not identified. Breakdown products were quantified as *p*-coumaric acid equivalence.

Data Analysis. The data were analyzed using SAS© system (SAS Institute Inc., 1999) primarily employing procedures SAS/GLM, SAS/MIXED, and SAS/NLIN procedures. For each data set, models were developed with the joint goals of providing a parsimonious explanation of the data while maintaining maximum predictive power. Initial model screening and the computation of the r^2 statistic were handled using procedure SAS/GLM. However, for many of the analyses, the data contained observations on the same unit measured repeatedly over time. This created a violation of the assumptions for standard analysis of variance and the need to use methodology for handling linear models with repeated measures. The tool used in this case was the SAS/MIXED procedure (Littell et al., 1996). In one set of analyses, the models employed used the concept of relative potency of a compound relative to a standard. In this case, the resulting models were nonlinear and required use of procedure SAS/NLIN.

RESULTS

How Do Multiple Treatments of Phenolic Acids Affect Transpiration, Water Utilization, and Leaf Area Expansion?

Effects of Phenolic Acids. Seedlings treated with the cinnamic acid derivatives, ferulic acid and *p*-coumaric acid, had lower overall mean transpiration rates, water utilization, leaf areas, absolute and relative rates of leaf expansion, and shoot and root dry weights than the seedlings treated with the benzoic acid derivatives, *p*-hydroxybenzoic acid and vanillic acid (Table 1). Relative potencies for the phenolic acids expressed in terms of ferulic acid (1.00) ranged from 0.76 to 1.00, 0.10 to 0.33, and 0.03 to 0.42 for *p*-coumaric acid, *p*-hydroxybenzoic acid, and vanillic acid, respectively (Table 1). As treatment phenolic acid concentrations were increased, overall mean transpiration rates, water utilization, leaf areas, absolute and relative rates of leaf expansion, and shoot and root dry weights declined in a quadratic (concave up) or linear manner (Table 1). For comparative purposes, utilizing the models of Table 1, 0.5 mM of the cinnamic acids (ferulic acid and *p*-coumaric acid) and the benzoic acids

TABLE 1. PARSIMONIOUS MODELS FOR THE EFFECTS OF MULTIPLE PHENOLIC ACID TREATMENTS ON TRANSPIRATION, WATER UTILIZATION, LEAF AREA, ABSOLUTE AND RELATIVE RATES OF LEAF EXPANSION, AND SHOOT AND ROOT DRY WEIGHTS OF CUCUMBER SEEDLINGS GROWING IN NUTRIENT CULTURE, EXPERIMENT 1[a]

		Coefficients					
Day	Intercept	Linear	Quadratic	Relative potency		P	r^2
Transpiration (ml/cm^2/hr)							
8	0.031	−0.043	0.020	FER[c]	1.000	<0.001	0.55
	(0.002)[b]	(0.011)	(0.010)		(0.000)		
9	0.031	−0.023	0.003	PCO	1.004		
	(0.002)	(0.010)	(0.010)		(0.085)		
10	0.024	−0.011	−0.005	POH	0.331		
	(0.002)	(0.010)	(0.010)		(0.059)		
11	0.039	−0.033	0.007	VAN	0.415		
	(0.002)	(0.010)	(0.010)		(0.063)		
12	0.024	−0.013	−0.001				
	(0.002)	(0.010)	(0.010)				
Water utilization (ml/seedling/hr)							
8	0.411	−0.905	0.608	FER[c]	1.000	<0.001	0.84
	(0.038)	(0.216)	(0.221)		(0.000)		
9	0.622	−1.231	0.758	PCO	0.778		
	(0.039)	(0.218)	(0.224)		(0.060)		
10	0.678	−1.320	0.771	POH	0.212		
	(0.039)	(0.220)	(0.225)		(0.022)		
11	1.383	−2.824	1.645	VAN	0.318		
	(0.041)	(0.236)	(0.244)		(0.026)		
12	1.003	−1.940	1.120				
	(0.040)	(0.226)	(0.232)				
Leaf area (cm^2)							
8	13.853	−11.663	8.843	FER[c]	1.000	<0.001	0.91
	(0.717)	(4.089)	(4.223)		(0.000)		
9	20.499	−26.112	16.463	PCO	0.760		
	(0.724)	(4.141)	(4.283)		(0.048)		
10	28.468	−40.890	24.974	POH	0.173		
	(0.741)	(4.239)	(4.392)		(0.016)		
11	37.034	−54.711	32.946	VAN	0.335		
	(0.763)	(4.370)	(4.536)		(0.021)		
12	42.530	−65.496	40.680				
	(0.784)	(4.445)	(4.634)				
Absolute rates of leaf expansion (cm^2/seedling/24 hr)							
9	6.618	−13.893	7.117	FER[c]	1.000	<0.001	0.77
	(0.336)	(1.949)	(1.985)		(0.000)		
10	7.963	−14.357	8.092	PCO	0.826		
	(0.337)	(1.928)	(1.975)		(0.076)		
11	8.564	−13.396	7.513	POH	0.181		
	(0.334)	(1.915)	(1.960)		(0.027)		
12	5.524	−10.747	7.640	VAN	0.332		
	(0.328)	(1.842)	(1.896)		(0.033)		

TABLE 1. CONTINUED

Day	Intercept	Coefficients: Linear	Coefficients: Quadratic	Relative potency		P	r^2
Relative rates of leaf expansion ($cm^2/cm^2/24$ hr)							
9	0.396	−0.696	0.277	FER[c]	1.000	<0.001	0.71
	(0.018)	(0.104)	(0.105)		(0.000)		
10	0.331	−0.252	0.044	PCO	0.823		
	(0.015)	(0.091)	(0.094)		(0.079)		
11	0.265	−0.061	−0.016	POH	0.100		
	(0.014)	(0.087)	(0.091)		(0.042)		
12	0.138	−0.089	0.091	VAN	0.314		
	(0.014)	(0.087)	(0.091)		(0.049)		
Shoot dry weight (g)							
12	0.123	−0.171	0.102	FER[c]	1.000	<0.001	0.66
	(0.006)	(0.031)	(0.032)		(0.000)		
				PCO	0.804		
					(0.180)		
				POH	0.228		
					(0.064)		
				VAN	0.390		
					(0.084)		
Root dry weight (g)							
12	0.031	−0.017		FER[c]	1.000	<0.001	0.47
	(0.002)	(0.003)			(0.000)		
				PCO	0.978		
					(0.186)		
				POH	0.298		
					(0.165)		
				VAN	0.034		
					(0.181)		

[a] Where transpiration, water utilization, leaf area, absolute and relative rates of leaf expansion, and dry weights on a given day = Intercept for a given day + (linear coefficient for a given day × [concentration × relative potency of phenolic acid of interest]) + (quadratic coefficient for a given day × [concentration^2 × relative potency^2 of phenolic acid of interest]); transpiration and water utilization were based on water depletion during each 8 AM to 5 PM time period; leaf areas were determined at 8 AM on each day.

[b] Standard errors in parenthesis.

[c] Where FER = ferulic acid, PCO = *p*-coumaric acid, POH = *p*-hydroxybenzoic acid, and VAN = vanillic acid; concentrations ranged from 0 to 1 mM; phenolic acid treatments were given on d 6, 8, and 10.

(*p*-hydroxybenzoic acid and vanillic acid) inhibited transpiration by 36 and 15%, water utilization by 65 and 25%, leaf area by 42 and 16%, absolute rate of leaf expansion by 62 and 22%, and relative rate of leaf expansion by 32 and 8%, respectively. On d 12, shoot dry weight was inhibited by 46 and 20%, and root dry weight was inhibited by 27 and 5%, respectively.

Patterns of Percent Inhibition. With the exception of relative rates of leaf expansion, the relationships of the inhibitory effects for the four phenolic acids on transpiration, water utilization, leaf area, and absolute and relative rates of leaf expansion were fairly similar over concentration and time. The magnitude of inhibition, however, varied with phenolic acid, concentration, and time. The three-way interaction of phenolic acid × time × concentration was significant only for relative rates of leaf expansion. Because both cinnamic acid derivatives, ferulic acid and *p*-coumaric acid, reduced transpiration, water utilization, and leaf expansion of seedlings considerably more than the benzoic acid derivatives, *p*-hydroxybenzoic acid and vanillic acid, in nutrient culture (see relative potencies; Table 1) and *p*-coumaric acid was more inhibitory than ferulic acid in Cecil Ap horizon-soil culture (unpublished data) used in a subsequent study, we present only the patterns for *p*-coumaric acid (Figure 1). Percent inhibitions in Figure 1 were derived from the models of Table 1. For all but water utilization and leaf area, the levels of percent inhibition were greatest after the first *p*-coumaric acid treatment and then declined at various rates over time (Figure 1). Percent inhibition of water utilization was essentially constant over time. Percent inhibition of leaf area initially increased and then leveled off to a constant percent. A dramatic decline in percent inhibition occurred for relative rates of leaf expansion. In fact, relative rates of leaf expansion averaged over concentrations (0.25–1 mM) were significantly different from the control seedlings only for d 8 and 9, and d 9 and 10.

How Does a Single Treatment of p-Coumaric Acid Affect Transpiration, Water Utilization, and Leaf Area Expansion? How Do Concentrations of p-Coumaric Acid Influence p-Coumaric Acid Depletion from Solutions Surrounding Roots?

Experiment 2a (8 AM Treatment). As treatment concentrations of *p*-coumaric acid increased, transpiration and water utilization declined in a linear manner for morning, afternoon, day, night, and 24-hr period (Table 2). For comparative purposes utilizing the models of Table 2, 0.5 mM *p*-coumaric acid reductions ranged from 37 to 41% for transpiration and from 38 to 47% for water utilization. Average leaf area and absolute and relative rates of leaf expansion of 13-d-old cucumber seedlings declined in a linear and quadratic manner (concave up), respectively, as *p*-coumaric acid treatment concentrations were increased (Table 2). For comparative purposes utilizing the models of Table 2, 0.5 mM *p*-coumaric acid significantly reduced average leaf area and absolute and relative rates of leaf expansion of seedlings by 6, 71, and 69%, respectively.

Uptake of *p*-coumaric acid (mM/g root/hr) by seedlings was based on the depletion of *p*-coumaric acid from the nutrient solution and thus may include some microbial utilization of *p*-coumaric acid. The models relating to uptake

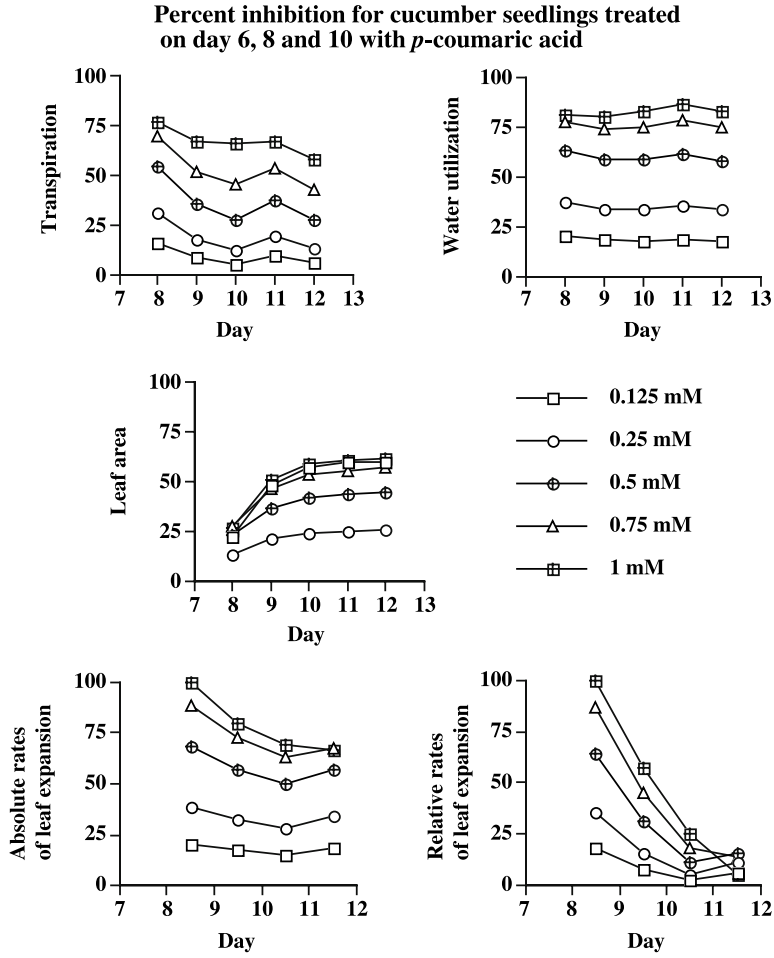

FIG. 1. Percent inhibition of 0.125 to 1 mM *p*-coumaric acid on cucumber seedling transpiration, water utilization, leaf area, absolute and relative rates of leaf expansion. Seedlings were treated to *p*-coumaric acid on d 6, 8, and 10. Nutrient solutions plus or minus *p*-coumaric acid were completely changed on alternate days starting with d 4.

had relatively low r^2 values indicating the existence of variability not explained by the models (Table 2). Uptake of *p*-coumaric acid increased linearly with increasing initial treatment concentrations of *p*-coumaric acid for the morning, and ranged from 0.013 mM/g root/hr for 0.125 mM to 0.035 mM/g root/hr for 1 mM. The increasing initial treatment concentrations of *p*-coumaric acid

TABLE 2. PARSIMONIOUS MODELS FOR THE EFFECTS OF A SINGLE *p*-COUMARIC ACID TREATMENT ON TRANSPIRATION, WATER UTILIZATION, AVERAGE LEAF AREA, ABSOLUTE AND RELATIVE RATES OF LEAF EXPANSION, AND *p*-COUMARIC UPTAKE OF 13-D-OLD CUCUMBER SEEDLINGS GROWING IN NUTRIENT CULTURE, EXPERIMENT 2a[a]

		Coefficients			
	Intercept	Linear	Quadratic	*P*	r^2
Transpiration ($ml/cm^2/hr$)					
Morning[b]	0.017 (0.001)[c]	−0.013 (0.002)		<0.001	0.70
Afternoon	0.014 (0.001)				
Day	0.016 (0.001)	−0.013 (0.002)		<0.001	0.86
Night	0.004 (0.001)	−0.003 (0.001)			
24-hr period	0.010 (0.001)	−0.008 (0.001)		<0.001	0.83
Water utilization (ml/seedling/hr)					
Morning	0.827 (0.052)	−0.633 (0.080)		<0.001	0.71
Afternoon	0.675 (0.053)				
Day	0.753 (0.049)	−0.636 (0.080)		<0.001	0.87
Night	0.176 (0.021)	−0.141 (0.034)			
24-hr period	0.464 (0.025)	−0.388 (0.040)		<0.001	0.84
Average leaf area (cm^2)					
	48.861 (1.016)	−5.392 (1.652)		0.004	0.37
Absolute rates of leaf expansion (cm^2/seedling/24 hr)					
	10.351 (0.762)	−14.669 (3.405)	6.711 (2.982)	<0.001	0.86
Relative rates of leaf expansion (cm^2/cm^2/24 hr)					
	0.215 (0.015)	−0.297 (0.069)	0.137 (0.060)	<0.001	0.86
p-Coumaric acid uptake (mM/g root/hr)					
Morning	0.010 (0.002)	0.026 (0.004)		<0.001	0.51
Afternoon	0.022 (0.003)	−0.004 (0.005)			
Day	0.016 (0.002)	0.011 (0.003)		<0.001	0.31
Night	0.027 (0.004)	0.005 (0.007)			

[a] Where transpiration, water utilization, average leaf area, absolute and relative rates of leaf expansion, and *p*-coumaric acid uptake = Intercept + (linear coefficient × *p*-coumaric acid concentration) + (quadratic coefficient × [*p*-coumaric acid concentration]^2); seedlings were treated at 8 AM to concentrations ranging from 0.125 to 1 mM.

[b] Where the time interval for Morning = 8 AM to 2 PM, Afternoon = 2 PM to 8 PM, Day = 8 AM to 8 PM, Night = 8 PM to 8 AM, and 24-hr period = 8 AM to 8 AM.

[c] Standard errors in parenthesis.

were not significantly related to the uptake of *p*-coumaric acid for the afternoon, night, or the 24-hr period. Actual overall mean uptake rates for 0.5 mM *p*-coumaric acid ranged from 0.023 ± 0.002 mM/g root/hr (3.75 mg/g root/hr) for the morning to 0.034 ± 0.006 mM/g root/hr (5.64 mg/g root/hr) for the night. Mean shoot and root dry weight for the seedlings on d 13 were 0.127 ± 0.003 and 0.030 ± 0.0011 g, respectively.

To calculate the uptake rates of the previous section, the mM of *p*-coumaric acid left in the nutrient solution was determined at 0, 6, 12, and 24 hr after addition of *p*-coumaric acid. The mM of *p*-coumaric acid declined in a curvilinear (concave down) manner (Table 3). The intercepts were different, but the linear and quadratic terms were identical for all *p*-coumaric acid concentrations. Initial treatment concentrations of *p*-coumaric acid were highly correlated ($r = 0.98$) with the *p*-coumaric acid concentrations for all time periods monitored. Finally, based on the models of experiment 2a (Table 3), a 25% depletion of *p*-coumaric acid from the nutrient solution by 13-d-old seedling was estimated to take approximately 8.5 hr for the 0.125-mM, 12 hr for the 0.25-mM, 19.5 hr for 0.5-mM, 25 hr for 0.75-mM, and 29.5 hr for the 1-mM treatments. Because the *p*-coumaric acid treatment was terminated after 24 hr, depletion times longer than 24 hr are extrapolated.

Experiment 2b (8 PM Treatment). As treatment concentrations of *p*-coumaric acid increased, transpiration and water utilization declined in a linear manner for night, morning, afternoon, day, and 24-h period (Table 3). For comparative purposes utilizing the models of Table 3, 0.5 mM *p*-coumaric acid reductions ranged from 34 to 46% for transpiration and from 36 to 45% for water utilization. Absolute and relative rates of leaf expansion of cucumber seedlings for night, day, and 24-hr period also declined in a linear manner as

TABLE 3. PARSIMONIOUS MODELS FOR CHANGES IN *p*-COUMARIC ACID SOLUTION CONCENTRATIONS (mM) OVER TIME FOR CUCUMBER SEEDLINGS GROWING IN NUTRIENT CULTURE, EXPERIMENT 2A AND 2B[a]

		Coefficients			
mM	Intercept	Linear	Quadratic	*P*	r^2
Experiment 2a[b]					
0.125	0.148 (0.008)[c]	−0.003 (0.001)	−0.0002 (0.000)	<0.001	0.99
0.25	0.251 (0.008)				
0.50	0.506 (0.008)				
0.75	0.742 (0.008)				
1.00	0.983 (0.008)				
Experiment 2b[d]					
0.125	0.154 (0.013)	−0.004 (0.002)	−0.0002 (0.000)	<0.001	0.99
0.25	0.247 (0.013)				
0.50	0.490 (0.013)				
0.75	0.745 (0.013)				
1.00	0.964 (0.013)				

[a] Where solution concentration = Intercept for a given concentration + (linear coefficient × time) + (quadratic coefficient × time^2), where time is in hours from treatment.
[b] Solution samples were taken a 0, 6, 12, and 24 hr from treatment; treatment was given at 8 AM.
[c] Standard errors in parenthesis.
[d] Solution samples were taken a 0, 12, 18, and 24 hr from treatment; treatment was given at 8 PM.

p-coumaric treatment concentrations were increased. Utilizing the models of Table 3, 0.5 mM *p*-coumaric acid reductions for rates of leaf expansion ranged from 39 to 43%. Average leaf areas of 14- to 15-d-old seedlings, however, were not significantly modified by the *p*-coumaric acid treatment.

As in the previous experiment (experiment 2a), the models relating to uptake had relatively low r^2 values indicating the existence of variability not explained by the models (Table 4). Uptake of *p*-coumaric acid increased linearly with increasing initial treatment concentrations of *p*-coumaric acid for the

TABLE 4. PARSIMONIOUS MODELS FOR THE EFFECTS OF A SINGLE *p*-COUMARIC ACID TREATMENT ON TRANSPIRATION, WATER UTILIZATION, ABSOLUTE AND RELATIVE RATES OF LEAF EXPANSION, AND *p*-COUMARIC UPTAKE OF 14-D-OLD CUCUMBER SEEDLINGS GROWING IN NUTRIENT CULTURE, EXPERIMENT 2B[a]

	Intercept	Linear coefficient	*P*	r^2
Transpiration (ml/cm^2/hr)				
Night[b]	0.0061 (0.001)[c]	−0.004 (0.001)	<0.001	0.80
Day	0.016 (0.001)	−0.013 (0.002)		
Morning	0.014 (0.001)	−0.013 (0.002)	<0.001	0.73
Afternoon	0.017 (0.001)			
24-hr period	0.011 (0.001)	−0.009 (0.001)	<0.001	0.80
Water utilization (ml/seedling/hr)				
Night	0.351 (0.046)	−0.250 (0.075)	<0.001	0.70
Day	0.971 (0.117)	−0.786 (0.190)		
Morning	0.869 (0.118)	−0.786 (0.190)	<0.001	0.58
Afternoon	1.072 (0.118)			
24-hr period	0.668 (0.071)	−0.526 (0.116)	0.001	0.61
Absolute rates of leaf expansion (cm^2/seedling/12 or 24 hr)				
Night	6.763 (0.657)	−5.300 (1.068)	<0.001	0.71
Day	9.375 (0.861)	−7.973 (1.401)		
24-hr period	16.138 (1.345)[b]	−13.273 (2.187)	<0.001	0.74
Relative rates of leaf expansion (cm^2/cm^2/12 or 24 hr)				
Night/day	0.198 (0.006)	−0.163 (0.009)	<0.001	0.82
24-hr period	0.394 (0.012)	−0.324 (0.019)	<0.001	0.96
p-Coumaric acid uptake (mM/g root/hr)				
Night	0.013 (0.002)	0.010 (0.003)	<0.001	0.50
Day	0.024 (0.003)			
Morning	0.028 (0.004)	−0.005 (0.006)	<0.001	0.37
Afternoon	0.019 (0.007)	0.028 (0.010)		
24-hr period	0.018 (0.002)	0.011 (0.004)	0.022	0.34

[a] Where transpiration, water utilization, absolute and relative rates of leaf expansion, and *p*-coumaric acid uptake = Intercept + (linear coefficient × *p*-coumaric acid concentration); seedlings were treated at 8 PM to concentrations ranging from 0.125 to 1 mM.

[b] Where the time interval for Night = 8 PM to 8 AM, Day = 8 AM to 8 PM, Morning = 8 AM to 2 PM, Afternoon = 2 PM to 8 PM, and 24-hr period = 8 PM to 8 PM.

[c] Standard errors in parenthesis.

night and for the afternoon. Uptake rates during the night ranged from 0.013 mM/g root/hr for 0.125 mM to 0.023 mM/g root/hr for 1 mM, and for the afternoon from 0.019 mM/g root/hr for 0.125 mM to 0.046 mM/g root/hr for 1 mM. The increasing initial concentrations were not significantly related to the uptake of *p*-coumaric acid for the morning period. Actual overall mean uptake rates for 0.5 mM *p*-coumaric acid ranged from 0.022 ± 0.001 mM/g root/hr (3.66 mg/g root/hr) for the night to 0.037 ± 0.009 mM/g root/hr (6.05 mg/g root/hr) for the afternoon. Mean shoot and root dry weight for the seedlings on d 14 were 0.18 ± 0.008 and 0.037 ± 0.002 g, respectively.

To calculate the uptake rates of the previous section, the mM of *p*-coumaric acid left in the nutrient solution was determined at 0, 12, 18, and 24 hr after addition of *p*-coumaric acid. The mM of *p*-coumaric acid declined in a curvilinear (concave down) manner (Table 3). The intercepts were different, but the linear and quadratic terms were identical for all *p*-coumaric acid concentrations. Initial treatment concentrations of *p*-coumaric acid were highly correlated ($r = 0.98$) with the *p*-coumaric acid concentrations for all time periods monitored. Finally, based on the models of experiment 2b (Table 3), a 25% depletion of *p*-coumaric acid from the nutrient solution by 14-d-old seedling was estimated to take approximately 8 hr for the 0.125-mM, 11 hr for the 0.25-mM, 18 hr for 0.5-mM, 23.5 hr for 0.75-mM, and 28.5 hr for the 1-mM treatments. Because the *p*-coumaric acid treatment was terminated after 24 hr, depletion times longer than 24 hr are extrapolated.

How Does Depletion of p-Coumaric Acid from Solutions Surrounding Roots Influence Transpiration, Water Utilization, and Leaf Area Expansion?

Experiment 3. Subsamples taken to determine *p*-coumaric acid concentrations left in the nutrient solution and solution pH for the no-solution change treatment were combined by treatment concentration for each time period. The average *p*-coumaric acid concentrations and percent remaining, in parenthesis, for this treatment were 0.126 mM (100%), 0.254 mM (100%), and 0.492 mM (100%) at 0 hr, 0.059 mM (47%), 0.181 mM (71%), and 0.418 mM (85%) at 12 hr, 0 mM (0%), 0.012 mM (5%), and 0.149 mM (30%) at 24 hr, and 0 mM (0%) at 36 hr for 0.125, 0.25, and 0.5 mM, respectively. The solution pH increased by approximately 1 pH unit from pH 5.00 ± 0.02 at the start of the treatment period to 6.04 ± 0.03 at the end of the 36-hr treatment period (Figure 2).

Subsamples taken to determine *p*-coumaric acid concentrations left in the nutrient solution and solution pH after each 4-hr period were also combined by treatment concentration. The average mM at the end of the 4-hr periods were 0.095, 0.220, and 0.450 for the 0.125-, 0.25-, and 0.5-mM treatment solutions, respectively. The actual starting solution concentrations in this instance were 0.128, 0.256, and 0.497 mM. The mean reductions over the 4-hr periods were

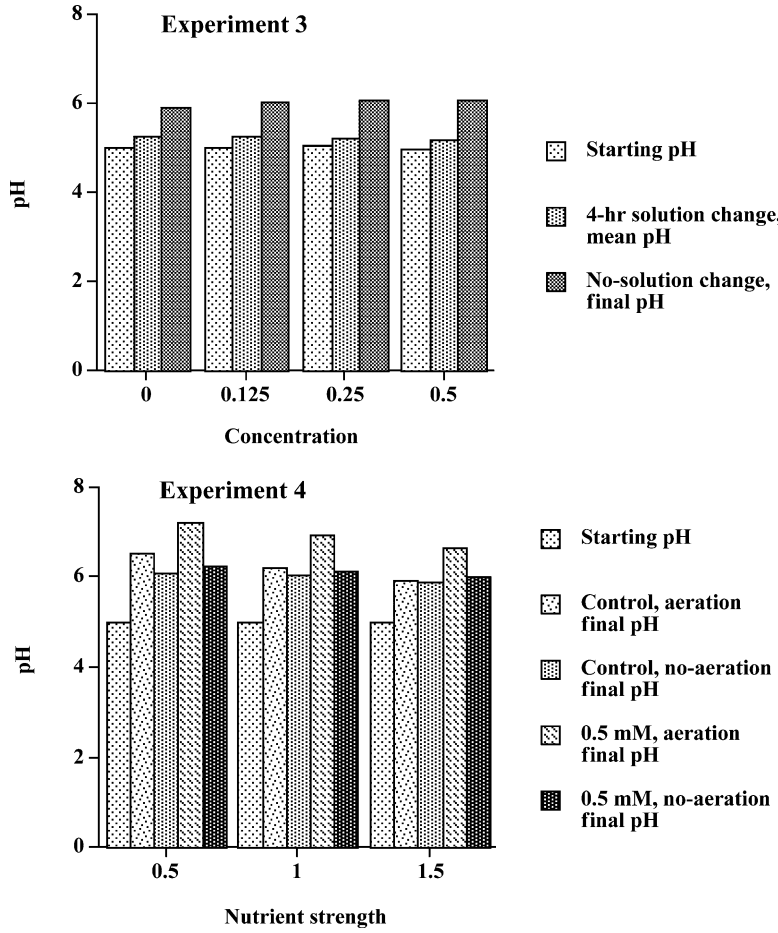

FIG. 2. Starting, mean, and final pH values for nutrient solutions of experiments 3 and 4.

thus 9, 14, and 5% for the 0.125-, 0.25-, and 0.5-mM treatments, respectively. The solution pH increased by approximately 0.25 pH unit from pH 5.00 ± 0.02 at the start of each 4-hr treatment period to 5.24 ± 0.02 at the end of each 4-hr treatment period (Figure 2).

The no-change solution treatment was used for all previous 24-hr treatment experiments. Thus, it was not surprising to find that over the first 24 hr, when effects were inhibitory, the inhibitory effects of 0.5 mM *p*-coumaric acid were consistent with previous experiments. The effects of *p*-coumaric acid on transpiration and water utilization, however, were inconsistently expressed over the

TABLE 5. PARSIMONIOUS MODELS FOR THE EFFECTS OF A SINGLE *p*-COUMARIC ACID TREATMENT ON TRANSPIRATION, WATER UTILIZATION, MEAN LEAF AREA, AND ABSOLUTE AND RELATIVE RATES OF LEAF EXPANSION OF 12+ D OLD CUCUMBER SEEDLINGS GROWING IN NUTRIENT CULTURE, EXPERIMENT 3[a]

			Coefficients			
	Solution change[b]	Intercept	Linear	Quadratic	*P*	r^2
Transpiration (ml/cm^2/hr)						
Day 1[c]		0.021	−0.016		<0.001	0.75
		(0.001)[d]	(0.003)			
Night		0.006	0.008			
		(0.001)	(0.003)			
Day 1		0.022	−0.018		<0.001	0.70
		(0.001)	(0.003)			
Day 2	No	0.022	−0.018			
		(0.001)	(0.003)			
	Yes	0.025	0.057	−0.131		
		(0.002)	(0.018)	(0.032)		
Water utilization (ml/seedling/hr)						
Day 1		0.984	−0.829		<0.001	0.70
		(0.052)	(0.154)			
Night	No	0.273	0.286			
		(0.057)	(0.166)			
	Yes	0.348				
		(0.057)				
Day 1		0.984	−0.829		<0.001	0.70
		(0.052)	(0.154)			
Day 2	No	1.288	−1.379			
		(0.099)	(0.270)			
	Yes	1.736				
		(0.092)				
Mean leaf area (cm^2)						
Day 1		46.164	−6.030		0.023	0.22
		(1.470)	(5.134)			
Night		51.800	−15.90			
		(1.723)	(6.015)			
Day 1		46.164	−6.030		<0.001	0.48
		(1.470)	(5.134)			
Day 2		59.600	−27.879			
		(2.026)	(7.075)			
Absolute rates of leaf expansion (cm^2/seedling/12 hr)						
Day 1	No	3.445	−19.612	28.795	<0.001	0.69
		(0.581)	(7.697)	(15.491)		
	Yes		−7.126	0		
			(2.290)			
Night	No	7.615	−10.661	0		
		(0.748)	(2.610)			
	Yes	8.942	−17.002	0		
		(0.748)	(2.610)			

TABLE 5. CONTINUED

	Solution change[b]	Intercept	Coefficients: Linear	Coefficients: Quadratic	P	r^2
Day 1	No	3.444	−19.612	28.795	<0.001	0.76
		(0.444)	(5.873)	(11.820)		
	Yes		−7.126	0		
			(1.747)			
Day 2	No	8.468	−10.122	0		
		(0.480)	(1.408)			
	Yes	6.175				
		(0.480)				
Relative rates of leaf expansion (cm^2/cm^2/12 hr)						
Day 1	No	0.074	−0.428	0.641	<0.001	0.73
		(0.010)	(0.132)	(0.265)		
	Yes		−0.149	0		
			(0.039)			
Night	No	0.150	−0.204	0		
		(0.013)	(0.045)			
	Yes	0.168	−0.294	0		
		(0.013)	(0.045)			
Day 1	No	0.074	−0.428	0.641	<0.001	0.75
		(0.008)	(0.102)	(0.205)		
	Yes		−0.149	0		
			(0.030)			
Day 2	No	0.147	−0.151	0		
		(0.008)	(0.024)			
	Yes	0.105				
		(0.008)				

[a] Where transpiration, water utilization, mean leaf area, and absolute and relative rates of leaf expansion = Intercept + (linear coefficient × *p*-coumaric acid concentration) + (quadratic coefficient × [*p*-coumaric acid]^2); seedlings were treated at 8 AM to concentrations ranging from 0 to 0.5 mM.
[b] Solutions not changed (No) or solutions were changed every 4 hr (Yes).
[c] Where the time interval for Day 1 = 8 AM to 8 PM, Night = 8 PM to 8 AM, and Day 2 = 8 AM to 8 PM.
[d] Standard errors in parenthesis.

36-hr experimental periods and thus are not described further (see Table 5 for details). The most consistent effects of solution treatments occurred for absolute and relative rates of leaf expansion. For the no-solution change treatment and the 4-hr change solution treatment, 0.5 mM *p*-coumaric acid inhibited absolute rates of leaf expansion by 76 and 100% for d 1 (first 12 hr), 70 and 95% for night (second 12 hr), and 60 and 82% for d 2 (final 12 hr), respectively. The values for relative rates of leaf expansion were 73 and 100% for d 1, 68 and 88% for night, and 51 and 72% for d 2, respectively.

Shoot dry weight was reduced by *p*-coumaric acid treatment but not by the solution change treatment [shoot dry weight (g) = 0.152–0.632 × mM *p*-coumaric acid; $r^2 = 0.30$; $P = 0.001$]. Shoot dry weight was reduced by 40% by 0.5 mM *p*-coumaric acid. Root dry weight was not significantly modified over the 36-hr treatment period. Control values for shoot and root dry weights were 0.154 ± 0.008 and 0.026 ± 0.001 g, respectively.

Experiment 4. Changing nutrient strength did not significantly modify *p*-coumaric acid depletion from the nutrient solution. Aeration, however, increased the depletion of *p*-coumaric acid (Table 6; Figure 3). Based on the models of Table 6, a 25% reduction of *p*-coumaric acid took approximately 14.5 hr for the aerated treatment and 24 hr for the nonaerated treatment. A 50% reduction took 16.5 and 31 hr, respectively. At 36 hr, the end of the treatment period, the aerated treatment had <1% (0.002 mM) left in solution and the nonaerated treatment had 36% (0.18 mM) left in solution. The solution pH for the aerated and nonaerated treatments increased from pH 5.00 ± 0.02 at the start of the treatment period to 6.12 ± 0.10 for the controls and to 6.54 ± 0.20 for the 0.5 mM *p*-coumaric acid treatment at the end of the 36-hr treatment period (Figure 2). An additional difference between these two treatments was the accumulation of breakdown products of *p*-coumaric acid (Figure 3). The nonaerated treatment had an accumulation of breakdown products over time ending with 0.27 mM *p*-coumaric acid equivalence at 36 hr (Table 6; Figure 3). There was little accumulation of breakdown products in the aerated treatment. The maximum concentration for this treatment was 0.018 mM at 12 hr. At all other times monitored, no breakdown products were detected (Figure 3).

Nutrient strength had only a minor impact on the inhibition of *p*-coumaric acid for transpiration, water utilization, mean leaf area, and absolute and relative rates of leaf expansion. However, a decline in the inhibition of 0.5 mM

TABLE 6. MODELS FOR SOLUTION CONCENTRATIONS OF 0.5 mM *p*-COUMARIC ACID OVER 36 HR, EXPERIMENT 4

Model	$\frac{a}{1+\exp[b+c\ln(\text{hour}+0.5)]}$			
		Coefficients		
Variable	Treatment	*a*	*b*	*c*
mM left in solution	No aeration	0.501	−15.381	4.432
	Aeration	0.511	−21.260	7.465
% left in solution	No aeration	100	−14.504	4.194
	Aeration	100	−21.257	7.464
Breakdown products (*p*-coumaric acid equivalence)	No aeration	0.394	11.804	−3.488

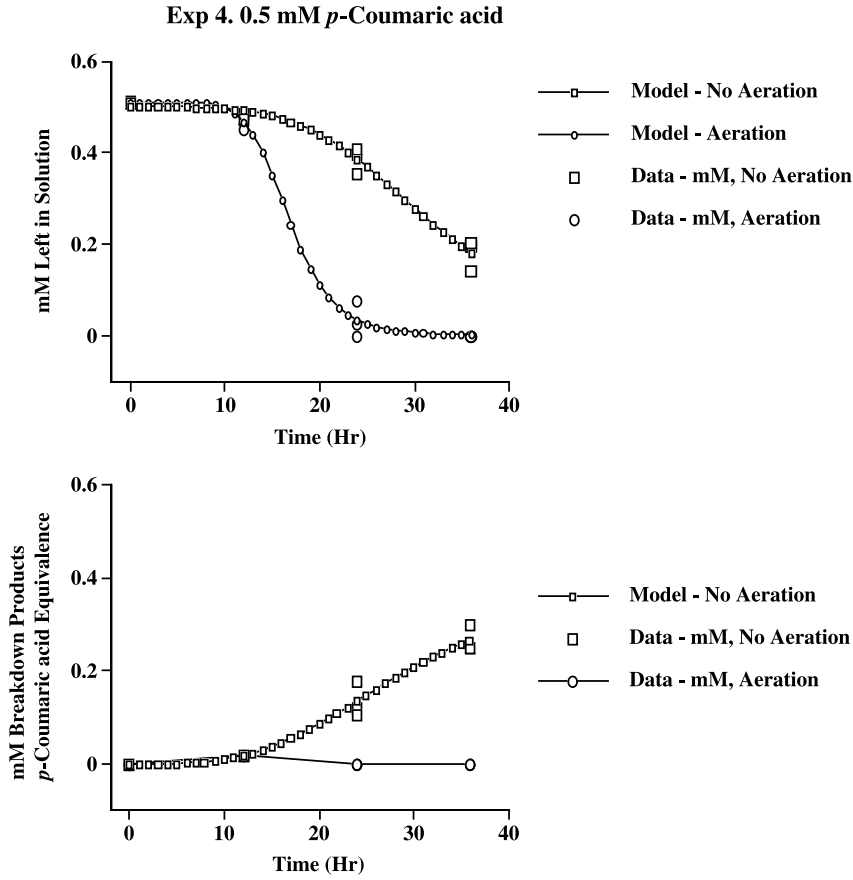

FIG. 3. Decline of 0.5 mM of *p*-coumaric acid and accumulation and/or decline of its breakdown products expresses as mM equivalents of *p*-coumaric acid in nutrient solutions surrounding roots of 12+ day-old cucumber seedlings. Nutrient solutions were aerated or not aerated. Experiment 4.

p-coumaric acid, because of the aeration of the nutrient solution, was evident after the first 12 hr of treatment. Utilizing the models of Table 7 and 8, 0.5 mM *p*-coumaric acid inhibited d 1 (first 12 hr) transpiration by 45 and 48%, water utilization by 48 and 51%, average leaf area by 5 and 4%, absolute rates of leaf expansion by 85 and 85%, and relative rates of leaf expansion by 84 and 85%, for aerated and nonaerated treatments, respectively. For night period (second 12 hr), the inhibitory effects for 0.5 mM *p*-coumaric acid were the following: transpiration by 9 and 13%, water utilization by 18 and 24%, average leaf area

TABLE 7. PARSIMONIOUS MODELS FOR THE EFFECTS OF NUTRIENT LEVELS, *p*-COUMARIC ACID, AERATION, AND TIME PERIOD (D 1 VS. NIGHT) ON TRANSPIRATION, WATER UTILIZATION, MEAN LEAF AREA, AND ABSOLUTE AND RELATIVE RATES OF LEAF EXPANSION OF 12+ D-OLD CUCUMBER SEEDLINGS GROWING IN NUTRIENT CULTURE, EXPERIMENT 4[a]

Variable	Transpiration	Water utilization	Mean leaf area	Rates of leaf expansion	
				Absolute	Relative
Intercept(s)		2.247 (0.351)	48.812 (0.975)		
Nut 0.5	0.056 (0.009)[b]			7.259 (0.358)	0.149 (0.007)
Nut 1	0.045 (0.009)			7.249 (0.358)	0.147 (0.007)
Nut 1.5	0.035 (0.009)			6.542 (0.358)	0.134 (0.007)
p-Coumaric acid (PCO)	−0.006 (0.010)	−0.549 (0.444)	−5.414 (1.379)	−5.434 (0.453)	−0.108 (0.009)
Aeration (AIR)	0.018 (0.007)	0.836 (0.314)	0.426 (1.126)	−0.513 (0.453)	−0.012 (0.009)
PCO × AIR				1.548 (0.640)	0.037 (0.012)
Nut 0.5			−0.804 (1.950)		
Nut 1			0.050 (1.950)		
Nut 1.5			−1.283 (1.950)		
Period (PER)	0.223 (0.010)	9.517 (0.444)	−5.006 (1.126)	−4.539 (0.453)	−0.087 (0.009)
PCO × PER	−0.124 (0.014)	−5.463 (0.629)	3.696 (1.593)	3.375 (0.640)	0.062 (0.012)
AIR × PER				2.062 (0.640)	0.047 (0.012)
PCO × AIR × PER				−2.891 (0.905)	−0.067 (0.018)
r^2	0.89	0.88	0.34	0.84	0.84
P	<0.001	<0.001	<0.001	<0.001	<0.001

[a] Where nutrient strength: 0.5, 1, or 1.5; *p*-coumaric acid concentration: control = 0 and 0.5 mM =1; aeration: no = 0 and yes = 1; period: d 1(8 AM to 8 PM) = 1 and night (8 PM to 8 AM) = 0; for the first 12 d, seedlings were grown in normal strength (1×) nutrient solution without aeration; nutrient, *p*-coumaric acid, and aeration treatments were initiated at 8 AM on d 12.

[b] Standard errors in parenthesis.

TABLE 8. PARSIMONIOUS MODELS FOR THE EFFECTS OF NUTRIENT LEVELS, *p*-COUMARIC ACID, AERATION, AND TIME PERIOD (D 1 VS. D 2) ON TRANSPIRATION, WATER UTILIZATION, MEAN LEAF AREA, AND ABSOLUTE AND RELATIVE RATES OF LEAF EXPANSION OF 12+ D-OLD CUCUMBER SEEDLINGS GROWING IN NUTRIENT CULTURE, EXPERIMENT 4[a]

Variable	Transpiration	Water utilization	Mean leaf area	Rates of leaf expansion	
				Absolute	Relative
Intercept(s)			55.950 (0.876)	7.444 (0.449)	0.131 (0.007)
Nut 0.5	0.270 (0.011)[b]	14.932 (0.557)			
Nut 1	0.253 (0.011)	14.361 (0.557)			
Nut 1.5	0.237 (0.011)	13.345 (0.557)			
p-Coumaric acid (PCO)	−0.084 (0.014)	−6.599 (0.705)	−9.466 (1.240)	−4.603 (0.635)	−0.068 (0.010)
Aeration (AIR)	0.028 (0.014)	1.523 (0.705)		−0.710 (0.635)	−0.011 (0.010)
PCO × AIR	0.062 (0.019)	3.256 (0.997)		3.682 (0.898)	0.070 (0.015)
Period (PER)	0.015 (0.014)	−2.411 (0.705)	−11.930 (1.240)	−4.966 (0.635)	−0.075 (0.010)
PCO × PER	−0.045 (0.019)	0.650 (0.997)	7.407 (1.753)	2.544 (0.898)	0.022 (0.015)
AIR × PER	−0.012 (0.019)	−0.761 (0.997)		2.259 (0.898)	0.046 (0.015)
PCO × AIR × PER	−0.064 (0.027)	−3.383 (1.409)		−5.025 (1.270)	−0.100 (0.021)
r^2	0.78	0.82	0.62	0.74	0.77
P	<0.001	<0.001	<0.001	<0.001	<0.001

[a] Where nutrient strength: 0.5, 1, or 1.5; *p*-coumaric acid concentration: control = 0 and 0.5 mM =1; aeration: no = 0 and yes = 1; period (8 AM to 8 PM): d 1 = 1 and d 2 = 0; for the first 12 d, seedlings were grown in normal strength (1×) nutrient solution without aeration; nutrient, *p*-coumaric acid, and aeration treatments were initiated at 8 AM on d 12.

[b] Standard errors in parenthesis.

by 12 and 11%, absolute rates of leaf expansion by 60 and 77%, and relative rates of leaf expansion by 54 and 75%, for aerated and nonaerated treatments, respectively. For d 2 (final 12 hr), the inhibitory effects for 0.5 mM *p*-coumaric acid were the following: transpiration by 8 and 33%, water utilization by 21 and 46%, average leaf area by 16 and 16%, absolute rates of leaf expansion by 14 and 62%, and relative rates of leaf expansion by 0 and 52%, for aerated and nonaerated treatments, respectively.

Final shoot and root dry weights were influenced by *p*-coumaric acid and aeration, respectively. The 0.5 mM *p*-coumaric acid treatment reduced shoot dry weight by 9% (shoot dry weight = 0.142–0.025 × *p*-coumaric acid concentration; $P < 0.001$, $r^2 = 0.49$). Aeration increased root dry weight by 23% [root dry weight = 0.024 + 0.006 × (1 for aeration and 0 for no aeration); $P < 0.001$, $r^2 = 0.23$].

DISCUSSION

Increasing concentrations of the cinnamic acids, ferulic acid, and *p*-coumaric acid, were approximately two to five times more inhibitory to cucumber seedlings transpiration, water utilization, leaf area, absolute and relative rates of leaf expansion, and shoot and root dry weights than the benzoic acids, *p*-hydroxybenzoic acid, and vanillic acid. Inhibitions ranged from 27 to 65% for the cinnamic acids and from 5 to 25% for the benzoic acids. These findings are consistent with previous studies utilizing cinnamic and benzoic acids and cucumber seedlings (Blum and Dalton, 1985; Blum et al., 1985a, b). As long as phenolic acid concentrations, for example, *p*-coumaric acid, were maintained at inhibitory concentrations (multiple treatments, experiment 1, Figure 1), the percent inhibition of water utilization remained relatively constant for a given concentration and phenolic acid, percent inhibition of leaf area increased and then leveled off to a constant percent, and percent inhibition of transpiration and absolute and relative rates of leaf expansion declined at various rates. Leaf area and leaf area expansion of these cucumber seedlings were primarily associated with the first true leaf, and thus as the leaf reached its maximum size, inhibition of leaf area leveled off and inhibition of leaf expansion declined. Final leaf area of individual leaves, however, was reduced permanently when inhibitory phenolic acid concentrations were maintained for a sufficient length of time during leaf development.

This is the first time that the relationships between treatment solution concentrations and uptake of phenolic acids by roots have been determined over an extended time period, i.e., 24 hr. Previous studies have determined uptake over a single time interval, e.g., mean uptake over 5 or 8 hr (Shann and Blum,

1987a; Lyu and Blum, 1990; Lehman and Blum, 1999). Uptake of *p*-coumaric acid from nutrient solutions by cucumber seedling roots occurred throughout the entire 24-hr periods monitored for experiments 2a and 2b. Relationships between initial treatment concentrations and uptake were, however, inconsistently expressed over 24 hr. For the 8 AM *p*-coumaric acid treatment (experiment 2a), the beginning of the photoperiod, significant positive relationships between uptake and initial *p*-coumaric acid concentration were only observed for the morning (8 AM to 2 PM), but not for the following afternoon (2 PM to 8 PM) and night (8 PM to 8 AM). For the 8 PM *p*-coumaric acid treatment (experiment 2b), the end of the photoperiod, significant positive relationships between uptake and initial *p*-coumaric acid concentrations were observed during the night, but not during the next morning. The relationship, however, reappeared in the afternoon. Periods without significant relationships between uptake and solution concentrations occurred, although initial concentrations of *p*-coumaric acid in both experiments were highly correlated (r = 0.98) with *p*-coumaric acid concentrations for all time periods monitored, including the end of the 24-hr treatment period. These findings suggested that periods with significant positive relationships between *p*-coumaric acid uptake and *p*-coumaric acid concentration may represent periods dominated by apoplastic movement of *p*-coumaric acid into the intercellular spaces of roots and the subsequent saturation of binding sites (e.g., covalent and noncovalent cross-links) on the cell walls in the apoplast (Fry, 1988), and that periods lacking significant relationships may represent periods dominated by symplastic movement (i.e., active transport across membranes and transfer by plasmodesmata from cell to cell). There is considerable evidence to suggest that absorption of secondary compounds, such as phenolic acid, by seedlings is an active process requiring energy and transporters (Martinoia et al., 2002; Taiz and Zeiger, 2002; Walker et al., 2003). Finally, the presence of periods without significant relationships between uptake and solution concentrations, although a broad range of solution concentrations was maintained over the 24-hr periods, suggested that even at 0.125 mM (lowest concentration used), the uptake sites on the root membranes of seedlings were saturated by *p*-coumaric acid.

We are not aware of any previous efforts to determine the influence of gradual phenolic acid depletion, i.e., declining concentrations, on the recovery of seedling processes, such as transpiration, water utilization, and leaf expansion. However, recovery after complete substitution of phenolic acid–nutrient solutions with just nutrient solutions has been determined (Blum and Dalton, 1985; Blum et al., 1985a, b; Blum and Rebbeck, 1989). In experiments 3 and 4, we attempted to manipulate *p*-coumaric acid concentrations over time by changing solutions at intervals to maintain roughly constant ranges of concentrations or increasing the rate of depletion by modifying nutrition and/or aeration. When *p*-coumaric acid was inhibitory, recovery (i.e., decline in inhibition) of

transpiration, water utilization, and rates of leaf expansion of actively growing leaves over a 36-hr period appeared to be related to the declining concentrations of *p*-coumaric acid.

To more fully characterize how cucumber seedlings responded to declining concentrations of *p*-coumaric acid, we chose to use the data for leaf area expansion, the most complete and consistent data set. To eliminate confounding due to the effects of treatment concentrations, only the data from the 0.5 mM *p*-coumaric acid treatments were used. Overall, there appeared to be a linear relationship between average mM *p*-coumaric acid at each interval [(mM at start + mM at end)/2] and percent inhibition of both absolute and relative rates of leaf expansion for 12+ d old seedlings (Figure 4). Based on the resulting regression, it would appear that for every decline in 0.1 mM, inhibition of absolute and relative rates of leaf expansion declined (i.e., recovered) by 12 and 14%, respectively.

One might, however, ask whether these differences in recovery were exclusively related to the physical decline of *p*-coumaric acid or whether other factors might also have been involved. For example, changes in solution pH could have been important in the observed differential rates of recovery

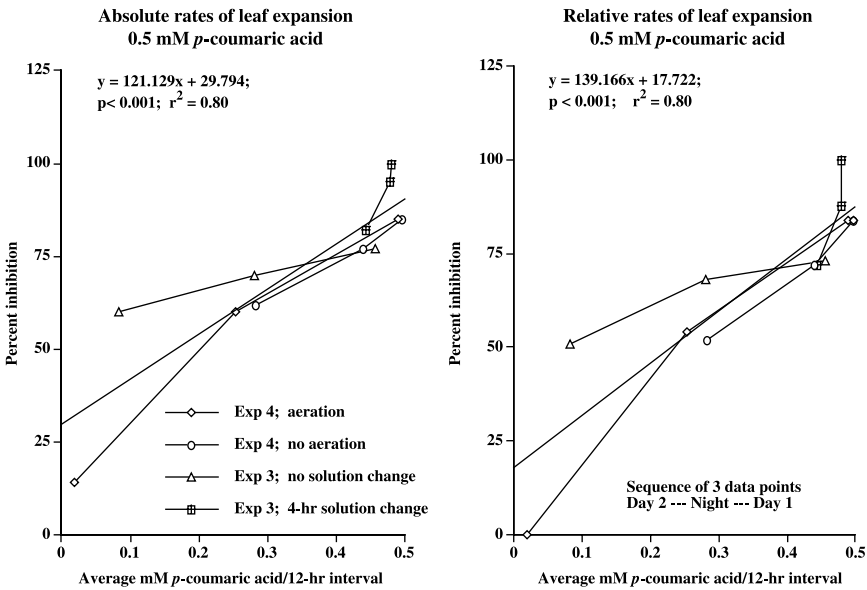

FIG. 4. Relationships between percent inhibition of absolute and relative rates of leaf expansion and the decline of 0.5 mM *p*-coumaric acid. Data derived from appropriate models in Tables 5–8. Each data point represents a 12-hr period, i.e., d 1, night, or d 2.

between the aerated and nonaerated treatments. It has been demonstrated that increasing solution pH will reduce the inhibitory activity of phenolic acids such as *p*-coumaric acid (Blum et al., 1985b); maximum inhibition and protonation occur under acidic conditions. *p*-Coumaric acid has a pK_a between 4.40 and 4.64 (Blum et al., 1999b). At the end of the 36-hr treatment period, the solution pH values for the aerated and nonaerated *p*-coumaric acid solutions were approximately 7 and 6, respectively. How important this may have been is not entirely clear; however, Blum et al. (1985b) observed that 0.5 mM *p*-coumaric acid had little inhibitory activity on cucumber seedlings above a pH of 6.25.

Finally, the range and magnitude of phenolic acid effects are determined by the sensitivity of seedling, seedling's ability to detoxify phenolic acids, the types and concentration of phenolic acids present, the rates of input and depletion of phenolic acids, and rhizosphere and rhizoplane pH (see Rice, 1984; Shann and Blum, 1987a, b; Blum et al., 1999b). Because cucumber seedling roots take up water and phenolic acids, detoxify phenolic acids, modify solution rhizosphere and rhizoplane pH, and regulate phenolic acid utilizing microbes within the rhizosphere, it would appear that cucumber seedlings themselves can readily change the active phenolic acid concentrations surrounding their roots and thus regulate the development, retention, and decline of both primary and secondary effects of phenolic acids through feedback regulation. This of course assumes that new input of phenolic acids does not replace or outpace losses. Feedback regulation is defined here as a response in which the product of one of the final steps in a chain of events affects one of the first steps in this chain; fluctuations in rate or concentration are minimized with negative feedback or amplified with positive feedback (Lambers et al., 1998). The findings here suggest that negative feedback regulation by seedling can be an important factor in determining the ultimate expression of inhibition by phenolic acid. It also would appear that unless care is taken in maintaining fairly constant phenolic acid concentrations and appropriate solution pH values surrounding roots over a given experimental period, the phytotoxicity of phenolic acids may be significantly over- or underestimated. Unfortunately, attempts to regulate solution pH values surrounding roots in nutrient culture by buffers for extended periods have met with little success (Blum et al., 1985a).

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DEGRADATION OF LEAF LITTER PHENOLICS BY AQUATIC AND TERRESTRIAL ISOPODS

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Abstract—To investigate species-specific decomposition rates of litter from native (*Quercus faginea*) and introduced (*Eucalyptus globulus*) tree species in Portugal, we monitored changes in the phenolic signature of leaf litter during decomposition as mediated by an aquatic, *Proasellus coxalis* (Isopoda: Asellota), and two terrestrial, *Porcellio dispar* and *Eluma caelatum* (Isopoda: Oniscidea), detritivores. Although the litter of *Eucalyptus* and *Quercus* did not differ in overall protein precipitation capacity, we detected differences in terms of contents of particular phenolic compounds and phenol oxidation products. Accordingly, we observed food-specific consumption rates in *Proasellus*, but not in the terrestrial isopods. *Proasellus* digested *Eucalyptus* at significantly higher rates than *Quercus*, whereas the opposite was the case for *Eluma*, and *Porcellio* digested both litter types equally well. Despite slight differences in detail, effects of *Proasellus* on changes in the signature of litter phenolics were similar for both litter types, whereas terrestrial isopods—*Porcellio* and *Eluma*, although they differed from each other—digestively degraded phenolic compounds in *Eucalyptus* and *Quercus* litter, respectively, in different ways. Overall, however, degradation of litter phenolics was similarly effective on both litter types. From these data, we conclude that decomposition of *Eucalyptus* litter does not proceed more slowly than of litter from native Portuguese trees.

Key Words—Animal–microbe interactions, decomposition, *Eucalyptus*, Isopoda, neophytes, phenolics, *Quercus*, tannin degradation.

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INTRODUCTION

Eucalyptus globulus Labill., native in Australia, has been planted during the last 170 yr in southern European countries, South America, and other parts of the world. In Portugal, *Eucalyptus* plantations nowadays occupy more than 20% of the forested area (Canhoto et al., 2002). Several studies demonstrated that *Eucalyptus* plantations affect invertebrate assemblages in soils (e.g., Pinto et al., 1997; Sousa et al., 1997) and freshwaters (e.g., Abelho and Graça, 1996; Graça et al., 2002). In freshwaters, invertebrate shredders may exert an important contribution to the decomposition of leaves entering streams (Graça, 2001); however, invertebrate shredders seem to have difficulties feeding on *E. globulus* leaves (Graça et al., 2002). In soil, terrestrial isopods apparently have the same difficulty using *E. globulus* litter (Sousa, personal communication). *Eucalyptus* litter is, therefore, considered a low-quality food for both freshwater (Mellilo et al., 1982; Campbell and Fuchshuber, 1995) and soil (Sousa, personal communication) detritivores. Such effects may be due to chemical and physical defense mechanisms in leaves. Canhoto and Graça (1999) showed that eucalyptus oils inhibit fungal growth and invertebrate feeding, and a thick cuticle retards microbial colonization of senescent leaves.

Another chemical defense of *E. globulus* leaves is polyphenolics. Canhoto and Graça (1996) observed a strong negative correlation between the phenol content of different native litter types and *Eucalyptus* litter and decomposition rates in a stream, whereas Canhoto and Graça (1999) showed that polyphenolics from eucalypt leaves decrease feeding by detritivores. Thus, effects of phenolics on detritivores may be one reason for the low decomposability of *Eucalyptus* litter. Overall, however, *Eucalyptus* litter decomposed at almost two times higher rates than the Portuguese oak, *Quercus faginea* Lam., the latter containing more than twice as much phenolics than the former but about the same amount of nitrogen (Canhoto et al., 2002). In the present study, we used leaf litter of the native Portuguese oak, *Q. faginea*, and the introduced *E. globulus* (both henceforth mentioned generically) to study in detail changes in the content of specific phenolic compounds during decomposition as mediated by aquatic and terrestrial detritivores.

Both terrestrial Oniscidea and freshwater Asellota had ancestors in the marine environment (that may have been closely related; Zimmer and Bartholmé, 2003). It has recently been discussed to what extent the ability to tolerate or even digest phenolic food compounds may have been important in the evolutionary step to utilizing terrestrial food sources (Zimmer et al., 2002a,b); despite their aquatic lifestyle, freshwater isopods mostly consume leaf litter of terrestrial origin (cf. Zimmer and Bartholmé, 2003) just like their terrestrial relatives do (cf. Zimmer, 2003). We were, thus, interested in whether representatives of different phylogenetic histories that are similar with respect to

available food sources are also similar in their digestive capabilities. Hence, we chose two terrestrial and one freshwater isopods as common Portuguese models for evolutionary ecological studies on digestive capabilities and detritivore–microbe interactions and their consequences for decomposition processes, in particular the degradation of phenolic leaf litter compounds.

METHODS AND MATERIALS

Leaf Litter and Detritivores. We collected leaf litter in spring 2002, when isopods were active in the field and started reproducing, in the Mata Nacional do Choupal, Coimbra (40°12′33″N, 8°90′27″W), Central Portugal. At this time of the year, with high feeding rates by isopods, *Quercus* litter had been lying on the ground for about 1–3 mo, whereas *Eucalyptus* litter was estimated to be up to 6 mo old. Thus, our choice of litter reflected the natural conditions detritivores face during their most active feeding period; many, but not all, species-specific compounds of the litter will have been leached at that stage. We selected leaves that did, upon visual inspection, not show heavy decomposition. In the laboratory, leaves were air-dried in the dark at room temperature and stored dry until needed to avoid further loss of phenolics through leaching or photoautoxidation. We did not test for differences in phenolic signatures of leaf litter before and after storage because we were interested in relative effects of detritivore activity on phenolic litter compounds during decomposition.

Terrestrial isopods, *Porcellio dispar* Verhoeff 1901 (Oniscidea: Porcellionidae) and *Eluma caelatum* [Miers 1877] (Oniscidea: Armadillidiidae), were collected in the Mata Nacional do Choupal by browsing through mixed leaf litter layers in spring 2002. In the laboratory, they were housed in plastic boxes with moist sand (16°C) feeding exclusively on *Quercus* or *Eucalyptus* for at least 5 d. *Proasellus coxalis* [Dollfus 1892] (Asellota: Asellidae) were collected in a small creek, Rio Pavia in Fail (40°36′17″N, 7°58′44″W), near Viseu, northeast of Coimbra, which belongs to the Mondego's drainage basin. In the laboratory, they were housed in plastic boxes with aerated water of the same origin (16°C) feeding exclusively on *Quercus* or *Eucalyptus* for at least 5 d.

Experimental Setup. During experiments ($N = 9$, each), groups of three (terrestrial) or five (aquatic) isopods were kept in small petri dishes, the lids of which were lined with moist filter paper to maintain high humidity or that were filled with filtered creek water, respectively. Feces were collected twice a day to minimize both coprophagy and postdigestive changes in phenolic compounds by picking up fecal pellets with forceps or by filtering the water from the petri dishes after removal of isopods and litter, respectively. Afterwards, isopods

were placed in fresh petri dishes with filter paper-lined lids or filtered creek water, respectively. Feces were immediately stored at −20°C until being used for HPLC analysis.

Single air-dried leaves, randomly chosen from the pool of hand-selected litter (see above), were cut in pieces, two of which were weighed and then placed in either isopod assays or isopod-free controls. Another piece of that same leaf was used to determine the initial phenolic signature of individual leaves as described below. An additional piece of each leaf was weighed, then oven-dried (60°C, 24 hr), and weighed again to obtain a factor to estimate the initial dry mass of litter (Zimmer and Huryn, in press). After the experiment, lasting for 5 d, litter remnants were weighed fresh to calculate mass loss. Litter obtained from parallel experiments that were not used for HPLC analysis was used to determine a factor for the estimation of final dry mass of litter (oven drying at 60°C for 24 hr) as described above.

Consumption rates [mg (mg d)$^{-1}$] were determined based on litter mass loss and mean isopod dry mass. Digestibility (%) was determined as percent of ingested litter that was not egested as feces (Zimmer and Huryn, in press). Direct effects of detritivores on litter chemistry through digestion were quantified by comparing the phenolic signature of litter prior to the experiment with that of isopod feces. Indirect effects of detritivores on litter chemistry (e.g., through mediating microbial decomposition; see Zimmer et al., 2002b, 2004) were estimated by comparing the phenolic signature of litter prior to the experiment with that of unconsumed litter remnants after the experiment. Such indirect effects could have occurred either through selective consumption of litter or through direct influences of detritivores (e.g., fecal amount and composition, mucus, urine, molt deposition, and so on) on type and abundance of microbiota (Zimmer et al., 2002b, 2004).

Leaf Litter Phenolics. For extraction of phenolics, 45–55 mg (dry mass) of leaf litter or 3–10 mg of feces were homogenized in 2 ml 50% aqueous methanol (cf. Van Alstyne, 1995) and shaken for 3 hr at 16°C. After centrifugation, the solvent was replaced by 2 ml 50% methanol, and samples were extracted overnight at 16°C. After centrifugation (10,000 × *g*, 10 min), supernatants were pooled and either used for the determination of protein precipitation capacity or for HPLC analysis after filtering (0.45 μm).

The capability of phenolics to precipitate proteins was measured using the radial diffusion assay (Hagerman, 1987). Portions of 9.5 ml of agarose (1%) containing 0.1% of bovine serum albumin (BSA) were dispensed in 8.5-cm diam petri dishes. Polyphenolics were extracted from 50 mg of leaf powder as described above. Aliquots of the supernatant (36 μl) were inoculated into 4-mm diam wells punched out from the agar plate. A protein precipitation ring was allowed to develop for 3 d at 16°C. The precipitation area was measured and compared to a standard curve using tannic acid (Merck; Ref.: 1.00773.0250;

Lot K18722673703), and the results were expressed in terms of "tannic acid equivalents." We are aware that different phenolic compounds will have different protein precipitation capacities, but because we could not determine most of the compounds, we refrained from using different standards.

For the detection of specific phenolic compounds in litter and feces extracts, we performed RP-HPLC using a Brownlee Column (Applied Biosystems) SPHERI-5 RP-18 (5 μm) 250 × 4.6 (OD-5A) with a NewGuard RP-18 (7 μm) 15 × 3.2 guard column. Peaks were detected at 280 nm. In preruns, no peaks were detected after 45 min; thus, we chose a run time of 45 min. According to previous studies (Scalbert et al., 1988; Streit and Fengel, 1994) and our own preexperiments, we chose a system of two eluants [A, 950:49:1, water/methanol/phosphoric acid (85%); B, 999:1, methanol/phosphoric acid (85%)] with a gradient of 0–5% B during the first 15 min and a subsequent gradient of 5–90% B during 15 min at a flow rate of 1 ml min^{-1} at 20°C.

Besides other phenolic compounds, tannic acid (mainly *penta*-galloylglucose), gallic acid, catechin, and gallocatechin have been isolated from *Quercus* leaves (e.g., Scalbert et al., 1988; Tanaka et al., 1995). We used these compounds (Sigma) as standards for HPLC in concentrations of 0.1, 0.5, and 1.0 mg ml^{-1}. Because tannic acid, rutin, quercetin, and catechol have, besides others, been determined in extracts of *Eucalyptus* leaves (e.g., Conde et al., 1997; Sasikumar et al., 2002), we used these compounds (Sigma) as standards in HPLC (0.1, 0.5, and 1.0 mg ml^{-1}). To detect oxidation products of these model phenolics in experimental extracts, we forced phenol oxidation in standards by both vigorously shaking these solutions for 60 min at RT and adding NaOH solution (32%, pH 12; 1 ml 100 ml^{-1}). After the generation of brown coloration, we determined the elution time of phenol-specific oxidation products by HPLC as described above.

Even with the slowly changing conditions during min 0–15 (see above), we could not unambiguously identify every single peak derived from a complex mixture of litter phenolics and their derivates (see Table 1). Difficulties in separating phenolic compounds by RP-HPLC with water–methanol gradients have previously been reported in flowers by Van de Casteele et al. (1983), in leaves by Koupai-Abyazani et al. (1992), and in wood by Streit and Fengel (1994). We, thus, refrain from assigning phenolic standards to particular HPLC peaks, but refer to phenolic compounds by the name of peaks in order of their elution.

From the areas (units ml^{-1}) of particular peaks as revealed by HPLC analysis of pre- and postexperimental litter and feces, we approximated changes in the content of particular phenolic compounds (%) in isopod-free controls, through isopod-mediated microbial activity, and through isopod feeding and digestion. Further, we estimated litter decomposition in terms of mass loss by calculating consumption rates of detritivores as described in Zimmer and Huryn

TABLE 1. ASSIGNMENT OF STANDARD PHENOLIC COMPOUNDS[a] TO HPLC PEAKS

	C	D	E	F	G	H	M	O	P	R	U
GA					X						
TA	X	X	X	X							
CAT	X										
CATol						X					
gCAT	X										
oxGA		X									
oxTA							X	X			
oxCAT									X		
oxCATol		X									
oxgCAT										X	X

[a] GA, gallic acic; TA, tannic acid; CAT, catechin; CATol, catechol; gCAT, gallocatechin; ox-, oxidation product of -. Other standard compounds could not be assigned to HPLC peaks from experimental samples.

(in press). Because most of our data deviated from normal distribution, we chose median ± median absolute deviation for graphical presentation, and nonparametric tests were used for statistical analysis. Comparison of different treatments (leaf litter or detritivore species) was performed through Mann–Whitney *U*-tests. Differences between initial values and values after experimental treatment were tested with Wilcoxon sign tests.

RESULTS

General Patterns. Although *Quercus* and *Eucalyptus* litter did not differ from each other quantitatively in their initial protein precipitation capacity (radial diffusion assay: *Eucalyptus*, 24 ± 4 mg TA equivalents g^{-1}; *Quercus*, 18 ± 8 mg g^{-1}; $P > 0.7$), they differed qualitatively in terms of contents of different phenolic species and their oxidation products (Figure 1).

While microbial degradation (in animal-free assays) of *Quercus* leaves was not detectable in the terrestrial environment, litter lost 2 ± 1 μg $(mg\ d)^{-1}$ in the aquatic environment ($P < 0.05$), presumably because of microbial degradation and leaching. After correction of data for these control values, *Porcellio* [4 ± 2 μg $(mg\ d)^{-1}$], *Eluma* [7 ± 3 μg $(mg\ d)^{-1}$], and *Proasellus* [3 ± 2 μg $(mg\ d)^{-1}$] did not differ from each other with respect to consumption rates ($P > 0.3$). Digestibility of *Quercus* litter was similar in *Porcellio* (33 ± 17%) and *Proasellus* (31 ± 24%), but significantly higher ($P < 0.05$) in *Eluma* (62 ± 22%).

Microbial degradation and leaching (in animal-free assays) of *Eucalyptus* resulted in litter mass loss of 4 ± 1 μg $(mg\ d)^{-1}$ in the terrestrial environment and 11 ± 9 μg $(mg\ d)^{-1}$ in the aquatic environment ($P < 0.01$). After correction

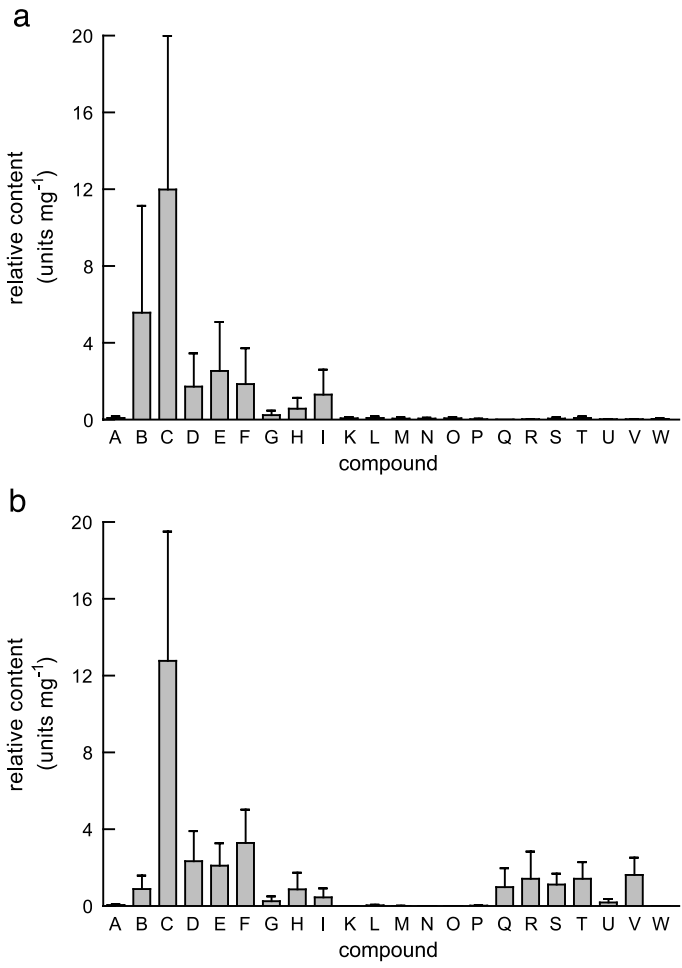

FIG. 1. Phenolic composition of leaf litter derived from *Quercus* (a) and *Eucalyptus* (b). Data are median ± median absolute deviation ($N = 9$).

of data for control values, *Porcellio* [5 ± 3 μg (mg d)$^{-1}$] and *Eluma* [3 ± 2 μg (mg d)$^{-1}$] did not differ from each other with respect to consumption rates ($P > 0.4$), whereas *Proasellus* [30 ± 20 μg (mg d)$^{-1}$] consumed about 10 times more than the terrestrial isopods. Thus, while the terrestrial isopods consumed about the same amounts of *Quercus* and *Eucalyptus*, *Proasellus* ingested significantly more *Eucalyptus* than *Quercus* ($P < 0.01$). Digestibility of *Eucalyptus* litter was similar in *Porcellio* (33 ± 13%) and *Eluma* (26 ± 20%), but significantly higher ($P < 0.05$) in *Proasellus* (71 ± 18%). Thus, *Porcellio* di-

gested both litter types equally well, whereas *Eluma* was significantly ($P < 0.01$) more efficient in digesting *Quercus*, and *Proasellus* digested *Eucalyptus* at significantly ($P < 0.01$) higher rates than *Quercus*.

According to these results, *Proasellus* ingested larger amounts of *Eucalyptus* than *Quercus*. Consumption of *Eucalyptus* differed between consumers, with *Proasellus* consuming the largest amounts, but *Quercus* was ingested in similar rates by all consumers. Accordingly, digestibility of *Eucalyptus* by *Proasellus* was higher than that of *Quercus*. By contrast, although *Eluma*, too, exhibited higher digestibility of *Eucalyptus* than *Porcellio*, the terrestrial species digested both litter types equally well.

General Changes in Phenolic Signatures

Quercus. In the aquatic environment, only compounds B, C, E, I, P, and W were not completely or at least mostly leached, degraded, or transformed in animal-free controls (Figure 2a); compounds E, P, and W were obtained in higher concentration after the experiment than before ($P < 0.05$). With isopods present, compounds C and I were microbially degraded or transformed in the leaf litter that was not consumed by isopods (Figure 3a); microbially processed litter was enriched in compounds N, P, U, and V ($P < 0.01$). On average, compounds B and I were digested completely by litter-feeding isopods (Figure 4a), whereas compounds E, F, H, M, P, T, and V were concentrated higher in feces than in the litter ($P < 0.01$).

In the terrestrial environment, only a few compounds were lost through leaching, degradation, or transformation in animal-free controls (Figure 5a); compounds D, P, and U were obtained in higher concentration after the experiment than before ($P < 0.001$). With *Porcellio* present, compounds I, V, and W were microbially degraded, and compounds E, M, and P became significantly reduced, probably through microbial activity, in the leaf litter that was not consumed by isopods (Figure 6a); microbially processed litter was enriched in compounds L and O ($P < 0.05$). With *Eluma* present, compounds D, E, F, O, and T were microbially degraded (Figure 7a), whereas compounds M, P, U, and V became enriched in the litter ($P < 0.001$). On average, compounds C, U, and V were digested completely in isopod guts, and about 90 and 50% of compounds E and I, respectively, was degraded digestively by *Porcellio* (Figure 8a), whereas compounds D, F, G, H, M, P, R, S, and T were concentrated higher in feces than in the litter ($P < 0.01$). *Eluma* digested all of the remaining compounds B, G, H, I, O, T, and V and reduced compound D by about 85% (Figure 9a), but led to a relative increase of compounds E, M, P, Q, R, U, and W ($P < 0.001$).

Eucalyptus. In the aquatic environment, only compounds B, C, E, H, P, U, and W were not completely or at least heavily leached or degraded in animal-free controls (Figure 2b); compounds H, P, and U were obtained in higher

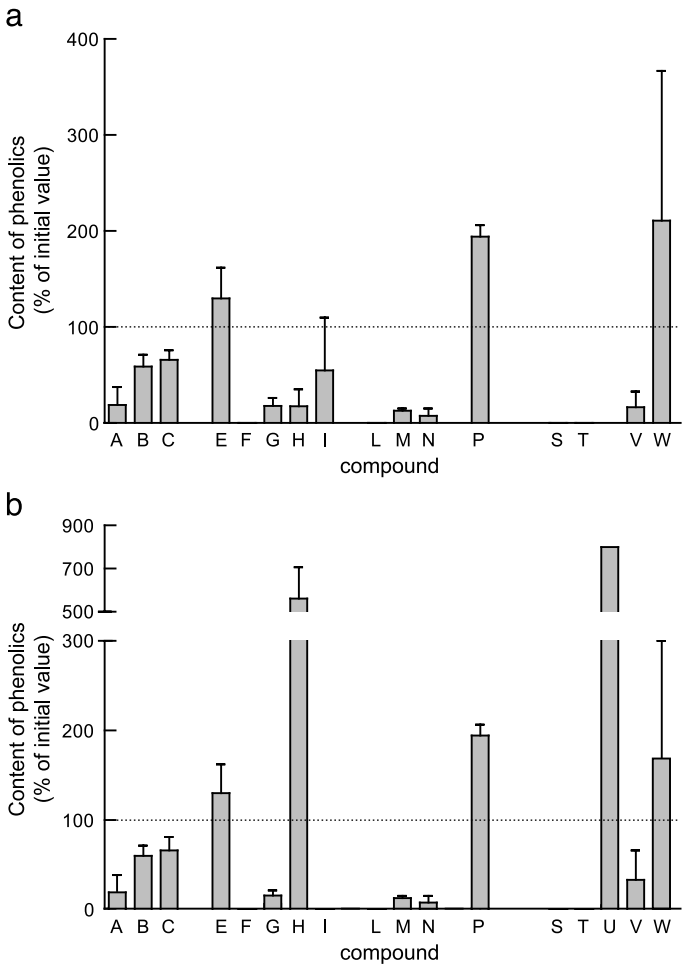

FIG. 2. Change in content of phenolic compounds in leaf litter derived from *Quercus* (a) and *Eucalyptus* (b) due to leaching and microbial processing within 5 d in aquatic animal-free control assays as compared with initial values of the litter (100%). Data are median ± median absolute deviation ($N = 9$). Only those peaks are listed along the *X*-axis that were found in the samples studied here.

concentration after the experiment than before ($P < 0.001$). With isopods present, compound A was microbially degraded or transformed in the leaf litter that was not consumed by isopods (Figure 3b); microbially processed litter was enriched in compounds N, P, and U ($P < 0.01$). On average, compound C was digested completely during isopod gut passage, and about 30% of compound E was

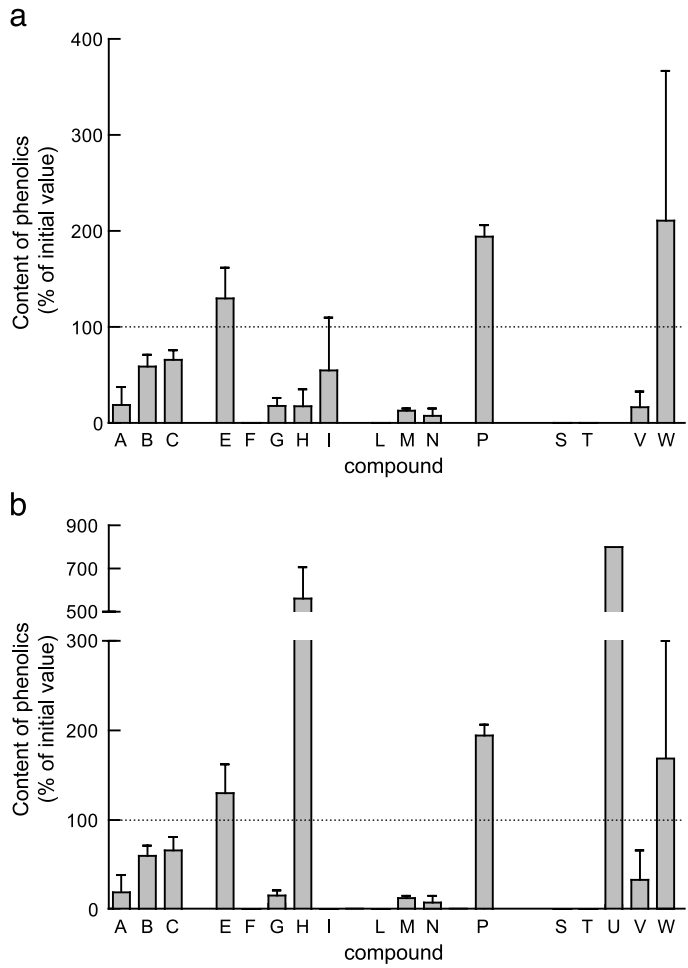

FIG. 3. Change in content of phenolic compounds in leaf litter derived from *Quercus* (a) and *Eucalyptus* (b) after 5-d incubation in aquatic environments, with *Proasellus coxalis* as detritivore, as compared with initial values of the litter (100%). Data are median ± median absolute deviation ($N = 9$). Only those peaks are listed along the *X*-axis that were found in the samples studied here.

degraded, although individual variation was high (Figure 4b), whereas compounds H, P, and U were concentrated higher in feces than in the litter ($P < 0.01$).

In the terrestrial environment, only a few compounds were completely leached, degraded, or transformed in animal-free controls (Figure 5b); only compound G was obtained in higher concentration after the experiment than

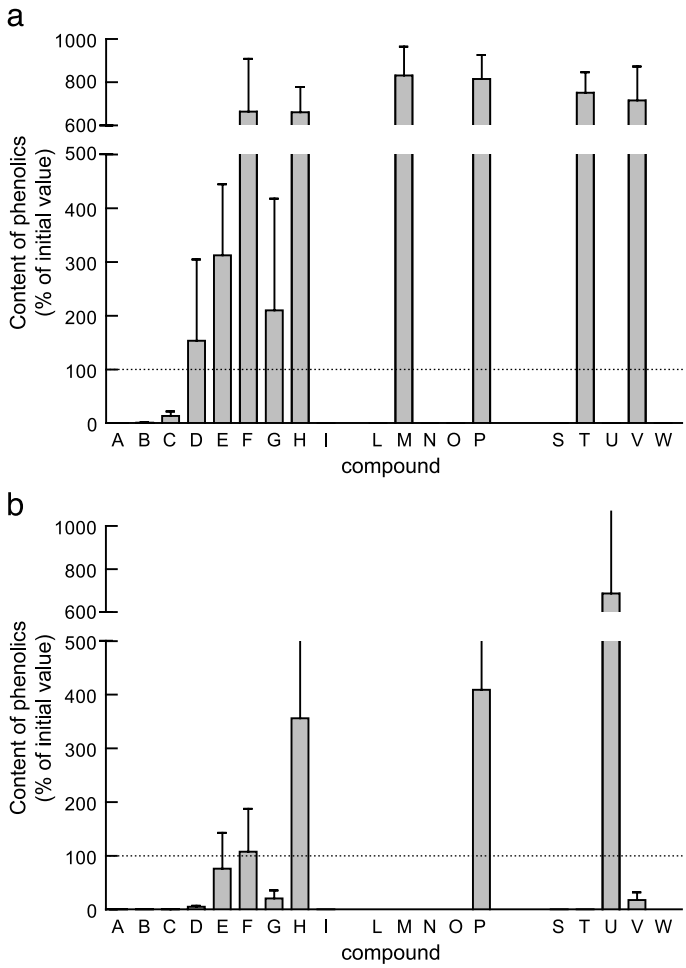

FIG. 4. Change in content of phenolic compounds of leaf litter derived from *Quercus* (a) and *Eucalyptus* (b) due to digestive processes by *P. coxalis* as deduced from comparing isopod feces with initial values of the litter (100%). Data are median ± median absolute deviation ($N = 9$). Only those peaks are listed along the *X*-axis that were found in the samples studied here.

before ($P < 0.001$). With *Porcellio* present (Figure 6b), microbially processed litter was enriched in compounds F, G, and T ($P < 0.01$). With *Eluma* present, compounds D, P, R, and V were microbially degraded or transformed in the leaf litter that was not consumed by isopods (Figure 7b), whereas compounds B, F, and T became enriched in the litter ($P < 0.01$). On average, compounds D, G, H,

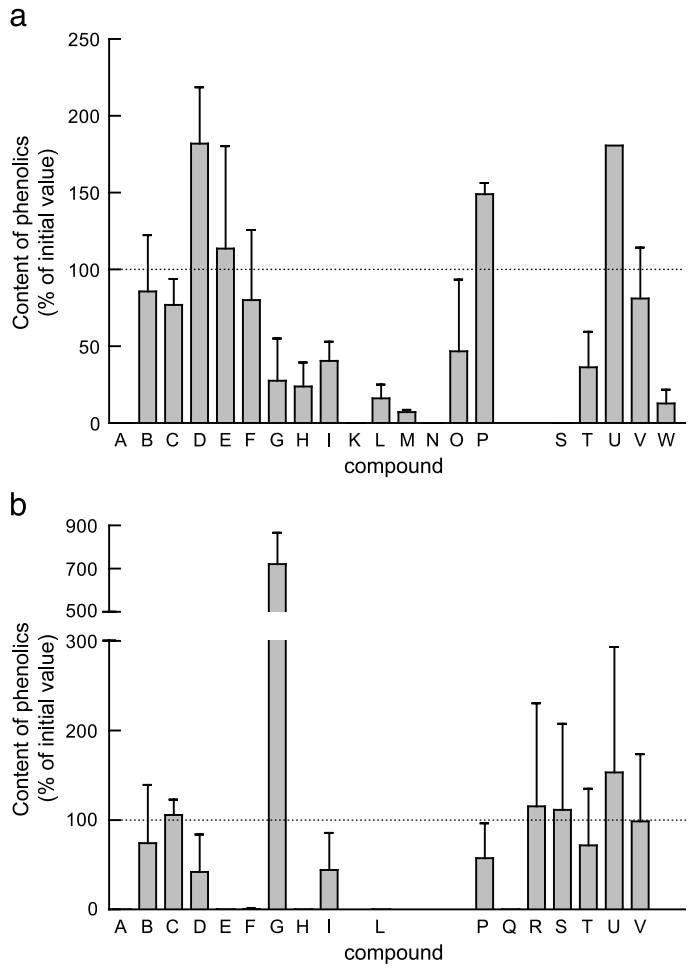

FIG. 5. Change in content of phenolic compounds in leaf litter derived from *Quercus* (a) and *Eucalyptus* (b) due to leaching and microbial processing within 5 d in terrestrial animal-free control assays as compared with initial values of the litter (100%). Data are median ± median absolute deviation ($N = 9$). Only those peaks are listed along the *X*-axis that were found in the samples studied here.

R, S, and V were digested extensively by *Porcellio* (Figure 8b), whereas compounds E, O, and T were concentrated higher in feces than in litter ($P < 0.01$). *Eluma* digested all of the remaining compounds B, G, I, P, R, T, and V, and degraded compound C almost completely (Figure 9b), but led to a relative increase of compounds D, O, and U ($P < 0.05$).

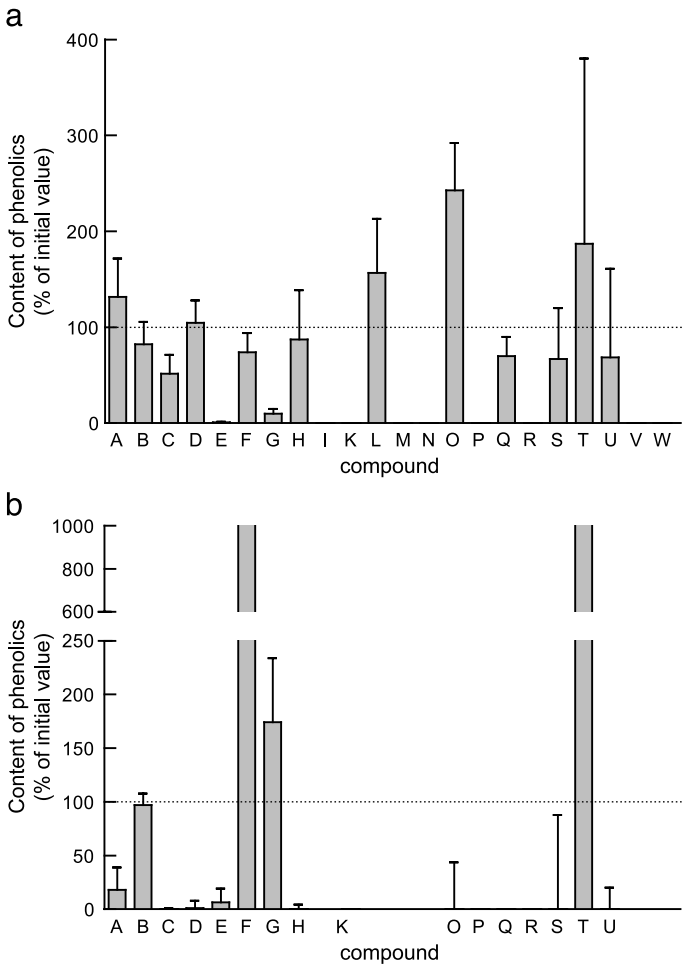

FIG. 6. Change in content of phenolic compounds in leaf litter derived from *Quercus* (a) and *Eucalyptus* (b) after 5-d incubation in terrestrial environments, with *Porcellio dispar* as detritivore, as compared with initial values of the litter (100%). Data are median ± median absolute deviation ($N = 9$). Only those peaks are listed along the *X*-axis that were found in the samples studied here.

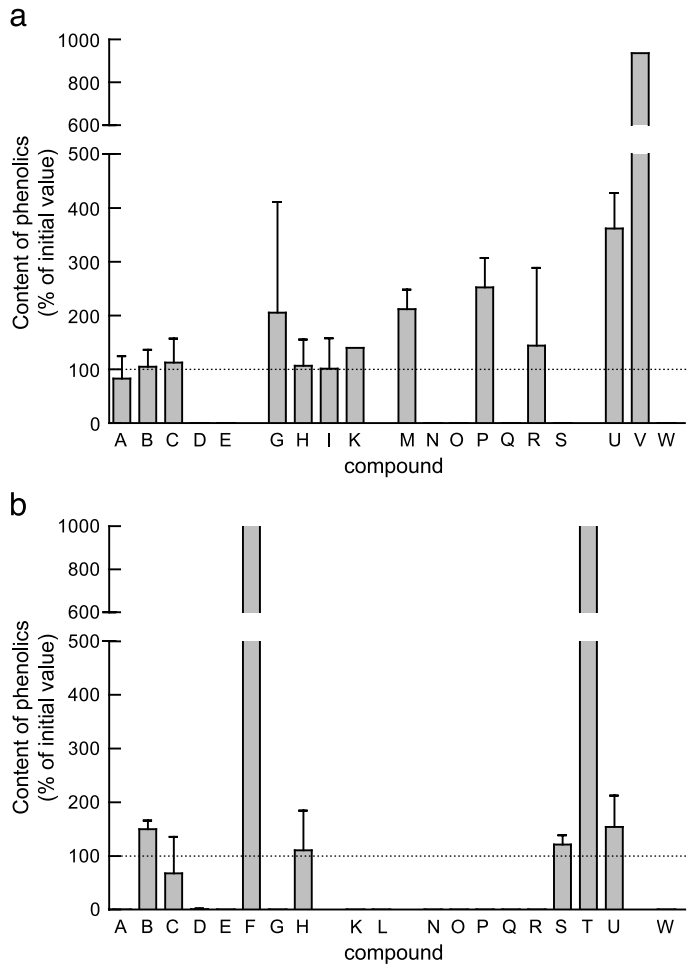

FIG. 7. Change in content of phenolic compounds in leaf litter derived from *Quercus* (a) and *Eucalyptus* (b) after 5-d incubation in terrestrial environments, with *Eluma caelatum* as detritivore, as compared with initial values of the litter (100%). Data are median ± median absolute deviation ($N = 9$). Only those peaks are listed along the *X*-axis that were found in the samples studied here.

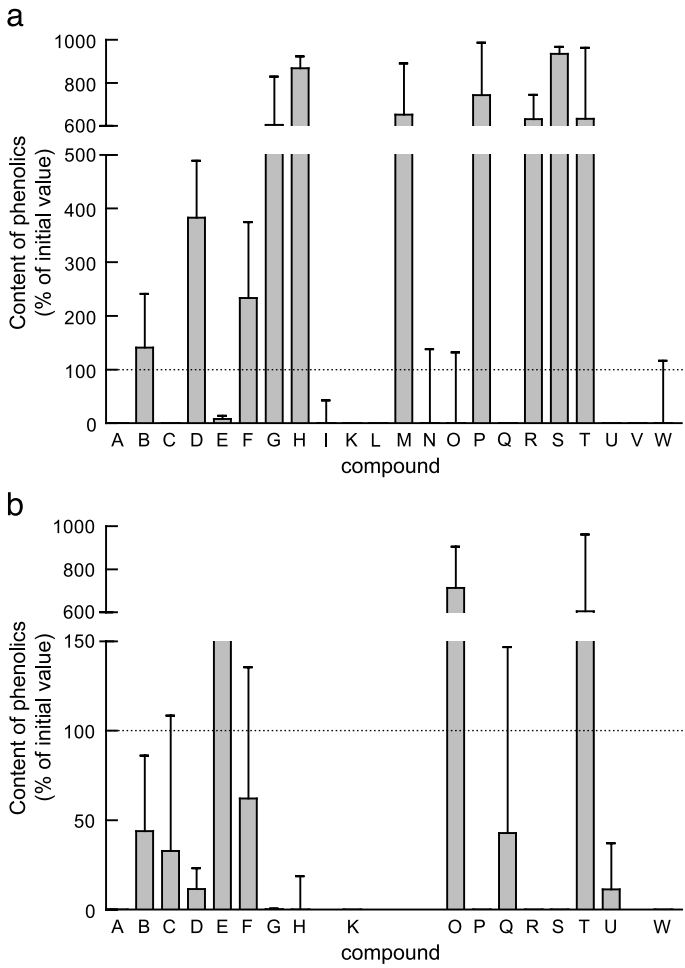

FIG. 8. Change in content of phenolic compounds of leaf litter derived from *Quercus* (a) and *Eucalyptus* (b) due to digestive processes by *P. dispar* as deduced from comparing isopod feces with initial values of the litter (100%). Data are median ± median absolute deviation ($N = 9$). Only those peaks are listed along the *X*-axis that were found in the samples studied here.

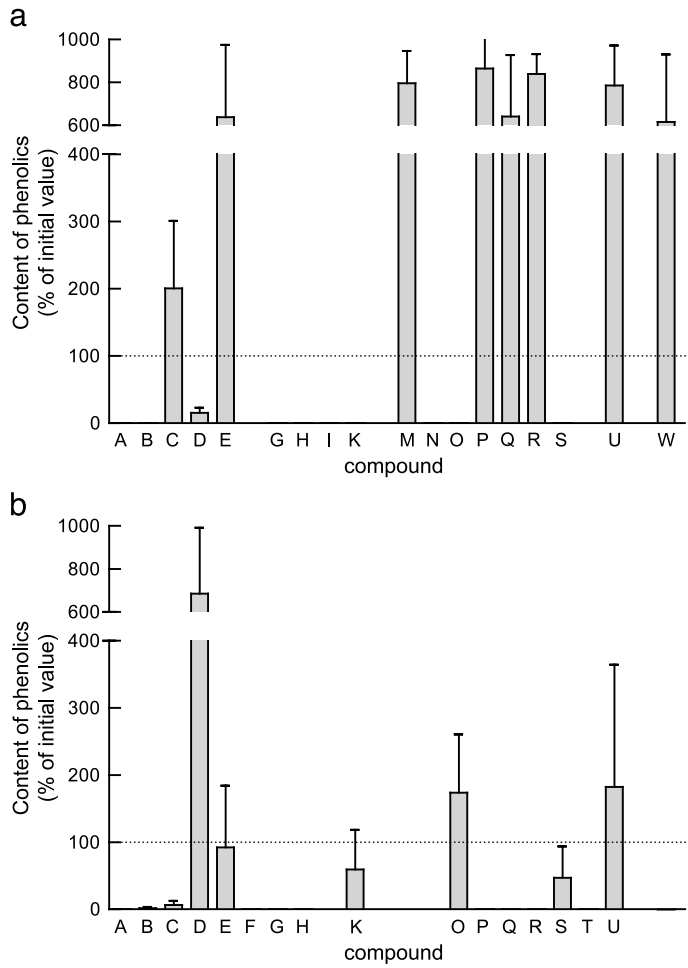

FIG. 9. Change in content of phenolic compounds of leaf litter derived from *Quercus* (a) and *Eucalyptus* (b) due to digestive processes by *E. caelatum* as deduced from comparing isopod feces with initial values of the litter (100%). Data are median ± median absolute deviation ($N = 9$). Only those peaks are listed along the *X*-axis that were found in the samples studied here.

DISCUSSION

Degradation of Particular Phenolic Compounds. Owing to the fact that we were unable to unambiguously assign different peaks of our HPLC analysis to distinct phenolic compounds (Table 1), we can only interpret our results based on rough interspecific differences between detritivores and tree species with respect to the reduction and/or increase in particular peaks. However, this information appears sufficient for a first ecosystemic approach to effects of interactions of different detritivores with different leaf litter types. Overall, however, interspecific differences deduced from our present results must be interpreted with caution because we do not know exactly whether the composition of single peaks we obtained by HPLC was the same in leaf litter from *Eucalyptus* and *Quercus*.

Ecosystemic Conclusions. The litter of *Eucalyptus* and *Quercus* we used in our study did not differ with respect to the overall content of phenolic compounds (protein-precipitating TA equivalents). The radial diffusion assay as used does not reveal phenol concentrations, but rather provides a measure of protein-binding capacity, and thus, one measure of potential biological activity of a mixture of phenolics that may or may not be correlated with phenol content. Further, tannic acid, which we used here, may not be an appropriate standard for protein precipitation by mixed phenolics because the species studied may differ in both amount and type of hydrolyzable tannins. Yet, comparison of data derived from this method still allows for an estimation of interspecific differences in phenol content or activity, and Domínguez (1994), using different methods, came to a similar conclusion of similar phenol concentrations in *Eucalyptus* and *Quercus*. Nevertheless, we found significant differences in consumption rates of these litter types by *Proasellus* but not in the terrestrial isopods. Thus, it is apparently not the total phenol content that determines detritivore consumption rates, but rather the phenolic signature.

In addition to phenolics (see Introduction), other compounds are considered responsible for mediating consumption and decay rates of plant tissue. While mainly phloroglucinol derivates, such as formylated phloroglucinol and acylphloroglucinol, are considered responsible for feeding deterrence towards mammals (e.g., Pass et al., 1998; Lawler et al., 1999; McLean et al., 2004), different essential oils of *Eucalyptus* spp. proved active against insect consumers (e.g., Lee et al., 2001; Wang et al., 2001). Whereas purely hydrocarbon components of essential oils (terpenes) are not water-soluble, and thus, will not be lost rapidly through leaching (and would have been present in the leaf litter we used in the present study if they had been so in freshly detached senescent leaves), their oxidized derivates may be leached at early decomposition stages. Thus, those essential oils that are common in *E. globulus* (e.g., 1,8-cineole and α-pinene; Li and Madden, 1995; Lee et al., 2001) are readily leached off litter (as can be deduced from, e.g., Krock et al., 2002; Rasmussen et al., 2003) and

probably were not present in our experimental litter. Those differences in consumption and digestibility of *Eucalyptus* and *Quercus* we observed herein were probably not due to essential oils. On the other hand, the lack of negative effects of *Eucalyptus* litter on decomposition through isopod feeding may be due to the lack of essential oils that had been lost from litter prior to collection.

According to our present results, phenolics in *Eucalyptus* appear to be less deterrent to the aquatic isopod, *P. coxalis*, than phenolics in *Quercus* (e.g., compounds B or I). In a previous study, *Eucalyptus* oils had stronger effects on aquatic fungal decomposers than phenolics (Canhoto et al., 2002). As expected, the loss of phenolics through leaching was almost complete and higher in the aquatic than in the terrestrial environment (but apparently had not been extensive under terrestrial conditions prior to litter collection). Thus, negative effects of *Eucalyptus* phenolics (if any exist) would be expected to be stronger for terrestrial detritivores than for aquatic, but we did not find any effect on consumption or digestibility by terrestrial isopods. From this, we conclude that decomposition of *Eucalyptus* litter does not proceed more slowly than that of litter from native Portuguese trees. In coincidence, Rezende et al. (2001) did not find significantly different decay rates of leaf litter derived from *Eucalyptus grandis*, introduced to Brazil, and *Dalbergia nigra*, a native Brazilian tree, after 1 yr.

As expected, we observed significant differences in phenol degradation, both between detritivores and between litter types. Digestive processes and effects on microbial activity obviously differ among detritivores, and both the identity of detritivores and the litter type affect phenol degradation (and probably also other decomposition processes) both directly and indirectly. Consequently, we cannot consider them functionally redundant, although they superficially belong to the same functional group (see Zimmer et al., 2002b, 2004; Chalcraft and Resetarits 2003a,b). Further, different litter types can obviously not substitute for each other in terms of their quality as food to detritivores (see Zimmer et al., 2004).

Overall, however, although the phenolic signature of *Eucalyptus* litter influences its degradation, we did not find consistent evidence for slowed-down decomposition processes of the litter of this introduced tree species that would have suggested impaired nutrient cycles. Thus, negative effects that *Eucalyptus* plantation may have on Portuguese forests and freshwater systems are not due to differences in tannins of *Eucalyptus* litter relative to common native trees.

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EFFECTS OF JASMONATE-INDUCED DEFENSES ON ROOT-KNOT NEMATODE INFECTION OF RESISTANT AND SUSCEPTIBLE TOMATO CULTIVARS

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Abstract—Jasmonates, such as jasmonic acid (JA), are plant-signaling compounds that trigger induced resistance against certain pathogens and a broad range of arthropod herbivores. One goal of this study was to determine the effects of JA-dependent defenses in tomato on root-knot nematodes. Another was to determine if the artificial induction of these defenses could enhance nematode control on plants that carry *Mi-1.2*, a nematode resistance gene that is present in many tomato cultivars. At moderate soil temperatures, *Mi-1.2* can effectively suppress reproduction of most isolates of the common root-knot nematode species *Meloidogyne javanica*, *M. incognita*, and *M. arenaria*. *Mi*-mediated resistance has its limitations, however. *Mi-1.2* is reported to lose its effectiveness at soil temperatures above 28°C, and certain virulent nematode isolates can overcome resistance even at moderate soil temperatures. This study used a foliar application of JA to activate induced resistance in two near-isogenic lines of tomato (*Lycopersicon esculentum*) with and without *Mi-1.2*, and evaluated the effects of induced resistance at moderate soil temperatures on one avirulent nematode isolate (*M. javanica* isolate VW4) and two virulent isolates (*M. javanica* isolate VW5 and *M. incognita* isolate 557R). In addition, the effects of induced resistance on avirulent nematode performance were examined at a high temperature (32°C). The results indicate that JA application induces a systemic defense response that reduces avirulent nematode reproduction on susceptible tomato plants. Furthermore, JA-dependent defenses proved to be heat-stable, whereas the effects of *Mi*-mediated resistance were reduced but not eliminated at 32°C. JA

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treatment enhanced *Mi*-mediated resistance at high temperature, but did not suppress either of the virulent nematode isolates tested.

Key Words—Induced resistance, octadecanoid pathway, jasmonic acid, *Mi*, temperature-sensitive resistance, nematode resistance, *Meloidogyne*, virulence, avirulence.

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp. Chitwood) are soil-borne roundworms that parasitize the root systems of a wide variety of crops, including cultivated tomato (*Lycopersicon esculentum* Mill). Symptoms include root galls, stunted growth, and increased susceptibility to drought stress and pathogen attack (Williamson, 1998). Heavy root-knot nematode infestations cause substantial yield reduction, making them among the most damaging agricultural pests worldwide (Williamson and Hussey, 1996). Furthermore, control of nematodes has become increasingly difficult due to the withdrawal of effective nematicides and soil fumigants such as methyl bromide from the market (Oka and Cohen, 2001). As a result, there is an increasing need for new nematode management techniques. Potentially, host plant defenses could be utilized to develop alternative control strategies for root-knot nematodes. The overall goal of this study was to determine the effects of two forms of host plant resistance, broad-spectrum induced resistance and highly specific heritable resistance, on root-knot nematode infection in tomato.

The term "induced resistance" (IR) is used to describe plant defenses that are induced by feeding damage, and that render plants less susceptible to subsequent attack by a broad range of herbivores (Karban and Baldwin, 1997). Activation of IR against caterpillars and other chewing insects is dependent on induction of oxylipins such as jasmonic acid and methyl jasmonate (Howe et al., 1996; McConn et al., 1997). These signaling compounds induce expression of plant defenses such as proteinase inhibitors and polyphenol oxidase, as well as volatile organic compounds that attract predators and parasitoids of herbivores (reviewed in Ryan, 2000; van Poecke and Dicke, 2004). We do not yet know the full range of pests against which jasmonate-dependent defenses are effective, and this remains an active area of research. A recent study demonstrated that jasmonates help suppress infestation by mites and thrips, which are classified as cell-content feeders (Li et al., 2002). Artificial induction of jasmonate-dependent defenses can also deter phloem-feeding insects such as aphids (Omer et al., 2001; Ellis et al., 2002; Bruce et al., 2003; Cooper et al., 2004; Cooper and Goggin, 2005). In addition to their role in arthropod resistance, jasmonates are also involved in plant defenses against certain pathogens, including several soil-borne fungi that invade root systems (Staswick et al., 1998; Vijayan et al.,

1998; Thaler et al., 2004). Furthermore, recent studies demonstrated that treating spinach and oats with methyl jasmonate reduced their susceptibility to plant-parasitic nematodes (Soriano et al., 2004a, b). The first objective of our study was to determine if induction of jasmonate-dependent defenses could reduce root-knot nematode infection on tomato. A second was to determine if these induced defenses could enhance nematode control on tomato cultivars that carry the nematode resistance gene *Mi-1.2*.

Mi-1.2 is a single dominant gene that confers resistance against three major species of root-knot nematodes (*M. incognita*, *M. javanica*, and *M. arenaria*), as well as the potato aphid (*Macrosiphum euphorbiae*) and the sweet potato whitefly (*Bemisia tabaci*) (Milligan et al., 1998; Rossi et al., 1998; Nombela et al., 2003). Based on its nucleotide sequence, *Mi-1.2* is categorized in a family of plant resistance genes (R-genes) characterized by leucine-rich repeats and nucleotide-binding sites (Milligan et al., 1998). R-gene-mediated resistance is thought to depend upon rapid, local defense responses triggered by direct or indirect interactions between the R-gene product and highly specific elicitors from the pest (Takken and Joosten, 2000). The signaling pathway(s) and proximal defenses responsible for *Mi*-mediated resistance have not yet been elucidated, but this form of resistance appears to require the plant signaling compound salicylic acid (Branch et al., 2004). Induction of *Mi*-mediated nematode resistance is correlated with increased activity of several enzymes implicated in defense, including phenylalanine ammonia-lyase, peroxidases, and polyphenol oxidase (Brueske, 1980; Bajaj et al., 1985; Zacheo et al., 1993). In addition, resistance is associated with rapid localized cell death around the invading nematode (Riggs and Winstead, 1959; Dropkin, 1969; Paulson and Webster, 1972).

Mi-1.2 is the only known source of heritable root-knot nematode resistance in cultivated tomato, and has been introduced through traditional breeding into many tomato cultivars used by commercial growers and home gardeners (Medina-Filho and Tanksley, 1983). Although it is widely used and is typically highly effective, *Mi*-mediated nematode resistance has several important limitations. Like many pathogen resistance genes, *Mi-1.2* is temperature-sensitive, and is reported to lose effectiveness at soil temperatures above 28°C (Holtzman, 1965; Dropkin, 1969). This could limit the use of resistant cultivars in warm growing regions such as Florida (Noling, 2002). It is also not effective against certain species of root-knot nematodes, such as *M. hapla*, a common species in temperate climates (Brown et al., 1997). Furthermore, virulent biotypes of *M. incognita*, *M. javanica*, and *M. arenaria* that can colonize resistant (*Mi*+) tomato cultivars have been identified in several tomato-growing regions around the world (Riggs and Winstead, 1959; Tzortzakakis and Gowen, 1996). These limitations suggest the need for supplemental control measures for root-knot nematodes. If jasmonate-dependent defenses in tomato were effective against root-knot nematodes, induction of these defenses could potentially

enhance nematode control on resistant cultivars. It is necessary, however, to determine if JA-dependent and *Mi*-dependent resistance are compatible. Signaling conflicts are known to exist between JA and salicylic acid, and induction of one signaling compound can, in some cases, inhibit induction of the other (Pena-Cortes et al., 1993; Doares et al., 1995; Thaler et al., 1999). Given that salicylic acid is involved in *Mi*-mediated resistance, prior induction of JA could have a negative impact on the function of *Mi-1.2*.

To investigate the effects of JA-induced defenses on root-knot nematodes, this study applied a foliar treatment of jasmonic acid to tomato cultivars with and without *Mi-1.2*, and assessed nematode reproduction on treated *versus* untreated plants. Treatment with exogenous jasmonates previously has been shown to trigger induced arthropod resistance in tomato, and to mimic herbivore induction of plant defenses such as proteinase inhibitors (Farmer et al., 1992; Thaler et al., 1996). To determine if JA-induced defenses could supplement *Mi*-mediated resistance, this study examined the effects of JA-induced defenses on nematode infection at high soil temperatures, and on two virulent nematode isolates that can overcome *Mi-1.2*.

METHODS AND MATERIALS

Plants Materials. Two near-isogenic cultivars of tomato, Moneymaker (*Mi*−), and Motelle (*Mi*+), were used for bioassays. All plants were grown in 0.95-l Styrofoam cups of autoclaved sand (Quikrete, Atlanta, GA, USA) under stable greenhouse conditions (~24–27°C; 16:8 L:D photoperiod). Plants were watered daily with a nutrient solution containing 1,000 mg/l $CaNO_3$ (Hydro Agri North America, Tampa, FL, USA) 500 mg/l $MgSO_4$ (Giles Chemical Corp., Waynesville, NC, USA), and 500 mg/l Hydroponic 4-18-38 Gromore fertilizer (Gromore, Gardena, CA, USA).

Nematode Cultures. Two closely related isolates of *M. javanica*, designated VW4 and VW5, and a *M. incognita* isolate (557R) were obtained from Dr. V. M. Williamson (University of California, Davis). Isolate VW4 is a clonal population that was established from a single female. At moderate soil temperatures (≤28°C), this isolate has extremely low survivorship on resistant (*Mi*+) tomato cultivars, and is, therefore, classified as avirulent (Milligan et al., 1998). VW5 is a virulent isolate that can establish large populations on resistant tomato, and that was derived from VW4 by artificial selection (Milligan et al., 1998). Both *M. javanica* isolates were maintained on tomato plants in hydroponic culture in aerated Miller's Supreme 16-4-16 fertilizer solution (Miller Chemical & Fertilizer Corporation, Hanover, PA, USA) (Lambert et al., 1992). Infective second-stage juveniles for use in bioassays were collected from these hydroponic cultures by vacuum filtration of the nutrient solution using

Nuclepore polycarbonate Track-Etch Membranes (Whatman International, Maidstone, England, UK). Nematodes were then resuspended in a small volume of distilled water and quantified by examining aliquots of the sample with a light microscope. To assay *M. incognita* isolate 557R, which can survive and reproduce on plants that carry *Mi-1.2* (Yaghoobi et al., 1995), we used eggs provided by the Williamson laboratory. The eggs were extracted from root systems using 10% sodium hypochlorite (Hussey and Barker, 1973).

Jasmonic Acid Application. Jasmonic acid (JA) (Sigma, St. Louis, MO, USA) was dissolved in acetone at a rate of 1 g/ml and dispersed in water to achieve a 1.5-mM JA solution (Thaler, 1999). Carrier solution, which consists of acetone dispersed in water (315 μl/l), was used as a control treatment. Tomato plants were treated at approximately 4 wk after planting, when the plants had four fully expanded true leaves. Plants were sprayed with JA solution or control solution applied at a rate of 1 ml per leaf using an atomizer (~4 ml/plant), while shielding the root systems from treatment.

Effects of JA Application on Nematode Performance. The systemic effects of JA application on *M. javanica* infection were measured on tomato cultivars, Moneymaker (*Mi*−) and Motelle (*Mi*+), sprayed with either JA or carrier solution (7 plants/treatment group). Forty-eight hr after chemical treatment, the sand surrounding the roots of each plant was injected with ~3,000 second-stage juvenile (J2) avirulent nematodes (*M. javanica* isolate VW4). Plants were maintained in a greenhouse (~24–27°C; 16:8 L:D photoperiod) long enough for the nematodes to complete their life cycle (6.5 wk), and nematode establishment and reproduction was compared by measuring the number of egg masses produced per plant. Root systems were washed of all sand and immersed for 10 min in erioglaucine solution (0.1 g/l) (Sigma) to stain the egg masses, and the total numbers of egg masses per plant were counted (Yaghoobi et al., 1995). Each root system was then dried at 45°C in an incubator, and the dry root weights were recorded.

The effects of JA application on *M. javanica* isolate VW5 (virulent) were evaluated by using the same experimental design described above (6–7 plants/treatment group). Preliminary experiments in which we inoculated plants with 3,000 VW5 juveniles indicated that this inoculum level generated more egg masses than could reasonably be counted. Therefore, for this experiment, plants were inoculated with ~1,500 J2 nematodes. To test *M. incognita* isolate 557R, we performed a similar assay using eggs rather than juveniles as our inoculum. To achieve infection levels comparable to those obtained with infective VW4 and VW5 juveniles, plants were inoculated with approximately 9,000 eggs to compensate for the lower viability of eggs compared with juveniles. Plants (5–6 plants/treatment group) were inoculated 48 hr after chemical treatment, and egg mass numbers were counted 6.5 wk after inoculation.

Effects of Temperature on JA-induced Resistance and Mi-mediated Resistance. The effects of JA treatment and *Mi-1.2* on the performance of

M. javanica isolate VW4 (avirulent) were compared at 25 and 32°C (8 replicate plants/treatment group). Twenty-four hr prior to the experiment, Moneymaker (*Mi*−) and Motelle (*Mi*+) plants were placed in two separate Conviron growth chambers (Controlled Environments, Inc., Winnipeg, Canada) to achieve the desired sand temperatures. All plants were then treated with a foliar application of JA or carrier solution and inoculated with VW4 infective juveniles as described above. Plants were watered by hand twice a day with nutrient solution acclimated to the temperature of each growth chamber. Plants were maintained in growth chambers for 7 d after nematode inoculation to allow sufficient time for nematode establishment, and were then moved to the greenhouse (24–27°C). The critical period during which temperature can influence the effectiveness of *Mi*-mediated resistance occurs within the first 2–3 d after inoculation (Dropkin, 1969). Egg mass production was measured 6.5 wk after inoculation as described above.

A second assay with a larger number of replicate plants (20 plants/treatment for Motelle; 10 plants/treatment for Moneymaker) was also performed to assess the effects of JA treatment and *Mi-1.2* on nematode performance at 32°C. Due to limited growth chamber space, plants were grown in 200-ml Styrofoam cups. When the plants had approximately four true leaves, plants were transferred to a growth chamber at 32°C and treated with JA or control solution as described above. All plants were inoculated with *M. javanica* isolate VW4 48 hr after treatment. Because of the small size of the plants and their root masses, a low inoculum level (~600 infective juveniles/plant) was used for this assay. Plants were incubated at 32°C for 7 d and then gently transplanted into 1-l cups and transferred to the greenhouse. Egg mass numbers per plant were evaluated 6.5 wk after inoculation.

Data Analysis. We evaluated nematode performance using the number of egg masses/dry root weight/plant, because temperature can influence the size of root masses (Haroon et al., 1993), which can in turn influence nematode reproduction on resistant cultivars (Melakeberhan, 1998). Unequal variances were stabilized by log transformation using the equation "log (Y + 1)," where Y is egg masses/dry root weight/plant (Gomez and Gomez, 1984). Statistical comparisons were performed using JMP version 5.01 (SAS, Cary, NC, USA). For each assay, foliar treatment and genotype were compared as independent fixed factors by full factorial two-way ANOVA. Comparisons between treatment combinations were analyzed by Student's t test.

RESULTS

Effects of JA on Avirulent Nematodes. Egg mass production by *M. javanica* isolate VW4 differed among treatment groups (F = 102.23; df = 3,24;

TABLE 1. EFFECTS OF JA-INDUCED DEFENSES ON AVIRULENT *M. javanica* ISOLATE VW4

Genotype	Treatment	Avg. root mass (g)	Avg. egg masses/gram root tissue
(*Mi*−)	Control	4.66 ± 1.25 a*	170.0 ± 114.5 a
	Jasmonic acid	5.79 ± 2.67 a	42.11 ± 30.78 b
(*Mi*+)	Control	5.71 ± 2.47 a	0.27 ± 0.39 c
	Jasmonic acid	5.88 ± 3.27 a	0 ± 0 c

* Within each column, values followed by different letters are significantly different at $\alpha = 0.05$. Statistics were performed on log-transformed data.

$P < 0.001$) (Table 1). There was a significant interaction between tomato cultivars and chemical treatment ($F = 7.07$; $df = 1,24$; $P = 0.014$). Nematode performance was dramatically reduced on the resistant cultivar Motelle (*Mi*+) compared to the susceptible cultivar Moneymaker (*Mi*−) ($t = -10.11$, $df = 24$, $P < 0.001$ for plants sprayed with control solution; $t = 13.87$, $df = 24$, $P < 0.001$ for plants treated with JA). This confirms previous findings that *Mi*-mediated resistance is effective against isolate VW4 (Milligan et al., 1998; Lambert et al., 1999). On the susceptible cultivar Moneymaker, egg mass production was lower on plants treated with JA than on plants sprayed with control solution ($t = 4.35$; $df = 24$; $P < 0.001$). Egg mass numbers were low on Motelle plants treated with control solution, and no egg masses were observed on Motelle plants treated with JA. There was no statistically significant difference between these two treatment groups ($t = 0.59$; $df = 24$; $P = 0.559$). Neither JA treatment ($F = 0.46$; $df = 1, 24$; $P = 0.503$) nor cultivar ($F = 0.35$; $df = 1, 24$; $P = 0.559$) influenced root weights in this assay (Table 1).

TABLE 2. EFFECTS OF TEMPERATURE ON JA-INDUCED DEFENSES AND *Mi*-MEDIATED RESISTANCE

Temperature (°C)	Genotype	Treatment	Avg. root mass (g)	Avg. egg masses/gram root tissue
25	(*Mi*−)	Control	4.79 ± 1.11 a*	98.87 ± 40.47 a
		Jasmonic acid	5.99 ± 2.07 a	47.34 ± 29.91 b
	(*Mi*+)	Control	5.81 ± 1.73 a	0.20 ± 0.21 c
		Jasmonic acid	5.66 ± 1.63 a	0.27 ± 0.90 c
32	(*Mi*−)	Control	4.95 ± 1.23 a	106.89 ± 75.29 a
		Jasmonic acid	5.44 ± 1.41 a	54.18 ± 32.56 b
	(*Mi*+)	Control	5.63 ± 1.65 a	43.96 ± 24.4 d[§]
		Jasmonic acid	5.54 ± 1.24 a	23.34 ± 14.34 d[§]

* Within each column, values followed by different letters are significantly different at $\alpha = 0.05$. Statistics were performed on log-transformed data.
[§] These values differ significantly at the $\alpha = 0.1$ confidence interval.

TABLE 3. EFFECTS OF JA-INDUCED DEFENSES ON AVIRULENT NEMATODES AT 32°C

Genotype	Treatment	Avg. root mass (g)	Avg. egg masses/gram root tissue
(*Mi*−)	Control	8.16 ± 3.34 a*	17.98 ± 11.64 a
	Jasmonic acid	11.91 ± 5.48 a	9.80 ± 7.00 b
(*Mi*+)	Control	9.91 ± 3.94 a	0.88 ± 1.08 c
	Jasmonic acid	10.25 ± 3.83 a	0.24 ± 0.53 d

* Within each column, values followed by different letters are significantly different at $\alpha = 0.05$. Statistics were performed on log-transformed data.

Effects of Sand Temperature on JA-Induced Defenses and Mi-1.2. When the effects of JA treatment and *Mi-1.2* were compared at 25 vs. 32°C, egg mass production by *M. javanica* isolate VW4 differed significantly among treatment groups ($F = 70.58$; $df = 7,56$; $P < 0.001$). There was an interaction between temperature and plant genotype ($F = 107.62$; $df = 1, 56$; $P < 0.001$) but no interaction between genotype and chemical treatment ($F = 2.09$; $df = 1, 56$; $P = 0.154$), temperature and chemical treatment ($F = 0.23$; $df = 1, 56$; $P = 0.635$), or genotype, chemical treatment, and temperature ($F = 1.48$; $df = 1, 56$; $P = 0.229$) (Table 2). Nematode reproduction was lower on Motelle (*Mi*+) than on Moneymaker (*Mi*−) at both soil temperatures ($t = -14.57$, $df = 56$, $P < 0.001$ for plants at 25°C; $t = -2.97$, $df = 56$, $P = 0.004$ for plants at 32°C), although egg mass numbers on Motelle increased dramatically with temperature ($t = 11.34$; $df = 56$; $P < 0.001$). Trends in the data suggested that the impact of JA on resistant plants varies with temperature, and so Student's *t* tests were also used to compare the effects of chemical treatment on nematode reproduction. JA treatment reduced nematode performance on the susceptible cultivar Moneymaker at both soil temperatures ($t = -2.75$, $df = 56$, $P = 0.008$ for plants at 25°C; $t = 2.01$, $df = 56$, $P = 0.049$ for plants at 32°C). On the resistant cultivar Motelle, JA treatment had no effect at 25°C ($t = 0.09$; $df = 56$; $P = 0.933$). At 32°C, nematode performance was lower on Motelle plants treated with JA than on Motelle sprayed with control solution, but this difference was not significant at the $\alpha = 0.05$ confidence interval ($t = 1.78$; $df = 56$; $P = 0.081$).

TABLE 4. EFFECTS OF JA-INDUCED DEFENSES ON VIRULENT *M. javanica* ISOLATE VW5

Genotype	Treatment	Avg. root mass (g)	Avg. egg masses/gram root tissue
(*Mi*−)	Control	11.63 ± 1.75 a*	62.57 ± 3.26 a
	Jasmonic acid	17.34 ± 2.55 b	39.28 ± 9.85 a
(*Mi*+)	Control	10.07 ± 2.21 a	81.85 ± 26.52 a
	Jasmonic acid	11.66 ± 2.39 a	79.02 ± 42.39 a

* Within each column, values followed by different letters are significantly different at $\alpha = 0.05$. Statistics were performed on log-transformed data.

TABLE 5. EFFECTS OF JA-INDUCED DEFENSES ON VIRULENT *M. incognita* ISOLATE 557R

Genotype	Treatment	Avg. root mass (g)	Avg. egg mass/gram root tissue
(*Mi*−)	Control	7.38 ± 1.05 a*	193.81 ± 78.63 a
	Jasmonic acid	6.84 ± 1.57 a	213.73 ± 78.49 a
(*Mi*+)	Control	8.63 ± 2.23 a	171.80 ± 89.67 a
	Jasmonic acid	7.97 ± 1.25 a	143.20 ± 25.75 a

* Within each column, values followed by different letters are significantly different at $\alpha = 0.05$. Statistics were performed on log-transformed data.

In a second assay that tested the effects of JA and *Mi-1.2* at 32°C with a larger number of replicates, reproduction again differed significantly among treatments ($F = 92.84$; $df = 3,56$; $P < 0.001$), and there was no interaction between plant genotype and chemical treatment ($F = 0.85$; $df = 1,56$; $P = 0.362$) (Table 3). Egg mass numbers were lower on Motelle than on Moneymaker ($F = 266.74$; $df = 1,56$; $P < 0.001$), and JA treatment reduced nematode reproduction on both cultivars (for main effects of JA, $F = 11.72$; $df = 1, 56$; $P = 0.001$; for effects of JA on Moneymaker, $t = 2.66$; $df = 56$; $P = 0.010$; for effects of JA on Motelle, $t = 2.17$; $df = 56$; $P = 0.034$).

Effects of JA on Virulent Nematodes. Egg mass production by *M. javanica* isolate VW5 did not differ among treatment groups ($F = 1.03$; $df = 3,22$; $P = 0.399$) (Table 4), and there was no interaction between plant genotype and chemical treatment ($F = 5.13$; $df = 1,22$; $P = 0.481$). The overall effect of *Mi-1.2* was not statistically significant ($F = 1.95$; $df = 1, 22$; $P = 0.177$), which confirms previous findings that VW5 is virulent on resistant cultivars (Milligan et al., 1998; Lambert et al., 1999). The overall effect of JA treatment also was not significant ($F = 0.76$; $df = 1, 22$; $P = 0.393$), indicating that JA application is not effective against nematode isolate VW5.

Reproduction of *M. incognita* isolate 557R also did not differ among treatment groups ($F = 0.75$; $df = 3,18$; $P = 0.537$) (Table 5), and there was no interaction between plant genotype and chemical treatment ($F = 0.15$; $df = 1,18$; $P = 0.702$). This isolate established comparable numbers on resistant vs. susceptible plants ($F = 2.09$; $df = 1,18$; $P = 0.166$), confirming that it is indeed capable of overcoming *Mi*-mediated resistance (Yaghoobi et al., 1995). Reproduction of this isolate was also not deterred by JA treatment ($F = 0.05$; $df = 1,18$; $P = 0.825$).

DISCUSSION

This study demonstrates that application of synthetic JA to tomato foliage induces systemic effects that suppress root-knot nematode infestation. Relative-

ly few other studies have examined the effects of JA-induced defenses on belowground pests. Application of exogenous methyl jasmonate to roots is known to induce nematode resistance in spinach and oats (Soriano et al., 2004a, b), and treating the foliage of grapevines with jasmonic acid can reduce infestation by root-feeding phylloxera (Omer et al., 2000). Mutagenized *Arabidopsis* lines that are deficient in jasmonate signaling have increased susceptibility to the soil fungi *Pythium irregulare* and *P. mastophorum* (Staswick et al., 1998; Vijayan et al., 1998), and a tomato mutant that is deficient in JA synthesis is also highly susceptible to *Fusarium oxysporum* and *Verticillium dahliae* (Thaler et al., 2004). These studies indicate that jasmonates play a role in the protection of root tissues as well as in foliar defenses. The systemic nature of the induced response to foliar JA treatment in tomato and grape also supports the hypothesis that plant defenses can mediate indirect interactions between above- and belowground pests (van Dam et al., 2003).

As yet, we do not know the mechanism through which JA suppresses nematode infestation on tomato roots. JA is known to be transported from foliage to roots, where it can have a wide range of effects on development and metabolism (Baldwin et al., 1994; Zhang and Baldwin, 1997). Jasmonates influence root growth and nutrient partitioning (Tung et al., 1996; Staswick et al., 1998; Mader, 1999; Wang et al., 2002), which could potentially affect nematode parasitism. In this study, however, we did not observe a strong or consistent effect of JA treatment on the mass of our root systems (Tables 1–5). Jasmonates can also trigger synthesis of a wide range of secondary metabolites and defensive proteins in roots. For example, exogenous jasmonates stimulate production of 20-hydroxyecdysone in spinach (Soriano et al., 2004a), flavone-*C*-glycosides in oats (Soriano et al., 2004b), and nicotine in tobacco (Baldwin et al., 1994). All of these compounds are toxic to nematodes *in vitro*, and have been implicated in plant defenses against plant-parasitic nematodes (Davis and Rich, 1987; Soriano et al., 2004a, b). Exogenous jasmonates can also induce proteinase inhibitor (PI) expression in roots (Botella et al., 1996; Dammann et al., 1997; van Dam et al., 2001). When expressed at high constitutive levels in transgenic tomato, *Arabidopsis*, and rice, cysteine and serine PIs reduce the population growth of several species of plant-parasitic nematodes, including root-knot nematodes (Atkinson et al., 1996; Urwin et al., 1998; Atkinson et al., 2003). Furthermore, a transgene encoding a proteinase inhibitor from rice has successfully been pyramided with a source of partial nematode resistance in potato to achieve full resistance (Urwin et al., 2003). Potentially, PIs or other systemic defenses induced in tomato roots by JA treatment may be responsible for the partial resistance we have observed in treated plants.

This study also compared JA-induced resistance to *Mi*-mediated resistance, and examined the combined effects of these two plant defenses on nematodes. At a moderate temperature (25°C), *Mi-1.2* suppressed reproduction of avirulent

root-knot nematodes almost completely, whereas JA treatment imparted only partial resistance to a susceptible tomato cultivar (Tables 1 and 2). JA treatment did not inhibit *Mi*-mediated resistance, which suggests a lack of signaling conflicts between these two forms of defense (Tables 1–3). At a high temperature (32°C), *Mi-1.2* was significantly less effective than at 25°C, but nonetheless conferred partial nematode resistance (Tables 2 and 3). Despite many statements in the literature that *Mi-1.2* is inactive above 30°C, the majority of studies on the temperature sensitivity of *Mi-1.2* have compared nematode reproduction on resistant cultivars at different temperatures without making a statistical comparison between resistant and susceptible genotypes. Our results indicate that the effects of temperature on *Mi*-mediated resistance are quantitative rather than qualitative, and this finding is consistent with data presented by two other research groups (Araujo et al., 1982; Haroon et al., 1993). Unlike *Mi*-mediated resistance, JA-induced resistance was heat-stable (Table 2). Furthermore, at high temperature, JA treatment was capable of enhancing the control of avirulent nematodes on a resistant cultivar (Table 3). Thus, it appears that under certain conditions, *Mi*-mediated resistance and jasmonate-induced defenses can have an additive effect. JA treatment, however, was not able to suppress two nematode isolates that are virulent against *Mi-1.2* (Tables 4 and 5), including a virulent *M. javanica* isolate that is nearly isogenic to the avirulent isolate used in this study.

Further work is needed to understand the basis for virulence in resistance-breaking nematodes (Castagnone-Sereno, 2002; Williamson and Gleason, 2003). Because of its homology to many other plant genes that mediate gene-for-gene resistance against pathogens, it has been hypothesized that *Mi-1.2* may also act in a gene-for-gene manner (Milligan et al., 1998). In other words, *Mi*-mediated resistance may be due to a defense response that is triggered by the product of a single dominant avirulence (Avr) gene in the pest (Flor, 1955), and virulent nematodes may lack this elicitor. Alternatively, virulence in certain nematode isolates could be due to enhanced expression of pathogenicity factors, rather than to evasion of detection by the host plant. The enhanced pathogenicity hypothesis is consistent with the observation that nematodes can in some cases develop cross-resistance to more than one source of host-plant resistance. For example, some, but not all, root-knot nematode isolates that are virulent against *Mi-1.2* in tomato are also able to overcome the *Me3* resistance gene in pepper (Castagnone-Sereno et al., 2001). If virulence can be attributable to enhanced aggressiveness, this would more readily explain the ability of VW5 and 557R to overcome JA-induced defenses than the gene-for-gene hypothesis.

Our study demonstrates that artificial induction of JA-dependent defenses could play a role in the integrated management of root-knot nematodes on tomato. Furthermore, many other agricultural crops are heavily impacted by root-knot nematode infestations (Williamson and Hussey, 1996), and express

JA-dependent defenses (reviewed by Karban and Baldwin, 1997). Artificial induction of JA could potentially protect these crops from damage caused by nematodes, and be combined with sources of genetic resistance to reduce our current reliance on hazardous nematicides.

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RESPONSE OF *Plutella xylostella* AND ITS PARASITOID *Cotesia plutellae* TO VOLATILE COMPOUNDS

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Abstract—The effects of limonene, a mixture of limonene + carvone (1:1, v/v), and methyl jasmonate (MeJA) on diamondback moth (DBM) (*Plutella xylostella* L.) oviposition, larval feeding, and the behavior of its larval parasitoid *Cotesia plutellae* (Kurdjumov) with cabbage (*Brassica oleracea* L. ssp. *capitata*, cvs. Rinda and Lennox) and broccoli (*B. oleracea* subsp. *Italica* cv Lucky) were tested. Limonene showed no deterrent effect on DBM when plants were sprayed with or exposed to limonene, although there was a cultivar difference. A mixture of limonene and carvone released from vermiculite showed a significant repellent effect, reducing the number of eggs laid on the cabbages. MeJA treatment reduced the relative growth rate (RGR) of larvae on cv Lennox leaves. In Y-tube olfactometer tests, *C. plutellae* preferred the odors of limonene and MeJA to filtered air. In cv Lennox, the parasitoid preferred DBM-damaged plants with limonene to such plants without limonene. *C. plutellae* females were repelled by the mixture of limonene + carvone. In both cultivars, exogenous MeJA induced the emission of the sesquiterpene (*E*,*E*)-α-farnesene, the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and green leaf volatile (*Z*)-3-hexenyl acetate + octanal. The attractive effect of limonene and MeJA predicts that these two compounds can be used in sustainable plant protection strategies in organic farming.

Key Words—Limonene, methyl jasmonate, carvone, diamondback moth, *Cotesia plutellae*, cabbage, broccoli, vermiculite, olfactometer.

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INTRODUCTION

The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Yponometidae) is economically the most important pest of cruciferous crops throughout the world (Talekar and Shelton, 1993). DBM feeds on all cruciferous crop plants, cole crops, and several greenhouse plants (Reddy et al., 2004). In this context, one of the most important biological control agents for DBM is the braconid *Cotesia plutellae* (Kurdjumov) (Hymenoptera: Braconidae). *C. plutellae* is a potential larval parasitoid that can parasitize in the first three instars of DBM larvae (Reddy et al., 2002; Zu-hua et al., 2002).

Plants can defend themselves against herbivores by direct and indirect defense mechanisms. Direct defense affects the insect herbivore through toxic or antinutritional compounds or through repellents or deterrents (Karban and Baldwin, 1997). In indirect defense, after herbivore damage, plants produce volatiles that can attract predators or parasitoids, as has been shown in many plants (Dickens, 1999; Shiojiri et al., 2001; Dicke et al., 2003; Vuorinen et al., 2004a,b). These volatile compounds permit insect parasitoids and predators to discriminate between intact and damaged plants (Reddy and Guerrero, 2004; Vuorinen et al., 2004a). The parasitoid *C. plutellae* prefers the odor of damaged cabbage plants to that of intact cabbage in Y-tube olfactometer tests (Vuorinen et al., 2004a).

In previous studies, limonene alone or with other monoterpenes is deterrent (Ntiamoah et al., 1996; Ntiamoah and Borden, 1996) or repellent (Peterson et al., 1994; Nehlin et al., 1994; Ibrahim et al., 2001) toward insects. Chenier and Philogene (1989), for example, reported that monoterpenes, including limonene, attracted predators of conifer bark beetles. Carvone has been reported to inhibit the feeding of pales weevil (*Hylobius pales*) on *Pinus strobus* seedlings (Salom et al., 1996), of pine weevil (*Hylobius abietis*, Coleoptera: Curculionidae) on Scots pine (*Pinus sylvestris*) (Klepzig and Schlyter, 1999; Schlyter et al., 2004), and of the slug *Arion lusitanicus* on lettuce (Frank et al., 2002). Tripathi et al. (2003) reported feeding deterrence and contact and fumigant toxicity of carvone against stored product beetles, whereas Den Ouden et al. (1993) found a short-term oviposition repellence of carvone against cabbage root fly (*Delia radicum*).

Methyl jasmonate (MeJA) (a volatile derivative of jasmonic acid), which is involved in plant defense against herbivores, increases the activity of defense-related proteins (Thaler et al., 1996). MeJA was reported to protect genetically modified *Arabidopsis* plants (deficient in the jasmonate precursor linolenic acid) from attack by larvae of *Bradysia impatiens* (Diptera: Sciaridae) (McConn et al., 1997). The relative growth rate (RGR) of *Spodoptera exigua* larvae fed on MeJA-treated leaflets was found to be lower than that of those reared on control leaflets (Thaler et al., 1996). However, Oka et al. (1999) found that MeJA did

not show promising results as a nematocide to protect tomato from root-knot nematodes.

Natural compounds originating from plants might be potential alternative pesticide (Lee et al., 2001; Ibrahim et al., 2004) that are not persistent in the environment and are safe to natural enemies, nontarget organisms, and human beings for use in sustainable agriculture (Lacey and Shapiro-Ilan, 2003). Therefore, our aim was to investigate the role of limonene, a mixture of limonene + carvone, and MeJA in DBM control and the effects of these compounds on the parasitoid *C. plutellae*.

METHODS AND MATERIALS

Plant Material and Insects. Cabbage (*Brassica oleracea* L. ssp. *capitata*, cvs. Rinda and Lennox) and broccoli (*B. oleracea* subsp. *Italica* cv Lucky) seedlings, 4 to 5 wk-old, grown at 24/18°C (day/night) and relative humidity (RH) of 60% were used. Diamondback moth *P. xylostella* L. and the parasitoid *C. plutellae* (Kurdjumov) were from our own mass rearings (Vuorinen et al., 2004a). Second and third instars of *P. xylostella* feeding on broccoli plants were offered to *C. plutellae* females for egg laying. The emerged adults of *C. plutellae* were collected and released into a clean insect cage, and a honey–water solution (1:1) was provided for feeding. One- to three-d-old *C. plutellae* females were used in the behavioral assay.

Test Compounds. (*S*)-(+)-Carvone and (*R*)-(+)-limonene are the major compounds in the essential oil of caraway seeds (Bouwmeester et al., 1995, 1998; Hannukkala et al., 2002). Recently, there has been some interest in the use of caraway oil for plant protection purposes (Iacobellis et al., 2005). (*R*)-(+)-Limonene has been found to be more effective than (*S*)-(−)-limonene against the pine processionary caterpillar, *Thaumetopoea pityocampa* (Tiberi et al., 1999). (*R*)-(+)-Limonene (97% purity) and (*S*)-(+)-carvone (96%) obtained from Aldrich Chemical Co. Ltd. (Milwaukee, WI, USA), and methyl jasmonate (96%) provided by Bedoukian Research Inc. (Danbury, CT, USA) were used in this study.

DBM Egg-Laying Experiments. The following three experiments were conducted for the egg-laying study.

Experiment 1: Cabbage plants sprayed with 3% limonene solution in a 5% ethanol (99.5% purity) solution in water were offered to DBMs for egg laying in a two-choice test where the moths were allowed to choose between two treatments (control and treated) 24 hr after spraying in an acrylic polyester gauze cage (60 × 33 × 33 cm, external dimensions). Plants sprayed with 5% ethanol in 95 ml of water were used as controls. The amount of solution reaching the plant surface was estimated to be 3–4 ml. The test was replicated 15 times.

Experiment 2: Cabbage plants were exposed to limonene released from vermiculite (phyllosilicate mineral). Fifteen milliliters of limonene were mixed into 300 ml of vermiculite by vigorous stirring, and 25 ml of the mixture were placed at the base of each plant. For controls, 25 ml of vermiculite without limonene were placed at the base of the plants. Thereafter, the plants were introduced into the cages individually in a no-choice test where there was only one plant in a cage. The two tests were conducted simultaneously in two separate growth chambers, and each test was replicated ten times.

Experiment 3: As previously, 7.5 ml of limonene and 7.5 ml of carvone were mixed into 300 ml of vermiculite, and thereafter 25 ml of the mixture were placed at the base of each plant. For controls, 25 ml of vermiculite without test compounds were placed at the base of each cabbage and broccoli plant. Each test was replicated ten times.

Ten moths (1:1 sex ratio) were released into the cage and allowed to lay eggs for 48 hr for all three experiments. The moths were then removed from the cage and the eggs were counted.

Feeding Experiment. Cabbage (cv Lennox and Rinda) plants sprayed with 3% limonene (prepared as previously) or 4.5 mM MeJA in 5% ethanol (99.5% purity) in water were used. Plants sprayed with 5% ethanol in water served as controls. Twenty-four hr after spraying, the fully developed leaves of the sprayed plants were cut, and the petiole was inserted into a 1.5-ml Eppendorf tube filled with tap water. Thereafter, the leaf was placed into a plastic container (250 ml) with a lid. Late second or early third instars of DBM larvae were introduced onto the leaf and allowed to feed for 48 hr. The initial and final weights of larvae were measured to calculate the RGR, using the following formula: $\ln(W_f) - \ln(W_i)$, where W_f is the final weight and W_i the initial weight. The experiment was replicated 20 times for limonene and 15 times for MeJA treatments.

Olfactometer Experiments. Cabbage plants from the above-mentioned cultivars and broccoli were used. Ten microliters of limonene, 10 μl of MeJA, and 2 μl of a mixture of limonene + carvone (1:1 v/v) were applied by pipetting on Whatman filter paper (42.5 mm^2) and left in a fume hood for 10 min to evaporate. The filter paper with the elicitor was introduced into the glass container, as the odor source with a plant or without, depending on the test. The filter paper was placed on a piece of aluminum foil in the glass container. This experiment was conducted in a Y-tube olfactometer (main arm 10.5 cm, other arms 10 cm, inner diam 1.6 cm, angle between two arms ~90°). Plants were placed into 1-l glass containers closed with Teflon-sealed lids with two inlets. Pressurized air was filtered through activated-charcoal and passed through the glass container holding the plant with the odor source (with or without limonene, with or without limonene + carvone, and with or without MeJa), and then to one of the Y-tube arms. *C. plutellae* females were released individually into the opening of the main arm end of the Y tube and observed for 5 min or

until they made the final choice. This choice was recorded as the insect passed into the end of the Y-tube arm. The Y tube was rotated 180° after each test run. The source of the odor, the glass container, the Y-tube device, and the lids of the containers were replaced after testing eight *C. plutellae* females in the limonene and MeJA experiments. Ten females were used for each test in the limonene + carvone assays, the filter papers with the mixture, and the Y tube being replaced after testing five females.

Collection of Volatile Compounds. Volatiles emitted from the foliage of the two cultivars of cabbage plants were collected using the headspace collection technique and analyzed by gas chromatography–mass spectrometry (GC-MS) as described by Vuorinen et al. (2004a) 1 d after spraying with 3% limonene in 5% ethanol in water or 4.5 mM MeJA in 5% ethanol in water. Five and six (Lennox and Rinda, respectively) randomly selected seedlings per treatment were used for volatile collections. The roots of each sample plant were washed and pruned slightly before being inserted into a 15 ml vial filled with water. Thereafter, the whole plant was enclosed in a 1.5 l (Lennox) or 1-l (Rinda) glass vessel. The glass vessels had two inlets, one for purified air and one for sampling. Charcoal-filtered air was led through Teflon tubing at a flow rate of 200 ml min^{-1} to the Tenax TA adsorbent tubes (150 mg), where the volatiles were collected for 30 min per sample. Samples were analyzed by GC-MS (Hewlett Packard GC type 6890, MSD 5973). Compounds collected on the Tenax TA adsorbent tubes were released by thermodesorption at 250°C for 10 min. Compounds were cryofocused in a cold trap at −30°C and subsequently injected onto an HP-5 capillary column (50.0 m × 0.2 mm i.d. × 0.50 μm film thickness). The column temperature was first held at 40°C for 1 min. Thereafter, the temperature was programmed to increase from 40°C to 210°C at 5°C min^{-1} and finally to 250°C at 20°C min^{-1}. An interval of 30 to 300 *m*/*z* range was considered for the MS runs. Compounds were identified with different external standards, one for terpenoids and one for green leaf volatiles (GLVs), by comparing the mass spectra of a single compound with those of pure standards and those in the Wiley library. The amount of α-thujene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and (*E*,*E*)-α-farnesene was calculated by assuming that the responses to them were the same as those to α-pinene, (*Z*)-ocimene, and (*E*)-β-farnesene, respectively. The shoot biomass of sample plants was determined to calculate the volatile emissions as nanograms per gram dry weight per hour (ng g DW^{-1} h^{-1}). Volatiles were collected at a room temperature of 22°C and light intensity of 250 μmol m^{-2} s^{-1}. Light intensity was measured with Quantum Sensor LI-185B (LI-COR, inc. Lincoln, NE, USA).

Statistics. Statistical analysis was performed using the SPSS 11.5 for Windows statistical package. One-way ANOVA and general linear models (GLM) procedures followed by Tukey's or Dunnett's T3 multiple comparison tests were used to determine the RGR of larvae and for normally distributed

volatile compounds. Other compounds that were not normally distributed were tested with the nonparametric Kruskal–Wallis test. The Mann–Whitney test with Bonferroni correction was used to analyze differences between treatments. Data from the DBM egg-laying tests were analyzed with the independent-samples *t* test. The response of *C. plutellae* was analyzed with the non-parametric binomial test.

RESULTS

Egg-Laying Experiments. DBM females laid marginally significantly more eggs on cabbage (cv. Rinda) plants sprayed with limonene (Figure 1A) in the two-choice tests and when limonene was released from vermiculite (Figure 1B) in the no-choice test than on control plants. The number of eggs laid by females on cabbage plants treated with limonene + carvone released from vermiculite was lower in both cultivars in the no-choice tests (Figure 1C). On broccoli plants, the difference was not significant.

Feeding Experiment. Limonene had no deterrent effect on DBM larvae when detached leaves from plants sprayed with this compound were offered to them. Larval weight was not influenced by limonene (Figure 2A), but was significantly lower in cv Lennox sprayed with MeJA (Figure 2B).

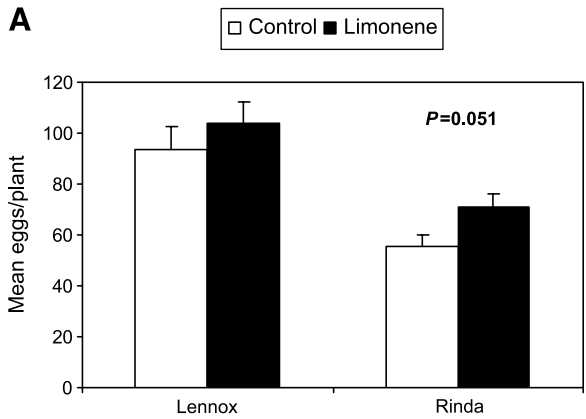

FIG. 1. *P. xylostella* ovipositioning results on (A) cabbage plants sprayed with 3% of limonene ($N = 15$) in 5% ethanol in water, (B) cabbage plants with limonene released from vermiculite ($N = 10$), and (C) cabbage (cv Lennox and Rinda) and broccoli plants exposed to the combination of limonene + carvone released from vermiculite ($N = 10$). The data were analyzed with independent samples *t* test, and error bars are from SE values. $^*P < 0.05$.

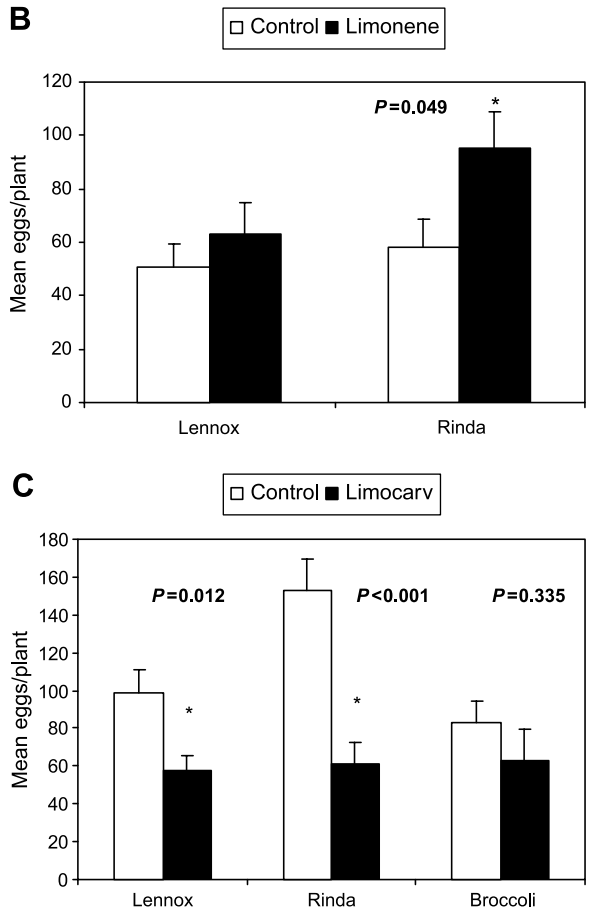

FIG. 1. CONTINUED.

Olfactometer Experiments. Females of *C. plutellae* preferred the odors of pure compounds (limonene and MeJA) to filtered air without plants in the Y-tube olfactometer tests (Figure 3A), but did not significantly discriminate between the limonene–carvone mixture and clean air. However, the number (N = 12) of *C. plutellae* females responding to any of the odor sources was lower ($P < 0.001$) than that of females (N = 38) that did not choose any of the odor source (Figure 3A). In another case, *C. plutellae* females showed a significant preference for the damaged plant with limonene over the damaged plant alone in cv Lennox (Figure 3B). In the tests with broccoli and with both cabbage cultivars, females preferred the damaged plant without limonene + carvone to

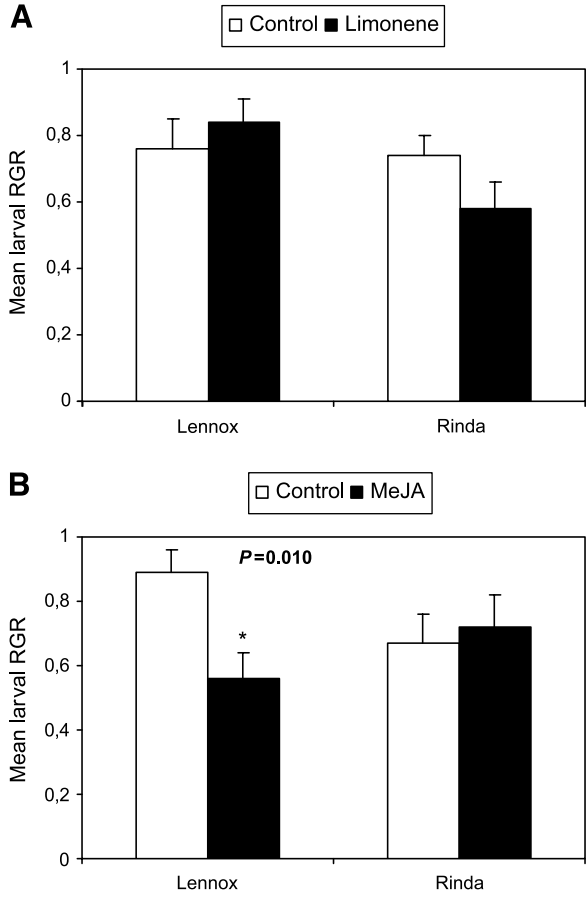

FIG. 2. Detached leaves from cabbage plants (cv Rinda and Lennox) sprayed with (A) 3% of limonene in 5% ethanol in water or (B) 4.5 mM of MeJA in 5% ethanol (99.5%) in water were fed to the DBM larvae for 48 hr and their RGR was calculated. Data were analyzed with one-way ANOVA and error bars are from SE values. $*P < 0.05$.

the damaged plant with the mixture of limonene + carvone (Figure 3C). Females were not able to differentiate between intact cabbage plants with limonene and those without it (Figure 3D). In the MeJA treatment, females did not show significant preference for any of the odor sources when tested with intact or damaged plants (data not shown).

Collection of Volatile Compounds. In both cultivars, emission of homoterpene DMNT and the sesquiterpene (*E*,*E*)-α-farnesene was induced in the

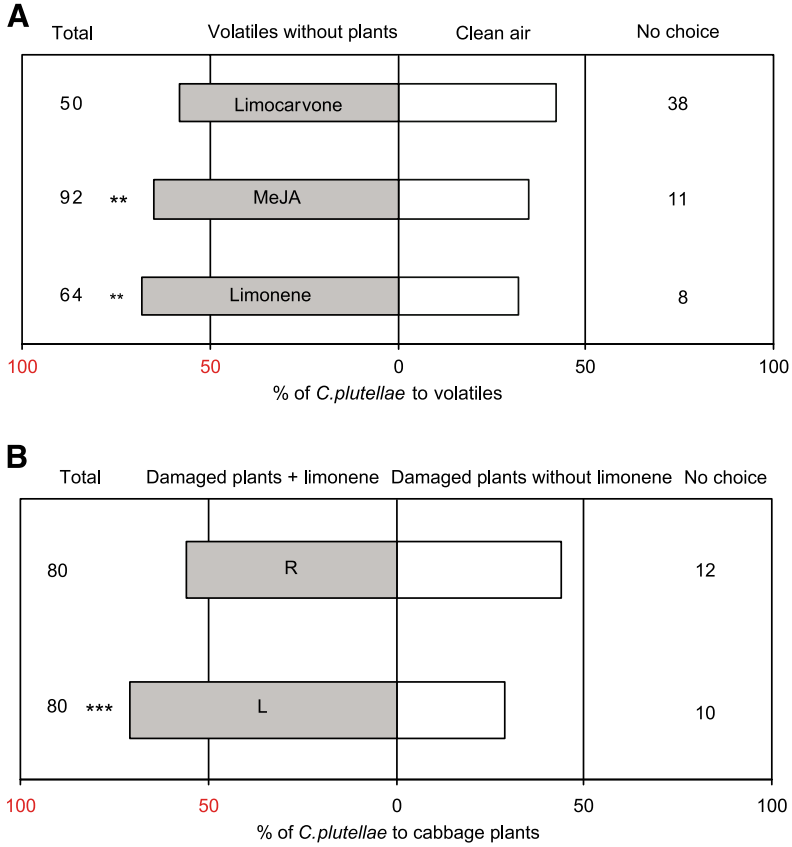

FIG. 3. *C. plutellae* responses in Y-tube olfactometer tests to (A) the odors of limonene, MeJA, and limonene + carvone without plants (treatment) and clean air; (B) damaged plants by DBM larvae + limonene (treatment) or damaged plants without limonene as control; (C) damaged plants with limonene + carvone as treatment or damaged plants without limonene + carvone serving as controls; and (D) intact plants + limonene (treatment) or without limonene as control. B = Broccoli, L = Lennox, R = Rinda. Total = the total number of females used in the experiment. No choice = the number of females that did not choose any of the odor sources. The data were analyzed using binomial tests: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

MeJA treatment (Tables 1 and 2). In cv Rinda, the concentration of total monoterpenes was higher in the limonene treatment than in the other two treatments (Table 2). In cv Lennox, sabinene concentration was lower in the MeJA treatment. In both cultivars, the GLV (*Z*)-3-hexenyl acetate + octanal was higher in

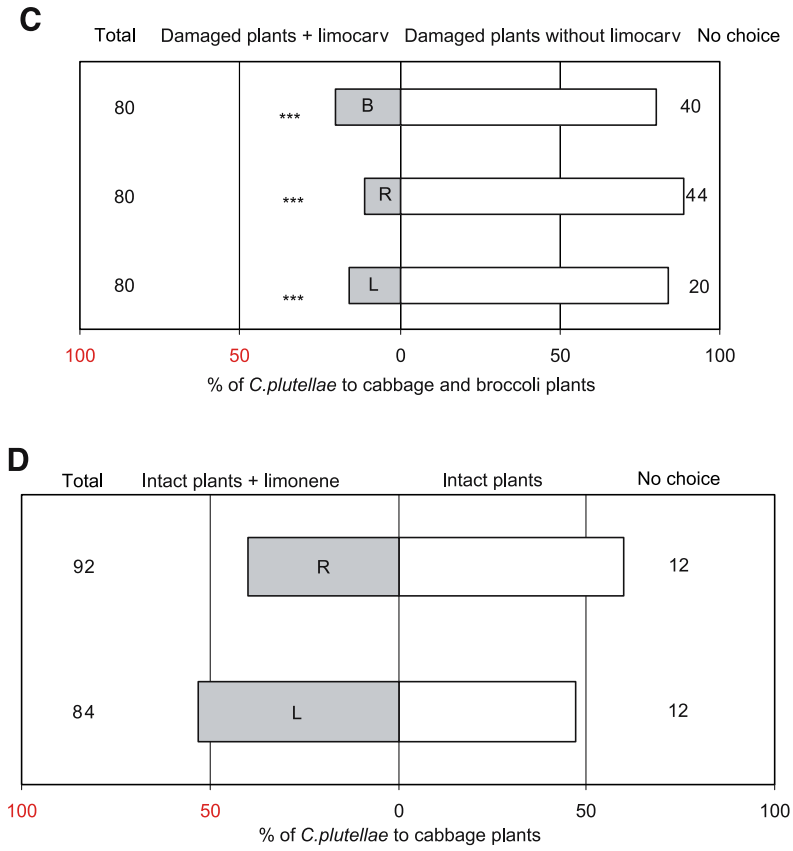

FIG. 3. CONTINUED.

the MeJA treatment than in the other two treatments. In cv Rinda, the total GLVs were higher in the MeJA treatment.

DISCUSSION

Limonene Attracts DBM and Its Parasitoid C. plutellae. We demonstrated for the first time that exogenous limonene alone attracts DBM females to cabbage cv Rinda for ovipositioning. Previously, Pivnick et al. (1994) showed that DBMs are highly sensitive to an uncharacterized combination of volatiles released by intact plants and probably dominated by terpenes. In this regard,

TABLE 1. CONCENTRATION (NG G DW^{-1} H^{-1}) OF MONO- AND SESQUITERPENES AND SOME GREEN LEAF VOLATILES IN THE HEADSPACE EMISSIONS OF CABBAGE CULTIVAR LENNOX 24 HR AFTER TREATMENT

Compounds	Control	Limonene	MeJA
Thujene	20.6 ± 1.7a	25.7 ± 4.6a	19.6 ± 5.5a
α-Pinene	11.6 ± 0.8a	14.5 ± 2.5a	8.5 ± 0.5a
Sabinene	61.6 ± 5.8a	77.1 ± 15.3ab	39.4 ± 2.1b
β-Pinene + myrcene	23.6 ± 2.2a	29.3 ± 5.7a	16.7 ± 0.7a
Limonene	40.3 ± 3.8a	254.9 ± 65.2a	35.5 ± 1.4a
1,8-Cineole	30.1 ± 3.4a	35.4 ± 6.4a	23.2 ± 0.8a
Total monoterpenes	187.9 ± 17.4a	436.8 ± 96.6a	142.9 ± 7.4a
DMNT [(*E*)-4,8-dimethyl-1,3,7-nonatriene]	0.0 ± 0.0a	0.0 ± 0.0a	10.9 ± 1.2b
(*E*,*E*)-α-farnesene	4.6 ± 4.6a	0.7 ± 0.6a	49.3 ± 5.3b
n-Heptanal	0.1 ± 0.1a	0.0 ± 0.0a	0.0 ± 0.0a
(*Z*)-3-Hexenyl acetate + octanal	0.2 ± 0.1a	1.0 ± 0.7a	29.8 ± 6.7b

Control = 5% ethanol in water; limonene = 3% of limonene in 5% ethanol in water; MeJA = 4.5 mM of methyl jasmonate in 5% ethanol in water ($N = 5$). Values are means ± SE. Means followed by different letters are significantly ($P < 0.05$) different.

TABLE 2. CONCENTRATION (NG G DW^{-1} H^{-1}) OF MONO- AND SESQUITERPENES AND SOME GREEN LEAF VOLATILES IN THE HEADSPACE EMISSIONS OF CABBAGE CULTIVAR RINDA 24 HR AFTER TREATMENT

Compounds	Control	Limonene	MeJA
Thujene	38.29 ± 4.48a	27.05 ± 6.93a	27.71 ± 7.45a
α-Pinene	23.78 ± 2.45a	22.88 ± 1.33a	20.38 ± 2.06a
Sabinene	131.19 ± 16.63a	125.96 ± 5.49a	103.04 ± 13.59a
β-Pinene + β-myrcene	48.29 ± 6.95a	47.34 ± 2.63a	43.27 ± 5.31a
Limonene	77.11 ± 10.65a	528.07 ± 157.89b	99.97 ± 11.87a
1,8-Cineole	52.23 ± 6.22a	58.13 ± 2.82a	53.58 ± 5.90a
γ-Terpinene	0.00 ± 0.00a	0.00 ± 0.00a	0.60 ± 0.60a
Total monoterpenes	370.89 ± 46.89a	809.422 ± 154.74b	348.55 ± 44.33a
DMNT [(*E*)-4,8-dimethyl-1,3,7-nonatriene]	0.00 ± 0.00a	6.46 ± 6.46ab	27.44 ± 3.86b
(*E*,*E*)-α-Farnesene	18.72 ± 15.17a	27.64 ± 21.03a	202.22 ± 38.74b
3-Hexen-1-ol	0.00 ± 0.00a	6.72 ± 4.35a	5.82 ± 2.47a
1-Hexanol	0.00 ± 0.00a	7.29 ± 3.00a	0.00 ± 0.00a
1-Octen-3-ol	0.00 ± 0.00a	17.09 ± 6.16a	0.00 ± 0.00a
(*Z*)-3-Hexenyl acetate + octanal	34.02 ± 15.38a	35.73 ± 9.81a	108.81 ± 20.57b
Nonanal	60.88 ± 3.90a	48.57 ± 12.75a	60.41 ± 3.22a
Total GLVs	94.91 ± 14.80a	115.42 ± 17.02ab	175.03 ± 24.06b

Control = 5% ethanol in water; limonene = 3% of limonene in 5% ethanol in water; MeJA = 4.5 mM of methyl jasmonate in 5% ethanol in water; GLV = green leaf volatile. ($N = 6$). Values are means ± SE. Means followed by different letters are significantly ($P < 0.05$) different.

limonene is one of the main volatile compounds in the headspace of intact cabbages (Shiojiri et al., 2001; Vuorinen et al., 2004b). However, DBM moths prefer the volatiles released from conspecific-damaged cabbage plants over undamaged plants (Shiojiri and Takabayashi, 2003). Vuorinen et al. (2004a) found that emission of total monoterpenes from cv Rinda was induced by the 48-hr feeding damage of DBM larvae. We found that the total monoterpene emission from limonene-treated cv Rinda was significantly higher than that in other treatments. This suggests that the volatile emission from exogenously limonene-treated cabbage cv Rinda resembles that of DBM-damaged cv Rinda.

Our results show that limonene has no deterrent effect on DBM larval feeding. This contrasts with the findings with other pest insects by Peterson et al. (1994), Nehlin et al. (1994), and Ntiamoah et al. (1996), who have shown that limonene deterred the oviposition of pickleworm moths (*Diaphania nitidalis*), carrot psyllids (*Trioza apicalis*), and onion maggots (*Delia antiqua* M.), respectively. In Y-tube olfactometer tests, *C. plutellae* females preferred infested plants with limonene to infested plants without limonene in cv Lennox. This observation agrees with our previous study, which indicated that limonene attracts the generalist predator *Podisus maculiventris* (Hemiptera: Pentatomidae) (Ibrahim and Holopainen, 2002).

Limonene + Carvone Improves the Repellent and Deterrent Effect. We found that a mixture of limonene and carvone reduces the number of eggs laid by DBMs on cabbage. These results are consistent with the findings of Ntiamoah et al. (1996) and those of Ntiamoah and Borden (1996), who have shown that a mixture of limonene, 3-carene, and *p*-cymene deters oviposition by the onion maggot (*D. antique* M.), and by the cabbage maggot (*D. radicum* L.), respectively. Similarly, limonene + carvone repels females of *C. plutellae* in Y-tube olfactometer tests. Although the use of this mixture in the Y-tube olfactometer has not been reported previously, our findings principally agree with those of Nehlin et al. (1994), Ntiamoah et al. (1996), and Ntiamoah and Borden (1996). This demonstrates that the deterrent effect of single monoterpene increases when it is combined with other terpenoids. In previous studies, Salom et al. (1996), Klepzig and Schlyter (1999), Frank et al. (2002), and Schlyter et al. (2004) reported a feeding deterrent effect of carvone on insects and slugs.

MeJA Induces the Emission of Volatiles. We found that MeJA treatment induces the emission of the sesquiterpene (*E*,*E*)-α-farnesene and the homoterpene DMNT, as previously reported by Rodriguez et al. (2001) for cotton plants treated with exogenous MeJA. Similarly, the exogenous application of MeJA on oilseed rape (*Brassica rapa* subsp. *oleifera*) increases the amount of these compounds (Loivamäki et al., 2004). In addition to constitutive monoterpenes (sabinene, limonene, β-pinene, myrcene, 1,8-cineole, α-thujene, and α-pinene), infested cabbage plants emit induced compounds such as DMNT, and (*E*,*E*)-α-farnesene, which are known to attract herbivores and their natural

enemies (Dicke et al., 1999; Rodriguez et al., 2001; Vuorinen et al., 2004a,b). Although the mechanism whereby MeJA induces DMNT and (*E*,*E*)-α-farnesene is not fully understood, Mandujano-Chavez et al. (2000) found that MeJA can induce the expression of sesquiterpene cyclase genes in tobacco cell cultures. However, MeJA has limited ability to induce later steps in the sesquiterpene pathway (Mandujano-Chavez et al., 2000). On the other hand, MeJA induces the expression of lipoxygenase in common bean (Porta et al., 1999) and in maize (Kim et al., 2003), which suggests that MeJA affects the emission of DMNT and (*E*,*E*)-α-farnesene via lipoxygenase pathway by inducing the accumulation of endogenous jasmonic acid.

As reported (Bogahawatte and van Emden, 1996; Potting et al., 1999; Vuorinen et al., 2004a), the volatile compounds emitted from plants damaged by DBM allow *C. plutellae* to discriminate between intact and damaged plants. In the present study, we demonstrated that the parasitoid *C. plutellae* is able to discriminate between limonene, MeJA, and clean air. The response of *C. plutellae* varies with the plant species and probably with the cultivar (Liu and Jiang, 2003), and the volatiles induced from infested plants seem to be important cues to *C. plutellae* (Shiojiri et al., 2001; Shiojiri and Takabayashi, 2003). Reddy et al. (2002) have also shown that DBM parasitoids, including *C. plutellae*, are attracted to a variety of chemical cues related to their host.

In summary, our results suggest that limonene and MeJA can be used as attractants for natural enemies of insect herbivores, particularly those of DBM, in organic agriculture. A mixture of limonene with carvone can act as a deterrent on crop plants, but deterrents have negative effects on natural enemies of DBM. Limonene can perhaps be used as an attractant of DMB on trap crop plants.

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EXOGENOUS APPLICATION OF JASMONIC ACID INDUCES VOLATILE EMISSIONS IN RICE AND ENHANCES PARASITISM OF *Nilaparvata lugens* EGGS BY THE PARASITOID *Anagrus nilaparvatae*

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Abstract—Jasmonate signaling pathway plays an important role in induced plant defense against herbivores and pathogens, including the emission of volatiles that serve as attractants for natural enemies of herbivores. We studied the volatiles emitted from rice plants that were wounded and treated with jasmonic acid (JA) and their effects on the host-searching behavior of the rice brown planthopper, *Nilaparvata lugens* (Stål), and its mymarid egg parasitoid *Anagrus nilaparvatae* Pang et Wang. Female adults of *N. lugens* significantly preferred to settle on JA-treated rice plants immediately after release. The parasitoid *A. nilaparvatae* showed a similar preference and was more attracted to the volatiles emitted from JA-treated rice plants than to volatiles from control plants. This was also evident from greenhouse and field experiments in which parasitism of *N. lugens* eggs by *A. nilaparvatae* on plants that were surrounded by JA-treated plants was more than twofold higher than on control plants. Analyses of volatiles collected from rice plants showed that JA treatment dramatically increased the release of volatiles, which included aliphatic aldehydes and alcohols, monoterpenes, sesquiterpenes, methyl salicylate, *n*-heptadecane, and several as yet unidentified compounds. These results confirm an involvement of the JA pathway in induced defense in rice plants and demonstrate that the egg parasitoid *A. nilaparvatae* exploits plant-provided cues to locate hosts. We explain the use

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of induced plant volatiles by the egg parasitoid by a reliable association between planthopper feeding damage and egg presence.

Key Words—Rice, jasmonic acid, *Nilaparvata lugens*, *Anagrus nilaparvatae*, plant volatiles, induced defense, host-searching behavior.

INTRODUCTION

It is widely accepted that plants respond to attack by specific herbivore species and tailor their induced direct and indirect defenses accordingly (Karban and Baldwin, 1997). Chemical defenses that target the herbivore directly result in herbivore death or retarded development (Barbosa et al., 1991; Karban and Baldwin, 1997; Agrawal, 1999; Lou and Baldwin, 2003; Sznajder and Harvey, 2003), whereas indirect defenses increase herbivore mortality through the recruitment of parasitoids and predators with volatile signals (Thaler, 1999; Kessler and Baldwin, 2001). Indirect plant defenses have been intensively studied since the late 1980s, and to date, this phenomenon has been reported in more than 23 plant species (see reviews in Vet and Dicke, 1992; Dicke, 1999; Sabelis et al., 1999; Turlings and Wäckers, 2004). Studies on the mechanisms leading to the production of herbivore-induced plant volatiles have revealed the role of herbivore-specific elicitors (Mattiacci et al., 1995; Alborn et al., 1997; Halitschke et al., 2001). These elicitors can activate various signaling pathways in the plant, causing an up-regulation of a large array of defense-related genes through cross-talk and resulting in accumulation or release of defense chemicals (Kessler and Baldwin, 2002).

Among these signaling pathways, the jasmonic acid (JA) pathway is the best studied and has been reported to play an important role in induced plant direct and indirect defenses (Hopke et al., 1994; Boland et al., 1995; Dicke et al., 1999; Schmelz et al., 2003). In the wild tobacco plant *Nicotiana attenuata*, for example, exogenous application of MeJA increases the release of volatile organic compounds (Halitschke et al., 2000), which enhances the mortality rates of the herbivores by attracting the natural enemies of herbivores (Kessler and Baldwin, 2001). Moreover, antisense suppression of a lipoxygenase gene LOX3, a specific wound- and herbivory-induced isoform involved in JA biosynthesis in *N. attenuata*, results in decreases in release of volatiles and nicotine and trypsin protease inhibitor levels (Halitschke and Baldwin, 2003). Exogenous application of JA to tobacco and tomato plants promotes parasitism and predation of the herbivores by natural enemies in nature (Thaler, 1999; Kessler and Baldwin, 2001). Chemical and behavioral analyses demonstrate that spider mite damage and JA treatment have similar, although not identical, effects on volatile induction in Lima bean plants (Dicke et al., 1999). In maize, caterpillar-induced volatile emissions are positively correlated with increased

JA levels (Schmelz et al., 2003). Ozawa et al. (2004) reported that maize plants treated with JA attract specialist parasitoids under laboratory conditions. In rice, the world's most important food crop, the role of JA signaling has been mainly studied for direct defenses. Exogenous application of JA on rice plants elicits the productions of proteinase inhibitors, phytoalexins, PRs, and salt-induced proteins (Tamogamia et al., 1997; Rakwal and Komatsu, 2000; Rakwal et al., 2001; Kim et al., 2003), and it may increase the emission of volatiles (Obara et al., 2002).

In this study, we investigated the effect of JA application to rice plants on the host-searching behavior of the rice brown planthopper *Nilaparvata lugens* and its mymarid egg parasitoid *Anagrus nilaparvatae*. *N. lugens* is one of the most important rice pests. It feeds on the plant's phloem and causes a decrease in leaf area, plant height, dry weight, leaf and stem nitrogen concentration, chlorophyll contents, and photosynthetic rate, but an increase in free amino acids, sucrose, and leaf iron content (Rubia-Sanchez et al., 1999; Watanabe and Kitagawa, 2000). The parasitoid *A. nilaparvatae* is a major natural enemy of the rice planthoppers. Previous studies have shown that rice volatiles play an important role in host plant location by *N. lugens* (Liu et al., 2002), and the volatiles emitted from rice plants in response to *N. lugens* attack attract the parasitoid (Lou and Cheng, 1996; Lou et al., 2002). However, little to nothing is known about the effect of JA application on rice volatiles and in turn on host-searching behavior of *N. lugens* and the parasitoid.

To determine if JA induces emission of volatiles that affect the host-searching behavior of the herbivore and the parasitoid, we first measured their responses to JA-treated plants and control plants in the laboratory. In additional greenhouse and field experiments, we then tested if JA treatment of rice plants enhanced the parasitism of *N. lugens* eggs by the parasitoid. Finally, we collected and identified volatiles that were released from JA-elicited and the control plants.

METHODS AND MATERIALS

Plant Growth. The rice variety used was TN1, which is susceptible to *N. lugens* (Lou and Cheng, 2003). Pregerminated seeds were sown in a greenhouse, and after 20–25 d, the seedlings were transplanted into small clay pots (8-cm diam × 10-cm height) each with one plant or big clay pots (16-cm diam × 10-cm height) each with three or six plants. For three plants per pot, they were arranged in an equilateral triangle 8 cm apart; for six plants per pot, they were arranged in two rows each with three plants and 8 cm between rows and 2 cm between plants. Plants were watered daily, and each pot was supplied

with 10 ml of nutrient solution [$Ca(NO_3)_2 \cdot 4H_2O$, 0.5g/l; $K(NO_3)_2 \cdot 4H_2O$, 0.125g/l; $MgSO_4 \cdot 7H_2O$, 0.125g/l; K_2HPO_4, 0.125g/l; $FeCl_2$, 0.005g/l] every 3 d. All plants were placed in a controlled climate room that was maintained at 23 ± 2°C, 70% r.h., and 18 hr photophase (25,000 lx). The plants were used for experiments 25–30 d after potting. Plantings were continued at regular intervals so that enough plants of suitable age were available for experiments.

Insects. The *N. lugens* culture was originally obtained from the China National Rice Research Institute (CNRRI), Fuyang, Zhejiang, and maintained on TN1 rice plants in a greenhouse. Late instar nymphs of *N. lugens* were captured from the greenhouse and reared on potted TN1 rice plants, which were confined in plastic cages (11-cm diam × 40 cm high). The caged rice plants were maintained in a controlled climate room at 28 ± 2°C, 12-hr photophase, and 70–80% r.h. Newly emerged adults of *N. lugens* were collected daily and fed on potted fresh TN1 rice plants. Using this procedure, *N. lugens* adults of uniform age were obtained.

A laboratory colony of the egg parasitoid *A. nilaparvatae* was started from individuals trapped in rice fields in Hangzhou using TN1 rice plants with *N. lugens* eggs as bait. The colony was propagated on *N. lugens* eggs in rice shoots enclosed in glass tubes (2.5-cm diam × 20-cm height), which were kept in a controlled climate room at 28 ± 2°C, 12-hr photophase, and 70–80% r.h. Each day, the newly emerged wasps were collected into clean glass tubes (2.5-cm diam × 20-cm height), with access to both water and honey solution, and held for at least 2 hr to ensure mating. From the second generation onwards, female parasitoids were used in experiments less than 24 hr after emergence.

Plant Treatment. The potted plants were washed with running water and trimmed to leave one, three, or six plants for each pot. Plants were individually damaged with a needle at the lower and upper position of rice stems each with 200 pricks, and then each damage site was treated by applying 20 μl of 10 or 1 mM jasmonic acid in 50 mM sodium phosphate buffer (titrated with 1 M citric acid until pH 8) (JA). Control plants (BUF) were wounded the same way and treated with 20 μl of the buffer on each of the two damaged sites. Plants were treated at 1700 hr, and then the plants were placed in the controlled climate room that was maintained at 28 ± 2°C, 12-hr photophase, and 80% r.h. Fifteen hours after treatment, i.e., at 0800 hr the next day, plants were used for experiments.

Effect of JA Elicitation on Host Plant Choice by N. lugens. Pots with six plants each were used for this experiment. Three plants in one row were wounded and treated with either 10 or 1 mM JA, and plants in the other row were wounded and treated with the buffer, thus obtaining two types of treatment pairs: 10 mM JA-treated plants vs. the buffer-treated plants or 1 mM JA-treated plants vs. the buffer plants. The potted plants were then individually placed into a sleeve cage (25 cm long, 25 cm wide, 50 cm high) that was maintained in a

controlled climate room at 28 ± 2°C, 12-hr photophase, 80% r.h. Fifteen hours after treatment, 30 macropterous *N. lugens* females (2 d old) were introduced into each cage. Subsequently, the number of *N. lugens* on JA-elicited and buffer-elicited plants were recorded 1, 2, 3, 4, 6, 12, 24, 36, 48, 60, and 72 hr after their release, respectively. Each of the two experiments was replicated five times.

Effect of JA Elicitation on Host-Searching Behavior of the Parasitoids

Olfactometer Test. Responses of *A. nilaparvatae* females to rice volatiles were measured in a Y-tube olfactometer. The olfactometer consisted of a Y-shaped glass tube of 1-cm diam. The base and the two arms of the Y tube were all 10 cm in length. Each arm was connected to an odor source container (a glass box, 10 × 10 × 30 cm). An air stream was generated and was divided in two, and each secondary air stream was led through a flowmeter, a tube with active charcoal, a humidifier bottle, and one of the odor containers. Subsequently, the two airstreams were led through the two arms of the Y-tube olfactometer at 150 ml/min. The Y-tube olfactometer was placed in a box painted white with an artificial light source consisting of a single 25-W lamp placed above the arms of the Y tube. All bioassays were conducted between 0900 and 1700 hr. During experiments, the temperature in the room was maintained at 25–28°C.

A. nilaparvatae females had the choice between odors from 1 mM JA-treated plants vs. the buffer-treated plants or 10 mM JA-treated plants vs. buffer-treated plants. To test for a possible effect of the treatment solutions *per se*, we added an experiment without plants, but with the solutions applied to filter paper (40 μl of 10 mM JA vs. 40 μl of the buffer). Fifteen hours after treatment, 10 plants that were individually planted in pots of each treatment were cut off at soil level, the cut stem was wrapped with wet cotton, and the entire plants were placed into one of the odor source containers. Mated female parasitoids were introduced individually into the base tube of the Y-shaped olfactometer and given 10 min to walk toward the end of one of the arms. Choice for an odor source was defined as a female crossing a line 7 cm after the division of the base tube and remaining there for at least 1 min. If a parasitoid did not make a choice within 10 min, this was recorded as no response. After testing two females, the olfactometer tube was washed with 98% alcohol and then was heated at 80°C for several minutes. To remove any asymmetrical bias, connections of the two arms of the olfactometer to the odor source containers were exchanged after testing two females, and the odor source containers were exchanged after testing eight females. The odor sources were replaced by a new set of 10 plants after testing 16 wasps, and for each odor source combination, at least 32 females were tested.

Greenhouse Experiment. Two plants, each with about 80–100 1-d-old *N. lugens* eggs, were transplanted into the center of a triangle of three plants that had been wounded 15 hr earlier and treated with either 10 mM JA, 1 mM JA, or with buffer. To obtain plants with *N. lugens* eggs, they were individually infested for 1 d with 10 gravid *N. lugens* females that were placed in two parafilm bags at the upper and lower position of the plant stems. After removal of the females, plants with 80–100 eggs were chosen. For each treatment, five pots were randomly placed into a cage (length 2.0 m, width 1.5 m, height 1.5 m) covered with nylon net into which 60 mated *A. nilaparvatae* females were introduced. The experiment was carried out in a greenhouse maintained at 24 ± 4°C. Two days later, the parasitoids were removed, and each pot was confined in a plastic cage (6.5 × 32 cm), all of which were placed into a controlled climate room at 28 ± 2°C, 12-hr photophase, and 80% r.h. Five days after placing them in the climate room, the plants were dissected, and the total of parasitized *N. lugens* eggs (the eggs become red) was recorded. The experiment was replicated five times.

Field Experiment. The treatments for plants were the same as the greenhouse experiment. Both JA (10 or 1 mM)-elicited plants and the buffer-elicited plants were placed at 10 locations in a rice field (20 × 30 m) in October 2000 (Figure 1). The field was surrounded by rice fields with plants in the "heading" stage. Each location included three pots of plants, each pot with one of the three treatments. The three-pot groups were arranged in two rows, each

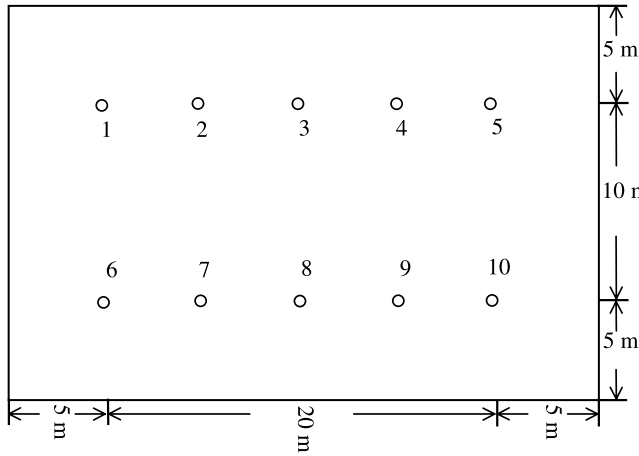

FIG. 1. Arrangement of the plants that were wounded and treated with 40 μl of either 10 mM JA in 50 mM sodium phosphate buffer (pH = 8), 1 mM JA in the buffer, or the buffer in a rice field. Numbers indicate locations, and each location includes three pots of plants, 10 mM JA-elicited plants, 1 mM JA-elicited plants, and the buffer-elicited plants.

included five groups placed 5 m apart. The distance between the two rows was 10 m (Figure 1). Two days after the plants were introduced into the rice field, the plants were transferred to the controlled climate room at 28 ± 2°C, 12-hr photophase, and 80% r.h., and each pot of plants was confined in an 11-cm diam × 40-cm-high plastic cage (herbivores, predators, and parasitoids on plants were all removed). Five days later, the plants were cut off at the soil level and dissected under a microscope to record the total, the parasitized, and the predated (sucked empty) *N. lugens* eggs. Parasitized eggs were carefully placed into petri dishes (6 cm in diam) that were lined with wet Whatman No. 1 filter paper. When the parasitoids were emerged, the species were identified.

Collection, Isolation, and Identification of the Volatile Compounds. The volatile collection system has been described in detail by Turlings et al. (1998). It consists of six vertically placed cylindrical glass tubes (9.5-cm i.d., 54 cm high). A split Teflon plate with a hole in the center at the base of a cylinder closed loosely around the stem of a plant, allowing the separation of the aerial part of a plant, in the cylinder, from the pot, which remained outside (Turlings et al., 1998). Purified and humidified air was pushed into each cylinder at a rate of 1 l/min and flowed over the plant. Around the base of each cylinder, just above the Teflon disk, eight openings served as ports that could hold the collection traps. Only one port was used during an experiment. For collections, air was pulled (0.8 l/min) through a Super-Q adsorbent trap (Heath and Manukian, 1994), whereas the rest of the air vented out through the hole in the bottom, thus preventing impure air from entering. The automated part of the collection system (Analytical Research System, Gainesville, FL, USA) controlled the flow through the trap. The climate chamber (CMP4030, CONVIRON, Winnipeg, Canada), in which the collection cylinders were housed, was kept at 17.5°C; because of the irradiation heat, the temperature inside the cylinders was 23 ± 3°C. During the light cycle, light intensity was about 20,000 lm/m^2.

Volatiles emitted from nonmanipulated plants and plants that were wounded and treated with either 10 mM JA or the buffer were collected. We also collected the volatiles from a blank, only a pot of soil without plants, to check if the system is clean. Collections started immediately after lights went on, 15 hr after treatment. Each collection lasted 4 hr. After each collection, traps were extracted with 150 μl methylene chloride (Lichrosolv., Merck, Whitehouse Station, NJ, USA), and 200 ng of *n*-octane and nonyl acetate (Sigma, Switzerland) in 10 μl of methylene chloride was added to the samples as internal standards. Each treatment was replicated six times.

Analyses were carried out with an HP 6890 series gas chromatograph equipped with an automated on-column injection system (HP G1513 A) and a flame ionization detector. Of each sample, a 3-μl aliquot was injected onto an apolar SE-30 capillary column (30 m, 0.25-mm i.d., 0.25-μm film thickness,

Alltech, Deerfield, IL, USA) preceded by a deactivated retention gap (5 m, 0.25-mm i.d.) and a deactivated precolumn (30 cm, 0.530 mm). Helium (24 cm/sec) was used as carrier gas. After injection, the column temperature was maintained at 40°C for 3 min, increased to 230°C at 8°C/min, and held at 230°C for 9.5 min. The detector signal was processed with HP GC Chemstation software.

To identify compounds, we collected volatiles emitted from 10 mM JA-elicited plants for 10 hr. A 3-µl aliquot from this sample was injected onto the same column and analyzed using the same temperature program. Volatiles were detected by a Hewlett-Packard 5973 mass selective detector (transfer line 230°C, source 230°C, quadrupole 150°C, ionization potential 70 eV, scan range 50–400 amu). Compounds were identified by comparison of GC retention times with those of authentic standards and by comparison of mass spectra with spectra of a NIST database.

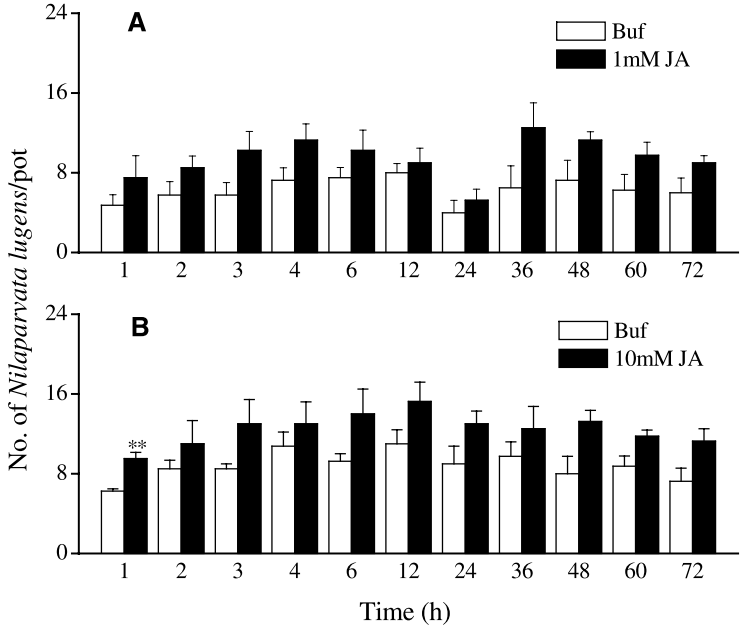

FIG. 2. Mean (±SE, $N = 5$) number of *Nilaparvata lugens* female adults on pairs of plants that were wounded and treated with 40 µl of 1 mM JA in 50 mM sodium phosphate buffer (pH = 8) (1 mM JA) vs. plants that were wounded and treated with 40 µl of buffer (Buf) (A), or that were wounded and treated with 40 µl of 10 mM JA in buffer (10 mM JA) vs. Buf (B), 1–72 hr after five replicated plant pairs were exposed to 30 insects. The exposure of the plants started 15 hr after the treatment. Asterisks indicate significant differences between members of a pair (JA vs. buffer, $P < 0.05$, *t*-test).

Data Analysis. Differences in behavioral responses of the parasitoid to JA-induced rice volatiles and the buffer-induced volatiles were determined by chi-square tests, whereas differences in behavioral responses of female *N. lugens* adults were determined by *t*-tests. To test for differences in parasitism among the treatments, we used the Fieldman rank sum test. Comparison of the data on plant volatiles was analyzed by MANOVA after the data were log transformed. If the MANOVA analysis was significant ($P < 0.05$), univariate ANOVAs for the individual effects and Fisher LSD *post hoc* tests to detect significant differences between groups were conducted. Data were analyzed with Statistica (Statistica, SAS Institute Inc., Cary, NC, USA).

RESULTS

Effect of JA-Elicited Plants on Host Preference of N. lugens. N. lugens female adults were recovered consistently more often from the JA-treated plants than from the buffer-treated plants, but this apparent preference was only

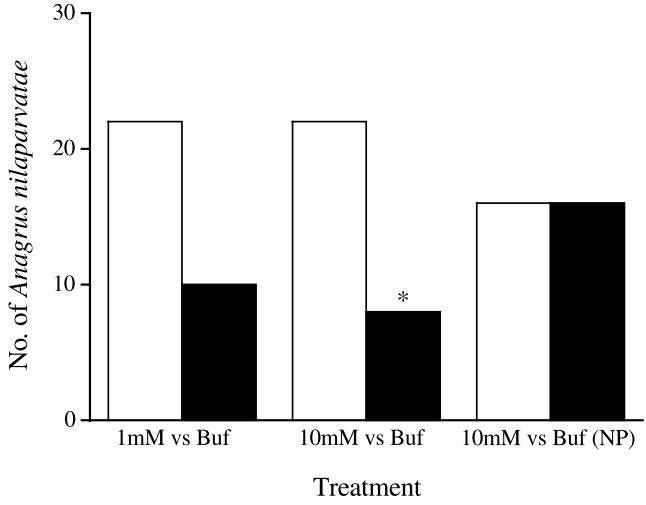

FIG. 3. Number of *Anagrus nilaparvatae* female adults attracted by volatiles released from either pairs of plants that were wounded and treated with 40 μl of 1 mM JA in 50 mM sodium phosphate buffer (pH = 8) (1 mM) vs. plants that were wounded and treated with 40 μl of the buffer (Buf), plants that were wounded and treated with 40 μl of 10 mM JA in the buffer vs. Buf, or a pair of chemicals, 40 μl of 10 mM JA vs. 40 μl of the buffer without plants [10 mM vs. Buf (NP)]. The plants were used 15 hr after the start of treatment. Asterisks indicate significant differences between members of a pair (JA vs. buffer, $P < 0.05$, chi-square test).

significant for the 10 mM JA dose at 1 hr after the start of the experiment (Figure 2). The data may not suffice to conclude that JA induction renders the plants attractive to *N. lugens* females, but it can be concluded that the induced volatiles are not repellent.

Effect of JA-Elicited Plants on Host-Searching Behaviors of the Parasitoids. In olfactometer tests, *A. nilaparvatae* preferred the volatiles emitted from JA-elicited plants to those emitted from the buffer-treated plants. JA itself did not attract the parasitoids (Figure 3).

In the greenhouse experiment, parasitism of *N. lugens* eggs by *A. nilaparvatae* on plants that were surrounded by JA-treated plants was higher than on the buffer-treated plants (Figure 4A, $Q = 8.40$, $P = 0.008$), especially for the 10 mM JA-treated plants on which the parasitism of *N. lugens* eggs by *A. nilaparvatae* was 2.35-fold higher than those on the control plants.

During the field experiment, only *A. nilaparvatae* wasp was observed parasitizing *N. lugens* eggs. As in the greenhouse experiment, JA treatments

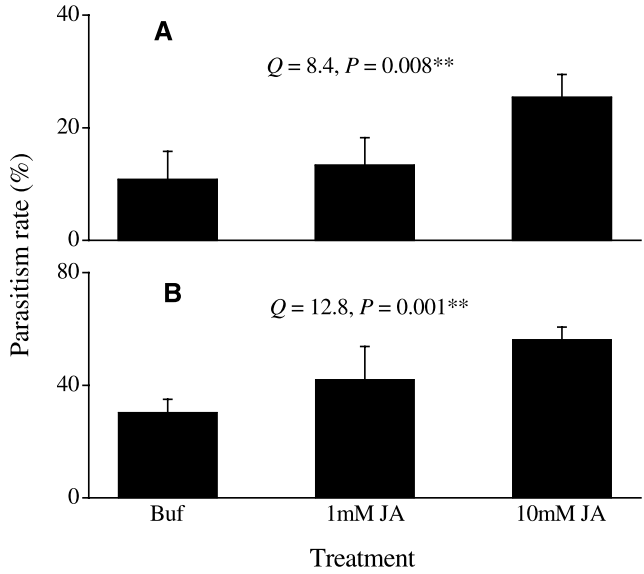

FIG. 4. Mean (±SE) parasitism rates (%) of *N. lugens* eggs by *A. nilaparvatae* in the greenhouse (A, $N = 5$) or field (B, $N = 10$) condition on rice plants surrounded by plants that were wounded and treated with either 40 μl of 1 mM JA in 50 mM sodium phosphate buffer (pH = 8) (1 mM), 40 μl of 10 mM JA in the buffer (10 mM), or 40 μl of the buffer (Buf). Fifteen hours after treatment, the plants were exposed to the parasitoid or the field for 2 d. The differences in parasitism among the treatments were determined by Fieldman rank sum test.

increased parasitism of *N. lugens* eggs by *A. nilaparvatae* (Figure 4B, Q = 12.80, P = 0.001). When 40 μl of 1 or 10 mM JA was applied to wounded plants, the parasitism of *N. lugens* eggs by *A. nilaparvatae* on plants that were surrounded by JA-treated plants were 1.39- and 1.85-fold higher than those on the control plants, respectively. *Cyrtorhinus lividipennis* Reuter (Hemiptera: Miridae) is the main predator of *N. lugens* eggs in the rice field. We found a tendency for predation rates of *N. lugens* eggs to be higher on JA-treated plants (1 mM JA 3.21 ± 1.00%; 10 mM JA 7.25 ± 5.07%; buffer 1.58 ± 0.66%), but the differences were not statistically significant (F = 0.944, df = 2,27, P = 0.402).

Analysis of Volatiles. Collection and analysis of the volatiles revealed that only small amounts were released by nonmanipulated rice plants, whereas the buffer- and JA-treated plants emitted 9.43 and 35.68 times larger amounts, respectively (Table 1; Figure 5). Most compounds were released in significantly larger amounts by JA-treated plants compared to buffer-treated plants: 2-heptanone, 2-heptanol, limonene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene,

TABLE 1. COMPARISON OF VOLATILE COMPOUNDS EMITTED FROM DIFFERENTLY-TREATED PLANTS[a]

Chemical	Nonmanipulation	Buffer-treated plants (pH = 8)	10 mM JA-treated plants (40 ml)
1. 2-Heptanone	7.65 ± 1.27 c	133.82 ± 53.04 b	776.75 ± 259.34 a
2. 2-Heptanol	13.63 ± 4.41 c	127.27 ± 56.52 b	471.79 ± 119.64 a
3. Unknown 1	– c	8.15 ± 2.24 b	25.83 ± 6.05 a
4. Unknown 2	– c	12.90 ± 3.16 b	40.01 ± 10.69 a
5. Limonene	24.18 ± 1.73 c	100.07 ± 23.46 b	243.26 ± 57.54 a
6. Unknown 3	5.27 ± 2.56 b	56.18 ± 21.94 a	134.02 ± 44.88 a
7. Unknown 4	2.72 ± 1.16 b	34.89 ± 13.14 a	219.54 ± 88.17 a
8. Linalool	– c	413.50 ± 129.78 b	1731.30 ± 489.07 a
9. $C_{11}H_{18}$[b]	10.50 ± 3.26 c	57.83 ± 18.88 b	146.91 ± 29.30 a
10. Methyl salicylate	22.34 ± 9.01 b	134.45 ± 49.23 ab	194.45 ± 55.45 a
11. β-caryophyllene	1.61 ± 0.93 b	5.84 ± 2.41 b	52.60 ± 13.37 a
12. (*E*)-α-bergamotene	– c	6.65 ± 0.85 b	15.72 ± 4.30 a
13. *n*-Heptadecane	– c	4.56 ± 1.65 b	22.16 ± 5.25 a
14. (*E*)-Nerolidol	9.70 ± 1.03 c	45.84 ± 14.55 b	272.06 ± 46.98 a
15. $C_{16}H_{26}$[c]	1.56 ± 0.21 c	11.15 ± 2.90 b	46.99 ± 7.24 a
Total	126.02 ± 18.99 c	1188.51 ± 390.26 b	4495.85 ± 1119.35 a

[a] For explanation of treatments and methodology see "Methods and Materials." Data represent the mean amount (% of IS peak area) of six replications. Letters in a same row indicate significant differences among treatments ($P < 0.05$, Fisher LSD *post hoc* tests).
[b] (*E*)-4,8-Dimethyl-1,3,7-nonatriene.
[c] (3*E*,7*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene.

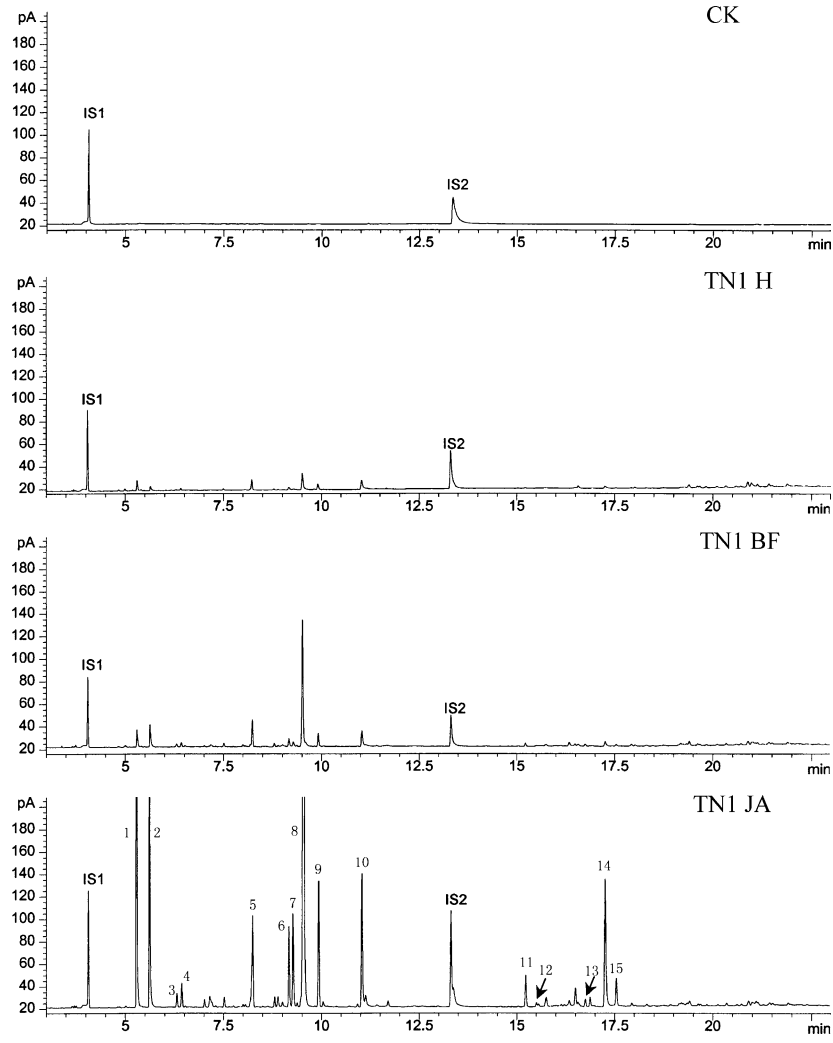

FIG. 5. Typical chromatograms obtained from headspace collections from an empty glass container (a pot of soil without plants) (CK), untreated rice plants (TN1 H), or rice plants 15 hr after they were wounded and treated with either 40 µl of 50 mM sodium phosphate buffer (pH = 8) (TN1 BF) or with 40 µl of 10 mM JA in the buffer (TN1 JA). (1) 2-Heptanone; (2) 2-heptanol; (3) unknown 1; (4) unknown 2; (5) limonene; (6) unknown 3; (7) unknown 4; (8) linalool; (9) (*E*)-4,8-dimethyl-1,3,7-nonatriene; (10) methyl salicylate; (11) beta-caryophyllene; (12) (*E*)-alpha-bergamotene; (13) *n*-heptadecane; (14) (*E*)-nerolidol; (15) (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

(3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, beta-caryophyllene, (*E*)-alpha-bergamotene, *n*-heptadecane, (*E*)-nerolidol, and two unknown chemicals (unknowns 1 and 2) (Table 1; Figure 5).

DISCUSSION

As reported for other plants, such as tobacco (Halitschke et al., 2000), maize (Schmelz et al., 2003), and lima bean (Dicke et al., 1999), wounding and application of JA to rice plants also resulted in an increase in volatiles emitted. The overall emission was almost fourfold higher than the emission of the buffer-treated plants. The increases involved aliphatic aldehydes and alcohols, monoterpenes, sesquiterpenes, methyl salicylate, *n*-heptadecane, and some unknown chemicals (Table 1; Figure 5). The composition of the odor blend was in part consistent with previous results reported for JA-treated rice plants (Obara et al., 2002). We too found a JA-mediated increase in the release of limonene, linalool, methyl salicylate, beta-caryophyllene, (*E*)-alpha-bergamotene, and several unknown chemicals. However, there are also some differences: the compounds 2-heptanone, 2-heptanol, *n*-heptadecane, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and (*E*)-nerolidol that we collected from the headspace of JA-treated plants were not reported by Obara et al. (2002). In turn, we did not detect the sesquiterpenes alpha-copaene, alpha-cadinene, alpha-humulene, and several others, which were identified by Obara et al. (2002). These differences may be due to treatment differences; they collected volatiles emitted from pieces of rice leaves (2.5 cm long) that were floating on a 0.5 mM JA solution for 7 or 48 hr. In maize, for example, it has been observed that excised leaves produced a 2.5- to 8.0-fold greater volatile in response to JA and the caterpillar produced elicitor volicitin than similarly treated intact plants (Schmelz et al., 2001). Herbivore- or elicitor-induced volatile releases also vary with time after treatment (Turlings et al., 1998), herbivore damage level (Gouinguene et al., 2003), and the applied elicitor amount (Halitschke et al., 2000). Compared to the nonmanipulated rice plants, wounding and application of the buffer also increased release of some volatiles (Figure 5), suggesting that wounding alone may be sufficient to induce a minor release of at least some of the compounds and that application of JA fortifies this effect.

The volatiles emitted from JA-treated rice plants were attractive to the parasitoid (Figure 2) and enhanced parasitism of *N. lugens* eggs in the greenhouse and field (Figures 3 and 4). This result is consistent with results reported from other plants (Thaler, 1999; Kessler and Baldwin, 2001). We have previously shown that linalool is attractive to *A. nilaparvatae* (Lou et al., 1999).

The higher linalool concentrations in the headspace of JA-induced plants compared to the buffer-induced plants might in part explain the difference in attractiveness. Which other compounds may be involved in the attraction remains to be elucidated.

We also studied the effect of treating rice plants with JA on the settling behavior of adult *N. lugens* females. A negative effect was expected, as JA is an important defense-related plant hormone. JA not only induces plants to release volatiles, but also elicits increases in many nonvolatile defense chemicals, such as phenolics, alkaloids, terpenoids, and proteinase inhibitors (Karban and Baldwin, 1997; Lou and Baldwin, 2003, 2004). These nonvolatile compounds are likely to affect the settling behavior of the planthoppers. Although numerous studies have shown that JA application results in reduced preference and performance of herbivores (Karban et al., 1997; Karban and Baldwin, 1997; Black et al., 2003), contrary effects have also been reported. For example, volatiles emitted by potato plants in responses to JA application enhanced the plant's attractiveness to female Colorado potato beetles (Landolt et al., 1999). In wild radish, responses induced by JA application increased feeding by some herbivores (Agrawal, 2000; Agrawal and Sherriffs, 2001). In addition, *N. lugens* female adults tended to prefer 10 mM JA-elicited plants to the buffer. The fact that this difference was only apparent 1 hr after release suggests that JA-induced rice volatiles were slightly attractive to female *N. lugens*, whereas JA-induced nonvolatile chemicals had no particular effect on host preference. The latter is somewhat surprising as JA elicitation increases the production of proteinase inhibitors, phytoalexins, and PRs in rice plants (Rakwal and Komatsu, 2000; Rakwal et al., 2001; Kim et al., 2003). *N. lugens* female adults are attracted to limonene (Lou et al., unpublished data). Hence, a slightly stronger attraction of JA-induced rice volatiles to *N. lugens* compared to the control can be related to higher levels of this and/or other compounds.

Thaler (1999) was the first to report that inducing plants with jasmonic acid increases parasitism of caterpillar pests in an agricultural field. Subsequent studies confirmed that JA treatment enhances the attractiveness of plants for parasitoids of lepidopteran larvae (Ozawa et al., 2004) and predatory mites that use induced plant volatiles to locate spider mite prey (Gols et al., 2003). These studies hold promise that field application of JA may enhance the efficacy of parasitoids and predators as biological control agents. Our results indicate that this may also be the case for egg parasitoids. The plants with *N. lugens* eggs that were used in our experiments were not elicited with JA; they were just placed near JA-treated plants. The supposed attraction of the wasps to the JA-treated plants did not prevent them from visiting neighboring plants, where they parasitized eggs. We have previously shown that the wasp does not discriminate between the volatiles emitted from *N. lugens*-infested plants and those from JA-induced plants (Wang et al., 2005). Therefore, it is likely that wasp uses only

few or a general blend of induced volatiles to locate plants that are potentially infested by its host, and that specific close-range attraction to the host is mediated by visual cues and/or kairomones released from *N. lugens*. Kairomones are attractive to the parasitoid at close range for all developmental stages of *N. lugens* (Lou and Cheng, 1994). A general attraction to induced plant volatiles is also evident from the fact that the volatile profiles from *N. lugens*-infested plants and JA-elicited plants are quite different, but both blends are attractive to wasps (Wang et al., 2005). In fact, the study by Wang et al. (2005) shows that *N. lugens* infestation does not trigger the JA pathway. However, some evidence is available showing the involvement of salicylic acid (SA) and ethylene in response to *N. lugens* feeding (Du et al., unpublished data). Hence, different defense pathways result in different volatile emissions, but all are attractive to wasps.

Egg parasitoids have been shown to respond to oviposition-induced plant volatiles in other studies, where herbivore feeding did not induce the volatiles attracting the egg parasitoids (Hilker et al., 2002; Hilker and Meiners, 2002; Meiners and Hilker, 2000). *A. nilaparvatae* females are equally attracted to volatiles emitted by rice plants infested by female *N. lugens* adults as to those from nymph-infested plants (Lou, unpublished data). Therefore, here we expect that the feeding damage is responsible for the induction. However, we do not rule out an effect of oviposition as well, especially because an ovicidal response of rice plants to oviposition by the rice white-backed planthopper *Sogatella furciferra* has been observed (Suzuki et al., 1996; Yamasaki et al., 2003). As the rice brown planthopper feeds and oviposits on the same plant (Cheng and He, 1996), the indirect association of feeding with egg presence is reliable and thus adaptive.

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THE ROLE OF FRESH *VERSUS* OLD LEAF DAMAGE IN THE ATTRACTION OF PARASITIC WASPS TO HERBIVORE-INDUCED MAIZE VOLATILES

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Abstract—The odor produced by a plant under herbivore attack is often used by parasitic wasps to locate hosts. Any type of surface damage commonly causes plant leaves to release so-called green leaf volatiles, whereas blends of inducible compounds are more specific for herbivore attack and can vary considerably among plant genotypes. We compared the responses of naïve and experienced parasitoids of the species *Cotesia marginiventris* and *Microplitis rufiventris* to volatiles from maize leaves with fresh damage (mainly green leaf volatiles) *vs.* old damage (mainly terpenoids) in a six-arm olfactometer. These braconid wasps are both solitary endoparasitoids of lepidopteran larvae, but differ in geographical origin and host range. In choice experiments with odor blends from maize plants with fresh damage *vs.* blends from plants with old damage, inexperienced *C. marginiventris* showed a preference for the volatiles from freshly damaged leaves. No such preference was observed for inexperienced *M. rufiventris*. After an oviposition experience in hosts feeding on maize plants, *C. marginiventris* females were more attracted by a mixture of volatiles from fresh and old damage. Apparently, *C. marginiventris* has an innate preference for the odor of freshly damaged leaves, and this preference shifts in favor of a blend containing a mixture of green leaf volatiles plus terpenoids, after experiencing the latter blend in association with hosts. *M. rufiventris* responded poorly after experience and preferred fresh damage odors. Possibly, after associative learning, this species uses cues that are more directly related with the host presence, such as volatiles from host feces, which were not present in the odor sources offered in the olfactometer. The results demonstrate the complexity of the use of plant

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volatiles by parasitoids and show that different parasitoid species have evolved different strategies to exploit these signals.

Key Words—*Cotesia marginiventris*, *Microplitis rufiventris*, *Spodoptera littoralis*, *Zea mays*, parasitoid, innate preference, associative learning, induced volatiles, green leaf volatiles, terpenoids, host location.

INTRODUCTION

Many parasitoid species make use of herbivore-induced volatiles to locate their herbivorous hosts. Various studies on host searching by parasitoids show that plant-provided cues are more important for the location of host habitats than odor cues from the host or host by-products (Turlings et al., 1990; Steinberg et al., 1993; McCall et al., 1993; Agelopoulos and Keller, 1994; Geervliet et al., 1994; Mattiacci et al., 1994; Takabayashi et al., 1995; Fukushima et al., 2002), but it remains largely unknown which compounds are the most crucial for the attraction.

That volatile blends can differ in their attractiveness is evident from various studies that show differential attraction mediated by odors of different plant species attacked by the same herbivore (Drost et al., 1988; McAuslane et al., 1991; Geervliet et al., 1996; De Moraes and Lewis, 1999). Also within one plant species, however, there can be considerable variation in attractiveness among genotypes. For instance, *Cotesia marginiventris*, a generalist parasitoid that attacks early instars of many Lepidoptera, prefers the caterpillar-induced odor of some specific maize varieties over others, and this preference is not simply a matter of differences in quantity of volatiles emitted (Hoballah Fritzsche et al., 2002). Moreover, several parasitoids show variable responses to odors emitted by the same plant species attacked by different herbivores, such as *Microplitis croceipes* (Zanen and Cardé, 1991), *Cotesia sesamiae* and *Cotesia flavipes*, (Ngi-Song et al., 1996), and *Cotesia glomerata* (Geervliet et al., 1997). Specialist parasitoids may use their ability to make such distinctions to focus only on plants that carry their specific hosts, as was found for *Aphidius ervi*, an aphid parasitoid with a limited host range (Du et al., 1998; Powell et al., 1998), and *Cardiochiles nigriceps*, which can only successfully develop in larvae of *Heliothis virescens* (De Moraes et al., 1998; De Moraes and Lewis, 1999). Equally intriguing is the observation that *Cotesia kariyai* can distinguish among odors produced by the same plant species fed upon by different larval stages of their respective host, *Pseudaletia separata* (Takabayashi et al., 1995). However, *Microplitis rufiventris*, which can also only develop in early instars of some Lepidoptera, does not show a preference for maize damaged by a particular larval stage of *Spodoptera littoralis* (Gouinguené and Turlings, 2003).

A review by Dicke (1999) shows that evidence for specificity in signals is highly variable, suggesting that different parasitoid species employ different strategies to exploit plant-provided signals to find hosts. As proposed by Vet and Dicke (1992), parasitoids use volatiles emitted by host-damaged plants rather than volatiles from the hosts themselves because plant volatiles are released in much larger quantities and are easier to detect. However, damaged plants are expected to provide limited information on the suitability as host of an herbivore that has been damaging it. This problem is partially solved by the ability of parasitoids to learn by association (Vet and Dicke, 1992). For various parasitoids, it has been shown that they learn to respond to a specific odor when they perceive it during contact with the host or host feces (Lewis and Tumlinson, 1988; Vet and Groenewold, 1990; Turlings et al., 1993; Vet et al., 1995). This ability may allow the wasps to learn subtle differences between odor blends and thus focus on odors that are more reliably associated with the presence of suitable hosts. *C. marginiventris*, for example, shifts its preference in favor of plant odors that it has experienced during an encounter with a host (Turlings et al., 1989, 1993). This wasp can even learn to distinguish between the odors released by maize plants fed on by two closely related *Spodoptera* species (Turlings et al., 1993).

What do generalist parasitoids such as *C. marginiventris* respond to before they have found their first host? One possibility is that they learn during emergence from the cocoon and initially focus on the same cues that guided their mother to hosts. Such early adult learning was found, for example, in *Microplitis demolitor* (Hérard et al., 1988), *Cotesia plutella* (Bogahawatte and Van Emden, 1996), and *Aphidius colemani* (Douloumpaka and Van Emden, 2003). A second possibility is that, as they emerge from the cocoon, the wasps will first cue on general plant odors, and that they will learn plant odors that are associated with the specific host only as soon as the first host is encountered and parasitized. Independent of plant genotype, the so-called green leafy volatiles (6-carbon aldehydes, alcohols, and acetates) are common and typically released from freshly damaged leaves, whereas herbivore-induced volatiles, such as blends of terpenoids, are more indicative of actual herbivore damage, and their composition varies among plant genotypes.

The aim of the current study was to test the hypothesis that inexperienced *C. marginiventris* females initially prefer blends with common fresh damage volatiles, and experienced females shift their preference toward a more specific blend. A second parasitoid, *Microplitis rufiventris* (Hymenoptera: Braconidae) with a more restricted host range, was included to determine a possible difference in exploitation of plant volatiles by generalists and specialists. These two parasitoids originate from different continents (near-arctic region and Northeast Africa, respectively). *C. marginiventris* parasitizes at least 25 Lepidoptera (including *Spodoptera* spp., *Helicoverpa* spp.) (Maes, 1989),

whereas *M. rufiventris* has been found only on *Spodoptera littoralis*, *Spodoptera exigua*, and *Heliothis armigera* (Hegazi and El-Minshawy, 1979). The hosts for both parasitoids can be found on many of the same crop plants (cotton, maize, cabbage, sweet potato, tomato, and other crops), implying that there is substantial overlap in potential plant cues that they can use.

Here, we used damaged maize plants as the odor sources. In all cases, plants were mechanically damaged and treated with caterpillar regurgitant. Such treatment results in the immediate release of several compounds, such as the green leafy volatiles, whereas the herbivore-inducible compounds, comprising mainly terpenoids and some aromatics, predominantly indole, appear at the earliest several hours after initial damage (Turlings et al., 1998). By offering the choice between odor of freshly damaged plants and the odor of plants with old damage to the wasps, the relative importance of the two volatile groups as attractants could be tested.

METHODS AND MATERIALS

Plants and Insects. Two-wk-old maize plants (var. Delprim) were used for all experiments. Two seeds per pot (200 ml, 6 cm high) were planted in commercial soil (COOP, Switzerland) and grown in a climate chamber (23°C, 60% r.h., 16D:8L, 50,000 lm m^{-2}). The day before an experiment was started, plants were transferred in glass pots that fit the olfactometer (250 ml, 4.5 cm diam, 11 cm high).

Spodoptera littoralis eggs were obtained from Syngenta (Stein, Switzerland). Rearing methods for hosts and parasitoids are described by Fritzsche Hoballah and Turlings (2001). Two parasitoids were used for the study, *C. marginiventris* and *M. rufiventris*. *C. marginiventris* was obtained from the USDA-ARS, Biological Control and Mass Rearing Research Unit (MS, USA), and *M. rufiventris* from the Faculty of Agriculture, Alexandria University (Egypt). Parasitized larvae and adult *C. marginiventris* were kept in an incubator (25°C and 16L:8D) until the day of the experiment, whereas parasitized larvae and adults of *M. rufiventris* were kept in the laboratory under ambient light and temperature conditions. Female parasitoids were used for the assays when they were 2–5 d old.

Six-arm Olfactometer Bioassay (Inexperienced Wasps). The six-arm olfactometer is described by Turlings et al. (2004). This system exposes wasps to six air streams entering a central choice chamber. Each stream, entering the chamber via a glass tube, can carry a different odor. In our bioassays, only three odor sources were offered to the parasitoids and each arm with odorous air stream was alternated with a neighboring arm carrying a stream of clean air. Charcoal filters purified and a bubbler humidified the air that was then pushed

into each odor source vessel at a rate of 1.2 l min^{-1}. During each bioassay, a portion of each odor was collected by pulling air from each vessel at a rate of 0.6 ml min^{-1} through Super-Q traps (see Heath and Manukian, 1992) that were attached to the vessels just above the treated pants (for details, see Turlings et al., 2004).

To induce volatile emissions in the plants that served as odor sources, we scratched the underside of two leaves over an area of 2–4 cm^2 on both sides of the central vein of the leaf with a razor blade, after which 10 μl of *S. littoralis* regurgitant were applied on each damaged site. The regurgitant had been collected from fourth and fifth instar *S. littoralis* caterpillars that had fed on maize leaves (var. Delprim) and had been stored at −70°C until just before use (for details, see Turlings et al., 1998). After treatment, the plants were placed under three fluorescent lamps (Sylvania standard F36W 133-T8 cool white, 5,000 lm m^{-2} at pot height) until used in bioassays.

Two combinations of three odor sources were offered as choice to inexperienced wasps. The combination of odors consisted of: (1) two maize plants treated 30 min before (fresh–fresh), two maize plants treated 6 hr before (old–old), and one plant treated 30 min before plus one 6 hr before (fresh–old) assaying; (2) two maize plants treated 30 min before (fresh–fresh), two maize plants treated 6 hr before (old–old), and two maize plants left unharmed, but treated with 20 μl of *S. littoralis* regurgitant on the leaf surface (spit) before assaying.

Wasps were released in groups of six into the choice chamber, and after 30 min, the wasps' choices for a particular arm were noted. Wasps that did not enter an arm after 30 min were considered a "no choice." For each replication day, the position of the odor sources was shifted. Between two and three replications per wasp species were carried out per day. On almost all replication days, both groups were tested using same odor sources (statistical tests confirmed that there was no influence of time of release even if some test days lasted 3 hr with three replications for each wasp species). After each experimental day, the olfactometer was washed with running tap water and soap, subsequently rinsed with acetone and pentane, and air-dried before being placed in the oven at 250°C for 3 hr. The Teflon tubes connecting the glass parts of the olfactometer were also rinsed with acetone and pentane.

A log-linear model fitted for the expected distribution of the wasps within the olfactometer (quasi-Poisson model) that do not conform to simple variance assumptions was used in statistical tests for odor preferences (Turlings et al., 2004). The numbers of wasps that chose one of the arms containing an odor source (three odor treatments plus three arms containing pure air as fourth treatment) for each released group (six wasps) were used as a replication. The numbers of replications for each test are given in the figure legends. We used the general linear model coefficients to determine preferences for one of the

treatments. *P* and *F* values are given only for the analysis of preference among the three odor cue treatments (not including the number of wasps that entered in the arm offering only clean air as odor cue). The software package R http://www.imsv.unibe.ch/cran) was used to test the model fitted by quasi-likelihood estimation, and its relative adequacy was assessed through likelihood ratio statistics and examination of residuals.

Six-arm Olfactometer Bioassay (Experienced Wasps). To provide them experience, the wasps were placed in a plastic box (9 cm top diam, 8.5 cm bottom diam, 3.5 cm high) containing maize leaves (var. Delprim) on which larvae of *S. littoralis* had been feeding for 24 hr. After females parasitized one or two larvae, they were considered experienced. This treatment is known to increase the responsiveness of parasitoids to the odors they perceive during the experience (Turlings et al., 1993). We gave only one oviposition experience to *M. rufiventris* to obtain approximately the same period of contact with the leaf–host complex for both parasitoid species, because *M. rufiventris* needs more time than *C. marginiventris* to parasitize the same number of hosts (Hoballah, personal observation).

Only one odor source combination was tested with experienced wasps: two maize plants treated 30 min before (fresh–fresh), two maize plants treated 6 hr before (old–old), and one plant treated 30 min before and one 6 hr before (fresh–old) assaying. Treatment of plants, odor collections, and statistical analyses were the same as for the bioassays with inexperienced wasps.

Volatile Collections and Analyses. Traps containing Super-Q (25 mg, 80/100 mesh; Alltech Associates, Inc., Deerfield State, USA) were used to collect odors from various treatments during the olfactometer experiments. Collections started 20 min before an olfactometer experiment and ended after a period of 3 hr had elapsed. After each collection, traps were rinsed with 150 μl methylene chloride. Two hundred ng of *n*-octane and nonyl acetate were added as internal standards. Three-μl aliquots of the samples were injected on column, with an automated injection system, into a Hewlett Packard model HP 6890 gas chromatograph equipped with a flame ionization detector. The HP-1 capillary column (30 m, 0.25 mm internal diam, 0.25 μm film thickness; Hewlett-Packard Company, USA) was kept at 50°C for 3 min and then programmed at 8°C min^{-1} to 230°C, where it was maintained for 9.5 min. The column was preceded by a deactivated retention gap (10 m, 0.25 mm internal diam; Alltech Associates) and a deactivated precolumn (30 cm, 0.53 mm internal diam; Alltech Associates). Helium (24 cm sec^{-1}) was used as carrier gas. HP GC Chemstation software was used to quantify all major components by comparison to the known quantity of internal standards. ANOVA and Student–Newman–Keuls *post-hoc* test were used to compare the quantities of collected volatiles (total of all compounds combined as well as of single compounds).

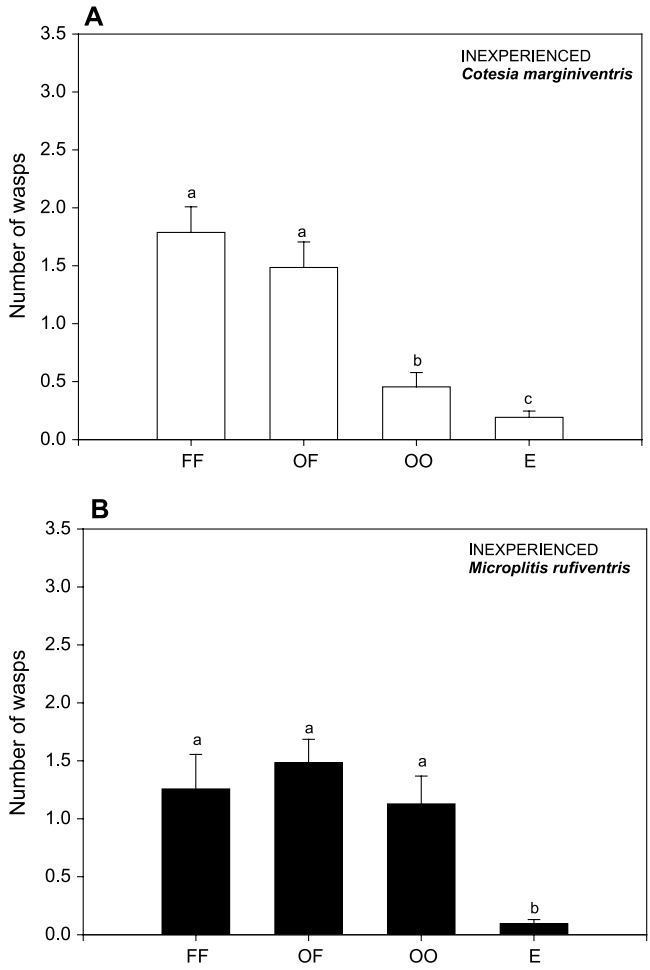

FIG. 1. Mean (±SE) of inexperienced *Cotesia marginiventris* (**A**, 33 replications, 26.8% "no choice") and *Microplitis rufiventris* (**B**, 31 replications, 30.1% "no choice") females that were attracted to a specific source in a six-arm olfactometer. FF: Two maize plants treated 30 min; OF: one plant treated 30 min and one plant treated 6 hr; OO: two plants treated 6 hr, respectively, before experiment started; E: clean air, mean (±SE) for the three empty olfactometer arms. Damage treatments consisted of scratching two leaves of a plant and adding *Spodoptera littoralis* regurgitant on the damaged sites. Different letters above bars indicate significant differences among total numbers of wasp that chose a particular odor source including clean air (preference for one of the three odor treatments: **A**, $P < 0.001$, $F = 15.87$, $df = 2$; **B**, $P = 0.608$, $F = 0.50$, $df = 2$).

RESULTS

Six-arm Olfactometer Bioassays (Inexperienced Wasps). C. marginiventris preferred the "fresh–fresh" and "fresh–old" odor to the odor of the "old–old" treatment, meaning that volatiles released after fresh damage are important for initial attraction (Figure 1A). Still, the "old–old" odor was more attractive than clean air (Figure 1A). In contrast, inexperienced *M. rufiventris* was equally attracted to all three treatments (Figure 1B). Both wasps rarely entered the three arms of the olfactometer carrying pure air, but almost one third of the wasps did not choose any arm (Figure 1).

The total amount of volatiles collected from the plants treated in different ways was not significantly different (mean ± SE for fresh–fresh: 613.6 ± 57 ng, fresh–old: 584.5 ± 72 ng, old–old: 610.6 ± 97 ng, ANOVA $P = 0.958$, $F = 0.043$). The odors collected during the olfactometer experiences show a

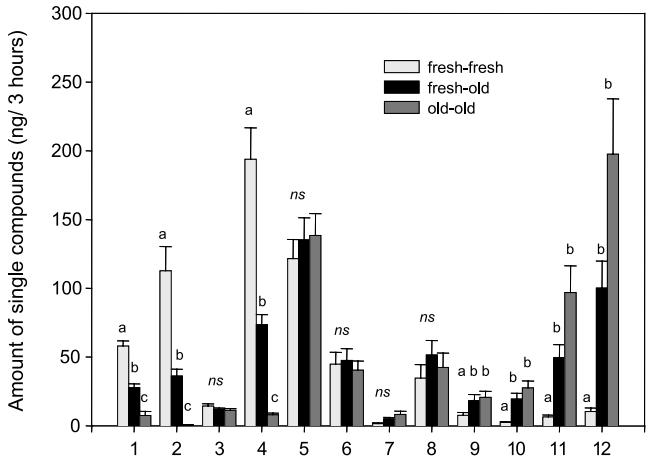

FIG. 2. Mean (±SE) amounts of major single volatile compounds of a blend collected for 3 hr from: two maize plants treated 30 min (fresh–fresh), one plant treated 30 min and one plant treated 6 hr (fresh–old), and two plants treated 6 hr (old–old) before experiments in a six-arm olfactometer started. Damage treatments consisted of scratching two leaves of a plant and adding *Spodoptera littoralis* regurgitant on the damaged sites. Different letters above bars indicate significant differences in amount of single compounds collected among treatments and *ns* indicate no significant difference among treatments. This graph includes the volatile collections of all experiments carried out with those treatments (experiments with inexperienced and experienced wasps, $N = 12$). **1**, (*E*)-2-Hexenal; **2**, (*Z*)-3-hexenol; **3**, β-myrcene; **4**, (*Z*)-3-hexenyl acetate; **5**, linalool; **6**, (*E*)-4,8-dimethyl-1,3,7-nonatriene; **7**, phenethyl acetate; **8**, 1-H-indole; **9**, geranyl acetate; **10**, (*E*)-β-caryophyllene; **11**, (*E*)-α-bergamotene; **12**, (*E*)-β-farnesene.

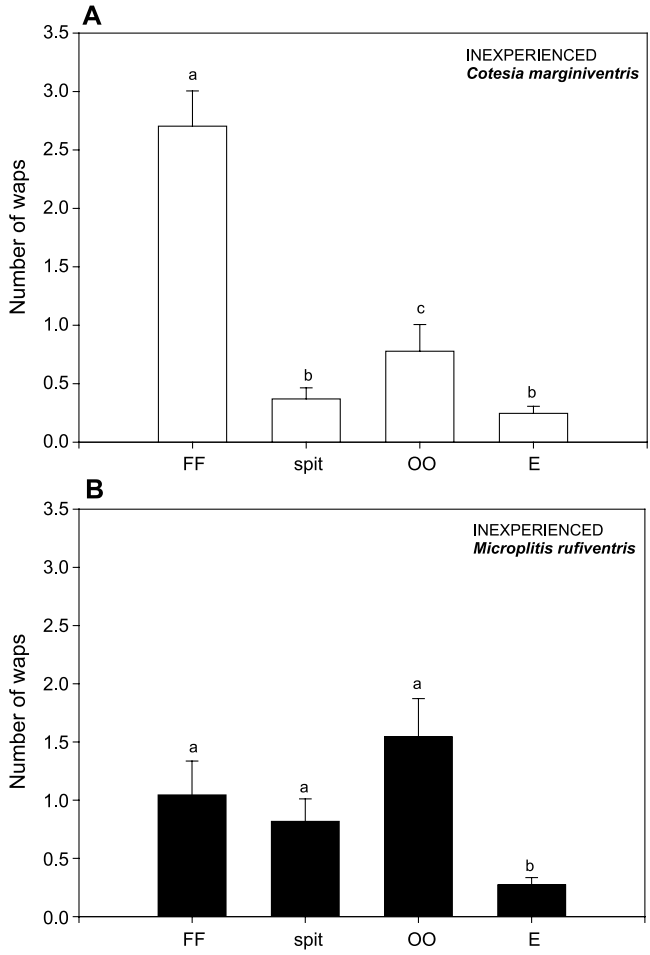

FIG. 3. Mean (±SE) of inexperienced *Cotesia marginiventris* (**A**, 27 replicates, 22.8% "no choice") and *Microplitis rufiventris* (**B**, 22 replicates, 27.3% "no choice") females that were attracted to a specific source in a six-arm olfactometer. FF: Two maize plants treated 30 min; spit: two undamaged plants on which *Spodoptera littoralis* regurgitant was smeared; OO: two plants treated 6 hr before experiment started; E: clean air, mean (±SE) for the three empty olfactometer arms. Damage treatments consisted of scratching two leaves of a plant and adding *Spodoptera littoralis* regurgitant on the damaged sites. Different letters above bars indicate significant differences among the numbers of wasp that chose a particular odor source including clean air (preference for one of the three odor treatments: **A**, $P < 0.001$, $F = 27.94$, $df = 2$; **B**, $P = 0.169$, $F = 1.83$, $df = 2$).

difference in the amounts of green leaf volatiles and terpenoids emitted by the treatments, with the "fresh–old" treatments showing intermediate release of both groups (Figure 2).

In the experiment where the wasps had a choice between (a) the odor of two freshly damaged plants, (b) the odor of two plants with 6-hr-old damage, and (c) the odor of two undamaged plants with regurgitant smeared on the leaves, *C. marginiventris* preferred the fresh damage (Figure 3A), whereas inexperienced *M. rufiventris* again showed no significant preference (Figure 3B). The relatively high number of *M. rufiventris* choosing the odor of undamaged plants treated with regurgitant was unexpected, because the amount of all major compounds released from this treatment was significantly lower than for the other two treatments (Figure 4).

Six-arm Olfactometer Bioassays (Experienced Wasps). When *C. marginiventris* wasps were given an oviposition experience on *S. littoralis* caterpillars feeding on maize (var. Delprim) leaves, their preference changed. Experienced

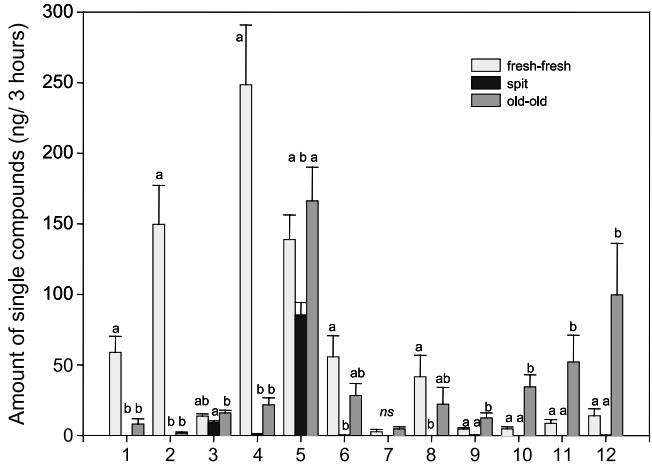

FIG. 4. Mean (±SE) amounts of major single volatile compounds of a blend collected for 3 hr from two maize plants treated 30 min (fresh–fresh), two plants smeared with *Spodoptera littoralis* regurgitant (spit), and two plants treated 6 hr (old–old), before experiments in a six-arm olfactometer started. Damage treatments consisted of scratching two leaves of a plant and adding *S. littoralis* regurgitant on the damaged sites. Different letters above bars indicate significant differences in amount of single compounds collected among treatments and *ns* indicate no significant difference among treatments. **1**, (*E*)-2-Hexenal; **2**, (*Z*)-3-hexenol; **3**, β-myrcene; **4**, (*Z*)-3-hexenyl acetate; **5**, linalool; **6**, (*E*)-4,8-dimethyl-1,3,7-nonatriene; **7**, phenethyl acetate; **8**, 1-H-indole; **9**, geranyl acetate; **10**, (*E*)-β-caryophyllene; **11**, (*E*)-α-bergamotene; **12**, (*E*)-β-farnesene.

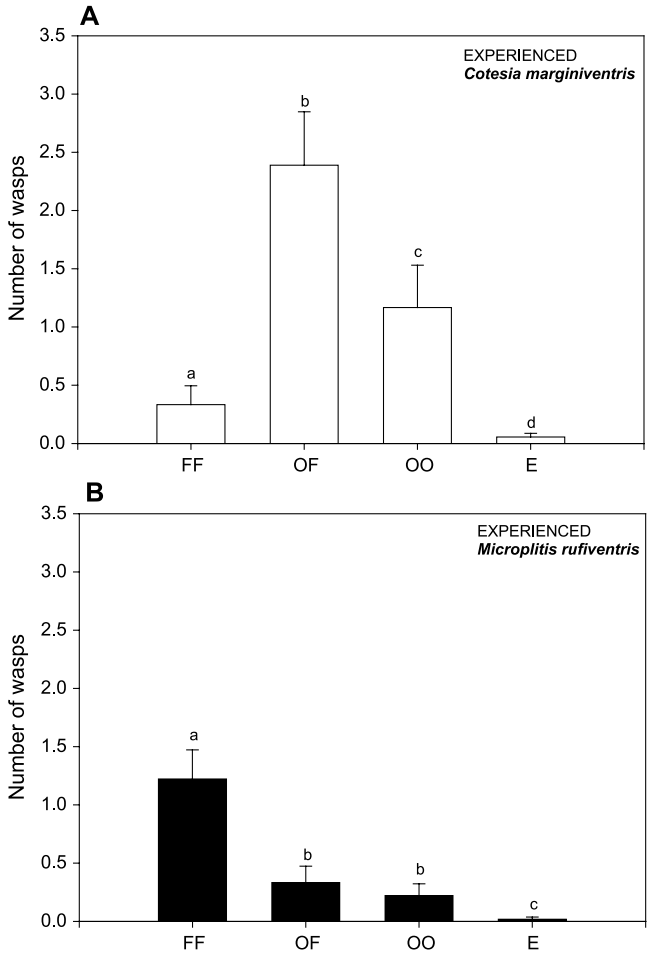

FIG. 5. Mean (±SE) of experienced *Cotesia marginiventris* (**A**, 18 replicates, 32.4 % "no choice") and *Microplitis rufiventris* females (**B**, 18 replicates, 69.4 % "no choice") that were attracted to a specific source in a six-arm olfactometer. Experience consisted of an oviposition in a host on maize leaves that had been damaged by hosts for 24 hr. FF: Two maize plants treated 30 min; OF: one plant treated 30 min and one plant treated 6 hr; OO: two plants treated 6 hr, respectively, before an experiment started; E: clean air, mean (±SE) for the three empty olfactometer arms. Damage treatment consisted of scratching two leaves of a plant and adding *Spodoptera littoralis* regurgitant on the damaged sites. Different letters above bars indicate significant differences among total numbers of wasp that chose a particular odor source including clean air (preference for one of the three odor treatments: **A**, $P < 0.001$, $F = 9.49$, $df = 2$; **B**, $P < 0.001$, $F = 9.16$, $df = 2$).

C. marginiventris females significantly preferred the complete blend (fresh–old) to blends containing larger amounts of green leaf volatiles (fresh–fresh), or terpenoids (old–old) (Figure 5A). Surprisingly, the majority of experienced *M. rufiventris* did not make any choice, and among those that did, the majority preferred the odor source containing more green leaf volatiles (Figure 5B). As was the case for inexperienced wasps, experienced wasps of both species rarely entered one of the three arms of the olfactometer that carried clean air (Figure 5).

DISCUSSION

Confirming our hypothesis, inexperienced *C. marginiventris* preferred blends containing a higher proportion of green leaf volatiles, indicating that, as expected, green leaf volatiles or compounds that are simultaneously released are important for initial attraction of inexperienced *C. marginiventris*. Odor released after recent damage is important also in attracting naïve *C. marginiventris* to cotton plants (Cortesero et al., 1997). Several parasitoids orient to individual green leaf volatile compounds in an olfactometer or flight tunnel tests (Whitman and Eller, 1990; Wickremasinghe and Van Emden, 1992; Reddy et al., 2002) or show strong electroantennogram responses to these compounds (Baehrecke et al., 1989; Li et al., 1992). However, there are exceptions. For instance, artificially damaged plants (an unspecific blend containing some green leaf volatiles) elicited little response in naïve *Cotesia kariyai* (Fukushima et al., 2002). The responses of *M. rufiventris* observed here show that for this species, green leaf volatiles are not crucial for initial attraction. In fact, inexperienced *M. rufiventris* responded well, but did not distinguish among the different odor blends offered. Surprisingly, even the undamaged plants that were treated with regurgitant were attractive to this wasp. It should be noted that several maize varieties, including Delprim, release some volatiles when they are not damaged (Turlings et al., 1998). It is possible that these compounds, which include linalool, are important for the attraction of *M. rufiventris*, and that some compounds in the other blends repel this wasp. A closely related species, *Microplitis croceipes*, is also equally attracted to the odors of artificially damaged plants and undamaged cotton plants (Röse et al., 1998). Apparently, these specialized wasps use cues other than the general volatiles emitted by the damaged plant, cues that are more specifically linked with their hosts. This also seems to be the case for the specialist *Cotesia rubecula*, which is more attracted by the odors of *Arabidopsis thaliana* damaged by its host (*Pieris rapae*) than by artificially damaged *A. thaliana* (van Poecke et al., 2001), and more by host-damaged plants compared to jasmonic acid treated plants (van Poecke and Dicke, 2002).

That inexperienced *C. marginiventris* and *M. rufiventris*, both solitary endoparasitic braconids, have different odor preferences may reflect differences in host location strategies between generalists and specialists. The generalist *C. marginiventris* oriented primarily toward odors released by freshly damaged plants, whereas the specialist *M. rufiventris* oriented to all treatment plants equally. A difference between the two species was also apparent after experience. *C. marginiventris* that experienced an oviposition on plants that had been damaged by host larvae overnight preferred the complete blend (fresh–old). Because the experience of oviposition was associated with the entire complex, host-damaged leaves, host larvae, and host by-products, it was to be expected that the wasps would be more attracted to the complete blend. Surprising was the preference of experienced *M. rufiventris* females for the blends containing a higher proportion of green leaf volatiles, as was the high proportion of experienced wasps that did not make a choice. It is possible that the experienced *M. rufiventris* searched for odors that are more specifically associated with the presence of hosts and that were not present among the cues offered.

It should be pointed out that we have emphasized green leaf volatiles and terpenoids as the groups of compounds associated with fresh and old damage; however, many other compounds are released in minor quantities, and these compounds may play a role in the attraction of the wasps as well. Electroantennogram studies have shown that some minor compounds elicit strong antennal responses in these parasitoids (Gouinguené et al., 2005), and current attempts to isolate and identify key attractants for *C. marginiventris* indicate an important role for minor compounds (M. D'Alessandro and T.C.J. Turlings, unpublished data). This does not invalidate the main conclusion from this study—that some generalist parasitoids use volatiles from fresh damage as general cues, and that truly herbivore-induced compounds, or other compounds directly associated with host presence, become more important after the wasps have experienced them while encountering hosts. Moreover, it can be concluded that parasitoids with comparable biologies, but different host ranges, employ different strategies in their use of plant-provided cues to locate hosts. Further insight into the host specificity and the circumstances under which wasps have to forage may provide explanations for these differences.

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THE INVOLVEMENT OF VOLATILE INFOCHEMICALS FROM SPIDER MITES AND FROM FOOD-PLANTS IN PREY LOCATION OF THE GENERALIST PREDATORY MITE *Neoseiulus californicus*

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Abstract—We investigated volatile infochemicals possibly involved in location of the generalist predatory mite *Neoseiulus californicus* to plants infested with spider mites in a Y-tube olfactometer. The predators significantly preferred volatiles from lima bean leaves infested with *Tetranychus urticae* to uninfested lima bean leaves. Likewise, they were attracted to volatiles from artificially damaged lima bean leaves and those from *T. urticae* plus their visible products. Significantly more predators chose infested lima bean leaves from which *T. urticae* plus their visible products had been removed than artificially damaged leaves, *T. urticae*, and their visible products. These results suggest that *N. californicus* is capable of exploiting a variety of volatile infochemicals originating from their prey, from the prey-foodplants themselves, and from the complex of the prey and the host plants (e.g., herbivore-induced volatiles). We also investigated predator response to some of the synthetic samples identified as volatile components emitted from *T. urticae*-infested lima bean leaves and/or artificially damaged lima bean leaves. The predators were attracted to each of the five synthetic volatile components: linalool, methyl salicylate, (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate. The role of each volatile compound in prey-searching behavior is discussed.

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Key Words—*Neoseiulus californicus*, *Tetranychus urticae*, lima bean, herbivore-induced volatiles, green leaf volatiles.

INTRODUCTION

Plant volatiles can be exploited by carnivorous natural enemies as cues for locating herbivorous victims (Lewis and Martin, 1990; Reddy et al., 2002). These infochemicals may originate from the food-plants of the herbivores, or from complexes of the herbivores and the food-plants (Steinberg et al., 1993). The importance of volatiles from the plant–herbivore complex (e.g., herbivore-induced volatiles) as foraging cues has been reported in many systems consisting of plants, herbivores, and carnivorous natural enemies (Takabayashi and Dicke, 1996; Dicke et al., 1998; Dicke, 1999a, b; Shiojiri et al., 2002). For example, laboratory studies have shown that volatiles from plants infested with spider mites are attractive to several species of insect predators and predatory mites, while those from spider mites, from uninfested plants, and from artificially damaged plants seldom or do not attract them (e.g., Sabelis et al., 1984; Maeda et al., 1999; Shimoda et al., 1997; Shimoda and Takabayashi, 2001).

There are relatively few studies that have identified predator attractants involved in volatile blends emitted from spider mite-infested plants despite the relevance of this topic from the ecological and biological control perspectives. Among the studies, for example, the predatory mites *Phytoseiulus persimilis* are attracted to volatiles from lima bean leaves infested with the two-spotted spider mite *Tetranychus urticae* (Sabelis and Van de Baan, 1983). In the volatile blend from *T. urticae*-infested lima bean leaves, each of the four herbivore-induced volatile compounds, linalool, methyl salicylate, (*E*)-β-ocimene, and (*E*)-4,8-dimethyl-1,3,7-nonatriene, is attractive to *P. persimilis*, whereas other volatile components in the blend, such as (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate, are not (Dicke et al., 1990). (*Z*)-3-Hexen-1-ol and (*Z*)-3-hexenyl acetate, called green leaf volatiles, are released by many plant species, including lima bean plants after physical damage as well as herbivory (Whitman and Eller, 1992; Reddy et al., 2002).

Neoseiulus (=*Amblyseius*) *californicus* is a predatory mite closely associated with spider mites, although it is regarded as a generalist predator that can exploit various foods such as pollen and small insects (Croft et al., 1998; Easterbrook et al., 2001). *N. californicus* is an effective biological control agent against *T. urticae* and other spider mite pests on agricultural crops (Garcia-Mari and Gonzalez-Zamora, 1999; Greco et al., 1999; Easterbrook et al., 2001). Although several studies have reported that *N. californicus* discriminates between plants infested with spider mites and uninfested plants (Janssen et al.,

1990; Llusia and Penuelas, 2001), the source of the predator attractants is still unclear. To clarify this point, we investigated possible volatile infochemicals involved in the location of *N. californicus* to spider mite-infested plants in a Y-tube olfactometer. We also focused on the predator response to single synthetic samples already identified as herbivore-induced volatiles in this tritrophic system (Dicke et al., 1990; Arimura et al., 2000), because no chemicals have been identified as attractants of *N. californicus*.

METHODS AND MATERIALS

Plants. Lima bean plants (*Phaseolus lunatus* cv. Sieva) were cultivated individually in plastic pots (9 cm diam, 7 cm depth) in a plant growth chamber (25 ± 1°C, 60 ± 10% r. h.). The fluorescent lights in the plant growth chamber (19,000 ± 3,000 lx) were switched on from 6:00 until 22:00 hours to maintain L16:D8 conditions. Young primary lima bean leaves of 14- to 16-d-old plants were used for rearing predators, bioassays, and headspace analysis. Kidney bean plants (*Phaseolus vulgaris* cv. Nagauzuramame) cultivated in a laboratory (20 ± 1°C, 60 ± 10% r. h.) were used for rearing the spider mites *T. urticae*.

Mites. N. californicus individuals were collected from Kudzu vine plants [*Pueraria lobata* (Wild.)] infested with *Tetranychus phaseolus* in a field at the National Agricultural Research Center in Ibaraki Prefecture, Japan, in 2001. The predatory mites were reared on lima bean leaf disks (8 cm diam) placed upside down on water-soaked cotton wool in plastic containers (14.5 × 19 cm, 5 cm in depth). To prepare *T. urticae*-infested lima bean leaves for rearing the predators, we first placed several kidney bean leaves infested with *T. urticae* on uninfested lima bean plants for several days in a climate-controlled room (25 ± 2°C, 60 ± 10% r. h. and 16L: 8D). Subsequently, a few lima bean leaves infested with *T. urticae* were offered to the predators in the lima bean leaf disks every 3 d. This predator culture had been maintained in the laboratory (23 ± 1°C, 60 ± 10% r. h., and L16:D8) for more than 1 yr. About 20 adult female predators after the final molting followed by mating were transferred from the leaf disk to a new one using a fine paintbrush. Fertilized adult female predators approx. 3–5 d after final molting were used for the experiments. To obtain starved predators for the bioassays, predators were individually placed in sealed plastic tubes (2 ml), which contained a small piece of moist filter paper, for 24 hr in the laboratory (25 ± 1°C and 16L: 8D).

Olfactometer Experiments. Each experiment was conducted in a Y-tube olfactometer with each arm connected to a glass container (1 l) containing an odor source. The airflow through each olfactometer arm (2.8 l/min) was checked with a flow meter. For details of the olfactometer set-up, see Takabayashi and Dicke

(1992). Predators were individually introduced at the start point on a steel wire that was positioned at the center of the glass tube, and observed until a predator reached the far end (finish line) of one of the arms. Predators that did not pass the finish line of either arm within 5 min (termed "no choice" subjects) were excluded from statistical analysis. We switched the arm containing the sample odor with that containing the control odor in the olfactometer every five bioassays. In every experiment, a total of 80 or more predators was tested over more than 4 d.

Experiment A: Origin of Volatiles that Attract Predators. To investigate possible volatile infochemicals involved in location of *N. californicus* to *T. urticae*-infested lima bean leaves, the following five experiments were conducted using six different types of odor sources: Experiment A1, uninfested leaves vs. clean air; Experiment A2, *T. urticae*-infested leaves vs. uninfested leaves; Experiment A3, artificially damaged leaves vs. clean air; Experiment A4, *T. urticae* plus visible products vs. clean air; Experiment A5: infested leaves without *T. urticae* vs. artificially damaged leaves, and *T. urticae* plus visible products.

(1) *T. urticae*-infested leaves. To prepare *T. urticae*-infested lima bean leaves, we placed several kidney bean leaves infested with *T. urticae* on primary leaves of uninfested lima bean plants; ca. 100 adult females together with larvae and nymphs were introduced to each leaf. The infested plants were incubated in a climate-controlled room (25 ± 1°C, 60 ± 10% r. h., and L16: D8) for 3 d. Just before the experiments, we cut five primary leaves infested with ca. 500 *T. urticae* individuals at all stages (approx. 100 of these were adult females) per leaf. The cut edge of the stem of each leaf was wrapped in a small piece of moist cotton wool and used as the odor source.

(2) Uninfested leaves. Several uninfested plants containing two primary leaves each were incubated in the climate-controlled room for 3 d. Five primary lima bean leaves were used as the odor source. Other conditions and procedures were the same as described above (1).

(3) *T. urticae* plus visible products. About 2,500 living spider mites at all stages (approx. 500 of these were adult females) and their visible products (feces, exuviae, eggs, egg shells, dead bodies, and silken webs) were carefully removed with a paintbrush from 5 infested leaves just before the experiments. The spider mites and their visible products were placed on moist cotton wool (10 × 10 cm) and used as an odor source. Other conditions and procedures were the same as described above (1).

(4) Infested leaves without *T. urticae*. All living spider mites and their visible products were removed from five lima bean leaves by washing. The water on each leaf was wiped with paper wipes (Kimwipe S-200), and then was used as an odor source. Other conditions and procedures were the same as described above (1).

(5) Artificially damaged leaves. Five lima bean leaves were damaged by rubbing with carborundum (Silicon carbide: 400) on a wet cotton wool pad.

After the treatment, the carborundum was removed by washing. Other conditions and procedures were the same as described above (1).

(6) Clean air. Five small pieces of moist cotton wool were used as the control odor source.

Experiment B. Predator-Response to Single Synthetic Samples. We investigated the olfactory responses of *N. californicus* to each of the five synthetic volatile components: linalool, methyl salicylate, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, and (*E*)-2-hexenal. The first two have been identified as minor components in a blend of herbivore-induced volatiles from *T. urticae*-infested lima bean plants (Dicke et al., 1990). The last three are also found in headspace samples of artificially damaged lima bean plants as well as in a blend of herbivore-induced volatiles from *T. urticae*-infested lima bean plants (Dicke et al., 1990; Arimura et al., 2000).

The synthetic samples were separately dissolved in hexane (10 μg, 100 μg, and 1 mg/10 μl hexane solutions). Ten μl of diluted chemicals were separately impregnated into a piece of cellulose sponge (2 × 2 × 0.5 cm). After evaporation of solvent for a few minutes, each sponge was doubly packed into two sealed polyethylene bags (Seinichi Unipack: 70 × 50 × 0.04 mm) and then used as a sample odor source. In the same way, a piece of cellulose sponge with pure solvent (10 μl of hexane) sealed in two polyethylene bags was prepared as a control odor source. The synthetic samples, of purity ranging from 96% to 98%, were obtained from Wako Pure Chemical Industries.

The following experiments were conducted: Experiment B1, linalool (10 μg); Experiment B2, linalool (1 mg); Experiment B3, methyl salicylate (10 μg); Experiment B4, methyl salicylate (1 mg); Experiment B5, (*Z*)-3-hexen-1-ol (10 μg); Experiment B6, (*Z*)-3-hexen-1-ol (100 μg); Experiment B7, (*Z*)-3-hexen-1-ol (1 mg); Experiment B8, (*E*)-2-hexenal (10 μg); Experiment B9, (*E*)-2-hexenal (100 μg); Experiment B10, (*E*)-2-hexenal (1 mg); Experiment B11, (*Z*)-3-hexenyl acetate (10 μg); Experiment B12: (*Z*)-3-hexenyl acetate (100 μg); and Experiment B13: (*Z*)-3-hexenyl acetate (1 mg). In each experiment, 10 μl of hexane were used as a control odor source.

Chemical Analysis of Volatiles. Headspace volatiles of each odor source used in Experiments A and B were collected by using a Tenax TA Thermal Desorption Tube (180 mg, 60/80 mesh; Gerstel) in a lidded glass bottle (2 l) for 30 min at a flow rate of 100 ml/min of clean air, purified by passing it through silica gel, molecular sieves, and activated charcoal (1 l each). Before collection of volatiles, a steady flow (200 ml/min) of purified air was circulated through the glass bottle containing each odor source for 10 min. Collected volatiles in each sample were analyzed with a GC-MS system [GC: Agilent Technologies 6890 with an HP-5MS capillary column (30 m long, 0.25 mm I.D., and 0.25 μm file thickness), injection temperature of 250°C; MS: Agilent Technologies 5973N mass selective detector, 70 eV] with Thermodesorption Autosampler

System and Cold Injection System (TDS A 2 and CIS 4: Gerstel). The oven temperature of the GC-MS was programmed to rise from 40°C (9 min hold) to 280°C at 10°C/min. The TDS A 2 and CIS 4 temperatures were programmed to rise from 20°C (1 min hold) to 280°C at 60°C/min and from −150°C (0.5 min hold) to 300°C at 12°C/min, respectively. The components were identified by comparing their spectra with mass spectra in the database (Wiley Library), along with our data regarding herbivore-induced volatiles from lima bean plants (Ozawa et al., 2000).

Statistics. The results from each bioassay experiment were subjected to a χ^2 test (Sokal and Rohlf, 1998). The null hypothesis was that predators exhibited a 50:50 distribution over the two odor sources. We applied the *t*-test or one-way analysis of variance (ANOVA) followed by Tukey–Kramer's test to the results of the chemical analysis. When necessary, data used in ANOVA were transformed using square root transformation of $\sqrt{(x + 0.5)}$ or logarithmic transformation of $\log_{10}(x + 1)$ to meet assumptions of normality.

RESULTS

Experiment A. When presented with a choice between uninfested leaves and clean air, predators did not discriminate (Exp. A1: $\chi^2 = 0.211$, $df = 1$, $P = 0.646$) (Figure 1). In contrast, the predators showed a strong preference for odors from *T. urticae*-infested leaves over uninfested leaves (Exp. A2: $\chi^2 = 23.027$, $df = 1$, $P < 0.001$). When artificially damaged leaves were offered vs. clean air, significantly more predators chose the former odor source (Exp. A3: $\chi^2 = 5.128$, $df = 1$, $P < 0.05$). The same tendency was observed when *T. urticae* plus visible products vs. clean air were compared (Exp. A4: $\chi^2 = 10.051$, $df = 1$, $P < 0.01$). Significantly more predators chose infested leaves without *T. urticae* than artificially damaged leaves, *T. urticae* plus visible products (Exp. A5: $\chi^2 = 20.513$, $df = 1$, $P < 0.001$).

Experiment B. When presented with a choice between linalool (10 μg) and hexane, predators did not discriminate (Exp. B1: $\chi^2 = 0.653$, $df = 1$, $P = 0.419$) (Figure 2). However, the predators showed a preference for linalool when offered at an amount of 1 mg (Exp. B2: $\chi^2 = 16$, $df = 1$, $P < 0.001$). They were also attracted to methyl salicylate when offered at an amount of 1 mg (Exp. B4: $\chi^2 = 16.327$, $df = 1$, $P < 0.001$). When the dose offered was only 10 μg, however, no positive response was observed (Exp. B3: $\chi^2 = 0.667$, $df = 1$, $P = 0.414$). This was also the case for (*Z*)-3-hexen-1-ol when offered at 10 μg (Exp. B5: $\chi^2 = 0.16$, $df = 1$, $P = 0.689$) (Figure 3). However, (*Z*)-3-hexen-1-ol attracted predators at 100 μg (Exp. B6: $\chi^2 = 7.364$, $df = 1$, $P < 0.01$) and 1 mg (Exp. B7: $\chi^2 = 5.343$, $df = 1$, $P < 0.05$). (*E*)-2-Hexenal was also attractive to predators at 1 mg (Exp. B10: $\chi^2 = 9.184$, $df = 1$, $P < 0.01$). However, no

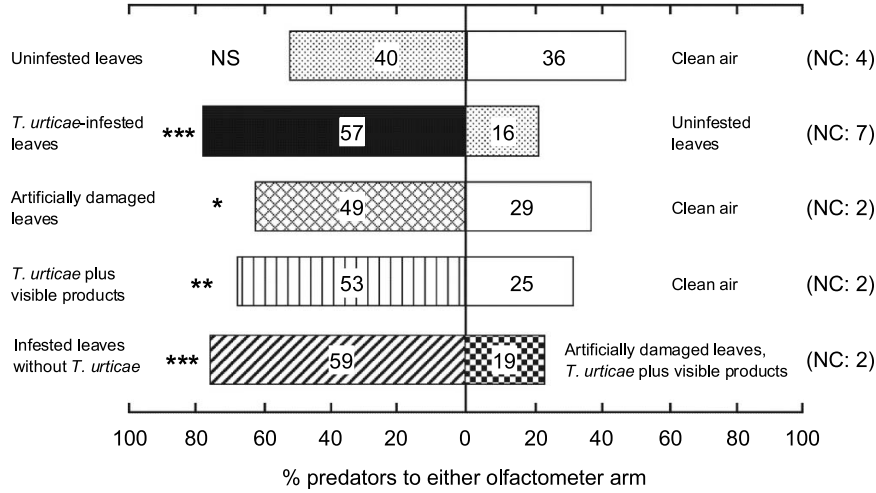

FIG. 1. Responses of adult females of *Neoseiulus californicus* in a Y-tube olfactometer when offered: Experiment A1, uninfested leaves vs. clean air; Experiment A2, *T. urticae*-infested leaves vs. uninfested leaves; Experiment A3, artificially damaged leaves vs. clean air; Experiment A4, *T. urticae* plus visible products vs. clean air; Experiment A5: infested leaves without *T. urticae* vs. artificially damaged leaves, *T. urticae* plus visible products. χ^2 test was used to evaluate whether the results differed from a 50:50 distribution between the two olfactometer arms (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, NS: $P > 0.05$). Predators that did not reach the end of either olfactometer arm within 5 min [no choice (NC)] were excluded from the statistical analysis. For further explanation, see text.

positive response was observed when the dose offered was 10 μg (Exp. B8: $\chi^2 = 1.779$, $df = 1$, $P = 0.182$) and 100 μg (Exp. B9: $\chi^2 = 3.375$, $df = 1$, $P = 0.066$). Similar results were obtained with (*Z*)-3-hexenyl acetate at 100 μg (Exp. B12, $\chi^2 = 0.163$, $df = 1$, $P = 0.686$) and 1 mg (Exp. B13: $\chi^2 = 2.56$, $df = 1$, $P = 0.11$). However, (*Z*)-3-hexenyl acetate was attractive to predators at 10 μg (Exp. B11: $\chi^2 = 8$, $df = 1$, $P < 0.01$).

Chemical Analysis of Volatiles. Uninfested leaves emitted trace amounts of (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, and α-copaene (Table 1). Artificially damaged leaves emitted small amounts of (*Z*)-3-hexenyl acetate and (*E*)-β-ocimene in addition to these three components. *T. urticae*-infested leaves emitted (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, methyl salicylate, and eight terpenoids. Linalool, methyl salicylate, (*Z*)-β-ocimene, (*Z*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, β-caryophyllene, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene were found in the headspace of the *T. urticae*-infested leaves, but not found in that of the artificially damaged

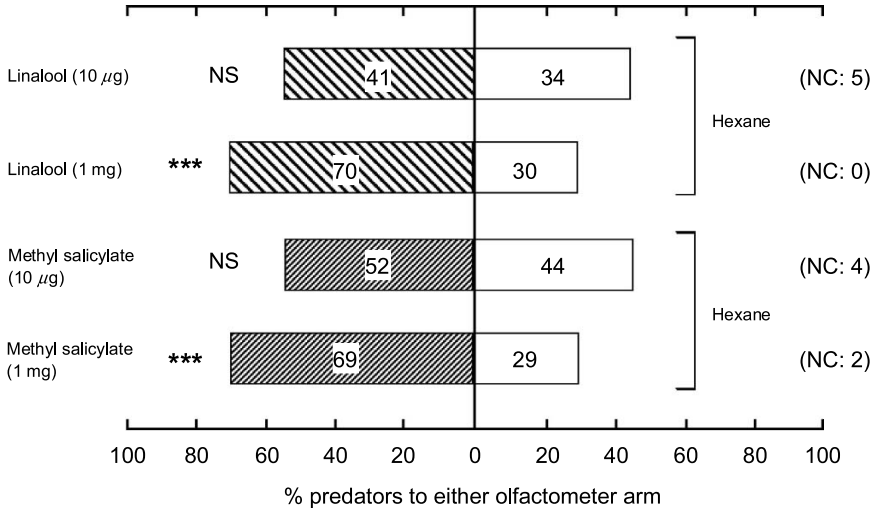

FIG. 2. Responses of adult females of *Neoseiulus californicus* in a Y-tube olfactometer when offered: Experiment B1, linalool (10 μg); Experiment B2, linalool (1 mg); Experiment B3, methyl salicylate (10 μg); Experiment B4, methyl salicylate (1 mg). The synthetic samples were separately dissolved in hexane to obtain different concentrations of each chemical (10 μg and 1 mg/10 μl hexane solutions). Aliquots (10 μl) of diluted chemicals were used as sample odor sources after evaporation of their solvent carriers. In each experiment, 10 μl of hexane were used as a control odor source. χ^2 test was used to evaluate whether the results differed from a 50:50 distribution between the two olfactometer arms (***$P < 0.001$, NS: $P > 0.05$). Predators that did not reach the end of either olfactometer arm within 5 min [no choice (NC)] were excluded from the statistical analysis. For further explanation, see text.

leaves. Furthermore, (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, and α-copaene were detected in significantly greater amounts within the former than within the latter odor source. Infested leaves without *T. urticae* emitted the same volatile components as detected in the headspace of *T. urticae*-infested leaves. There was no significant difference between these two odor sources as to the amount of each volatile component. No specific volatile components were found in the headspace of *T. urticae* plus visible products ($N = 3$), although this odor source was attractive to *N. californicus* in Experiment A.

A significantly greater amount of (*E*)-2-hexsenal was detected in the headspace of the glass bottle in which was placed either 10 μg, 100 μg, or 1 mg of the synthetic (*E*)-2-hexsenal in a cellulose sponge, double-packed in sealed polyethylene bags, than in the headspace of the glass bottle in which either artificially damaged leaves, *T. urticae*-infested leaves, or infested leaves without *T. urticae* were placed. We did not detect (*Z*)-3-hexen-1-ol when treated with

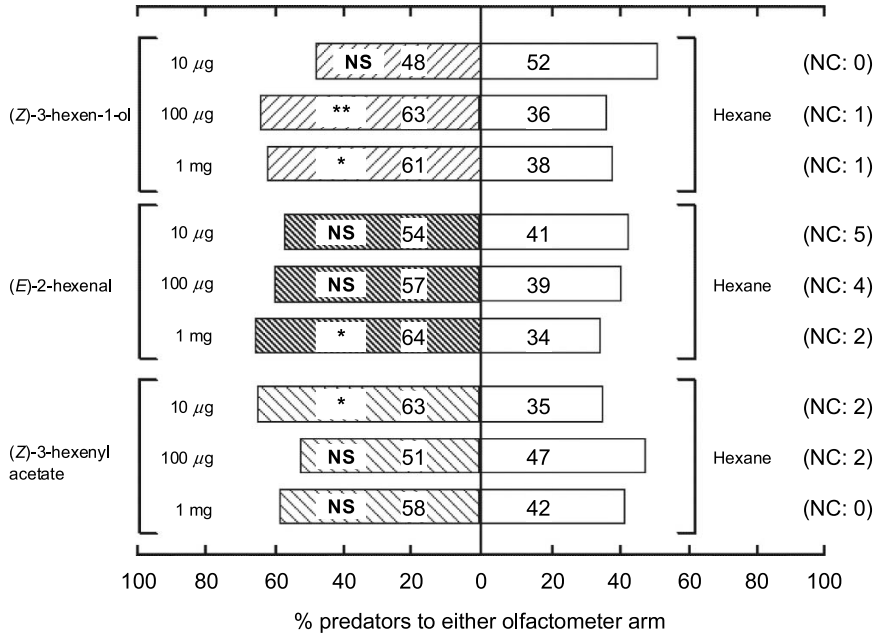

FIG. 3. Responses of adult females of *Neoseiulus californicus* in a Y-tube olfactometer when offered: Experiment B5, (*Z*)-3-hexen-1-ol (10 μg); Experiment B6, (*Z*)-3-hexen-1-ol (100 μg); Experiment B7, (*Z*)-3-hexen-1-ol (1 mg); Experiment B8, (*E*)-2-hexenal (10 μg); Experiment B9, (*E*)-2-hexenal (100 μg); Experiment B10, (*E*)-2-hexenal (1 mg); Experiment B11, (*Z*)-3-hexenyl acetate (10 μg); Experiment B12, (*Z*)-3-hexenyl acetate (100 μg); Experiment B13: (*Z*)-3-hexenyl acetate (1 mg). The synthetic samples were separately dissolved in hexane to obtain different concentrations of each chemical (10 μg, 100 μg, and 1 mg/10 μl hexane solutions). Aliquots (10 μl) of diluted chemicals were used as sample odor sources after evaporation of their solvent carriers. In each experiment, 10 μl of hexane were used as a control odor source. χ^2 test was used to evaluate whether the results differed from a 50:50 distribution between the two olfactometer arms (*$P < 0.05$, **$P < 0.01$, NS: $P > 0.05$). Predators that did not reach the end of either olfactometer arm within 5 min [no choice (NC)] were excluded from the statistical analysis. For further explanation, see text.

synthetic (*Z*)-3-hexen-1-ol (10 μg). When synthetic (*Z*)-3-hexen-1-ol (100 μg) was used as an odor source, a small amount of (*Z*)-3-hexen-1-ol was detected, which was at a similar emission level as that made by uninfested leaves, artificially damaged leaves, *T. urticae*-infested leaves, and infested leaves without *T. urticae*. A small amount of (*Z*)-3-hexenyl acetate was detected when treated with synthetic (*Z*)-3-hexenyl acetate (10 μg), which was an emission level similar to that made by artificially damaged leaves. When synthetic

TABLE 1. MEAN ION INTENSITIES (±SE, $\times 10^8$) OF VOLATILE COMPOUNDS FOUND IN THE HEADSPACE OF LIMA BEAN LEAVES WITH DIFFERENT TREATMENTS AND THOSE OBTAINED WHEN DIFFERENT CONCENTRATIONS OF SYNTHETIC CHEMICALS WERE OFFERED

Compound	Uninfested ($N = 4$)	Damaged ($N = 4$)	Infested 1 ($N = 4$)	Infested 2 ($N = 4$)	Synthetic samples			Statistics
					10 μg	100 μg	1 mg	(ANOVA or t test)
(*E*)-2-Hexenal	0.03 ± 0.01^a	0.04 ± 0.01^a	0.05 ± 0.01^a	0.04 ± 0.01^a	0.32 ± 0.06^b ($N = 6$)	2.22 ± 0.29^c ($N = 6$)	64.56 ± 7.7^d ($N = 4$)	$F = 131.317$, $df = 6$, $P < 0.001$
(*Z*)-3-Hexen-1-ol	0.49 ± 0.29^a	2.23 ± 0.73^{ab}	1.6 ± 0.28^{ab}	3.9 ± 1.06^b	ND ($N = 6$)	2.41 ± 0.64^{ab} ($N = 5$)	11.22 ± 0.71^c ($N = 4$)	$F = 39.419$, $df = 5$, $P < 0.001$
(*Z*)-3-Hexenyl acetate	ND	1.68 ± 0.27^a	13.41 ± 2.79^c	19.95 ± 6.57^{ab}	0.94 ± 0.07^a ($N = 6$)	4.75 ± 0.98^b ($N = 5$)	31.29 ± 4.4^d ($N = 4$)	$F = 54.33$, $df = 5$, $P < 0.001$
(*Z*)-β-Ocimene	ND	ND	0.38 ± 0.17^a	0.4 ± 0.09^a				$t = -0.091$, $df = 2$, $P = 0.93$
(*E*)-β-Ocimene	ND	0.02 ± 0.02^a	4.76 ± 1.63^b	5.91 ± 1.17^b				$F = 30.943$, $df = 2$, $P < 0.001$
DMNT 1	ND	ND	0.66 ± 0.13^a	0.66 ± 0.12^a				$t = -0.022$, $df = 2$, $P = 0.983$
Linalool	ND	ND	1.13 ± 0.3^a	1.71 ± 0.27^a	ND ($N = 6$)		0.97 ± 0.06^a ($N = 5$)	$F = 2.663$, $df = 2$, $P = 0.114$
DMNT 2	ND	ND	13.2 ± 1.81^a	11.8 ± 2.09^a				$t = -0.508$, $df = 2$, $P = 0.627$
Methyl salicylate	ND	ND	8.83 ± 0.92^b	12.62 ± 1.67^b	3.06 ± 0.31^a ($N = 6$)		10.25 ± 2.11^b ($N = 4$)	$F = 11.945$, $df = 3$, $P < 0.001$
α-Copaene	0.01 ± 0.01^a	0.01 ± 0.01^a	0.3 ± 0.02^b	0.25 ± 0.06^b				$F = 22.266$, $df = 3$, $P < 0.001$
β-Caryophyllene	ND	ND	0.27 ± 0.02^a	0.31 ± 0.05^a				$t = -0.722$, $df = 2$, $P = 0.494$
TMTT	ND	ND	4.54 ± 0.69^a	4.91 ± 0.84^a				$t = -0.341$, $df = 2$, $P = 0.743$

Uninfested = uninfested leaves; Damaged = artificially damaged leaves; Infested 1 = *T. urticae*-infested leaves; Infested 2 = infested leaves without *T. urticae*; ND = not detected in the chemical analysis (excluded from statistical analysis); DMNT 1 = (*Z*)-4,8-dimethyl-1,3,7-nonatriene; DMNT 2 = (*E*)-4,8-dimethyl-1,3,7-nonatriene; TMTT = (*E*, *E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

Values with the same letter of the same compound are not significantly different (Tukey–Kramer's test or t test, $P > 0.05$).

linalool (1 mg) was used, a small amount of this component was detected, which was at an emission level similar to that made by *T. urticae*-infested leaves and infested leaves without *T. urticae*. This was also true for synthetic methyl salicylate (1 mg), *T. urticae*-infested leaves, and infested leaves without *T. urticae*, although a large amount of methyl salicylate was emitted from each odor source.

DISCUSSION

We showed that the volatiles from lima bean leaves infested with *T. urticae* were attractive to *N. californicus*, whereas those from uninfested lima bean leaves were not. This parallels other studies in which plants infested with spider mites elicit olfactory preference from their carnivorous natural enemies, such as predatory mites and predacious insects (e.g., Dicke, 1999a; Shiojiri et al., 2002). Volatiles from artificially damaged lima bean leaves and from *T. urticae* plus their by-products were also attractive to *N. californicus*, suggesting that this generalist predatory mite is capable of exploiting a variety of volatile infochemicals originating from its prey, from the host plants of the prey, and from the interaction between the prey and the host plants. Similar results were reported for the generalist predatory bug *Anthocoris nemorum*, which feeds on spider mites and other small insects (Dwumfour, 1992). On the other hand, several species of specialist predatory mites and insect predators appear to (almost) exclusively exploit volatile infochemicals originating from the prey-host plant complex, i.e., herbivore-induced volatiles, to locate spider mite-infested plants (Shimoda et al., 1997; Maeda et al., 1999; Shimoda and Takabayashi, 2001). There may be differences between specialist and generalist predatory mites in terms of the use of volatile infochemicals originating from prey, plants, and plant–prey complexes.

Artificially damaged leaves and uninfested leaves led to different olfactory responses of *N. californicus*, which were presumably attributable to the differences in their emissions of volatiles (Table 1). (*Z*)-3-Hexenyl acetate appears to play a partial role in the olfactory discrimination of these leaves, because *N. californicus* showed olfactory preferences for the synthetic chemical [(*Z*)-3-hexenyl acetate; 10 μg], detected only in the volatile blend of the artificially damaged leaves. Positive olfactory responses to synthetic (*Z*)-3-hexenyl acetate were reported in several species of parasitoid wasps, such as *Microplitis croceipes*, *Netelia heroica*, and *Cotesia plutellae* (Whitman and Eller, 1992; Reddy et al., 2002). Furthermore, (*E*)-β-ocimene may be involved in attracting *N. californicus* to artificially damaged leaves, because this volatile component was not found in the headspace of uninfested leaves. (*E*)-*β*-Ocimene is a key compound in the herbivore-induced volatile blend that plays a role in

attracting *P. persimilis* to *T. urticae*-infested lima bean leaves (Dicke et al., 1990).

(*Z*)-3-Hexen-1-ol might also have contributed to predator attraction to the artificially damaged leaves, the infested leaves without *T. urticae*, and the *T. urticae*-infested leaves (Figures 1, 3, Table 1). However, (*Z*)-3-hexen-1-ol is unlikely be involved in olfactory discrimination between the infested leaves without *T. urticae* and the artificially damaged leaves because no significant difference was obtained in emissions between the two odor sources. Infested leaves without *T. urticae* produced a greater variety of volatile components, including two herbivore-induced volatile components identified as linalool and methyl salicylate, than did artificially damaged leaves. No specific compound was detected from *T. urticae* plus their by-products in the chemical analysis. We showed that synthetic samples of linalool (1 mg) and methyl salicylate (1 mg) were attractive to *N. californicus*. Emission levels of linalool and methyl salicylate obtained by these chemical treatments (1 mg each) were similar to those made by the infested leaves without *T. urticae* (Table 1). These two herbivore-induced volatile components probably contributed to *N. californicus*' preference for the infested leaves without *T. urticae* over the artificially damaged leaves. This argument may also apply to the strong olfactory response of *N. californicus* evoked by *T. urticae*-infested leaves, because there was no significant difference in volatile emissions between *T. urticae*-infested leaves and infested leaves without *T. urticae*. Both methyl salicylate and linalool play roles in attracting *P. persimilis* to lima bean plants infested with *T. urticae* (Dicke et al., 1990). Methyl salicylate is also attractive to the predatory bug *Anthocoris numoralis*, which can partly account for the observed preference for volatiles from pear leaves infested with *Psylla pyricola* (Scutareanu et al., 1997; Drukker et al., 2000). It remains an open question as to whether or not other components [e.g., α-copaene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, β-caryophyllene, and (*E*, *E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene] from infested leaves without *T. urticae* are also involved in attracting *N. californicus*.

We found that the five synthetic samples were all attractive to *N. californicus*. This finding prompts an interest in the potential of these synthetic samples for enhancement of their density and efficiency as biological control agents against *T. urticae* or other pest spider mites in agroecosystems. James (2003) reported the attraction of predaceous insects, such as *Orius tristicolor*, *Deraeocoris brevis*, and *Geocoris pallens*, to individual synthetic samples of (*Z*)-3-hexenyl acetate and methyl salicylate in a hop yard. Furthermore, James and Price (2004) recorded larger populations of natural enemies of spider mites, such as *Orius tristicolor* and *Stethorus punctum*, in a hop yard baited with methyl salicylate than those in an unbaited hop yard, which may account for a conspicuous reduction in the abundance of *T. urticae* in the baited yard. We are also interested in the use of synthetic predator attractants in, e.g., controlled

Japanese pear orchards in which *N. californicus* is an important natural enemy of *T. urticae*. To carry out field experiments of this type, it will be necessary to ascertain whether or not the five synthetic samples, individually or as synthetic blends, are equivalent to volatile blends from, e.g., *T. urticae*-infested lima bean leaves as predator attractants.

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HERBIVORE-INDUCED PLANT VOLATILES MEDIATE IN-FLIGHT HOST DISCRIMINATION BY PARASITOIDS

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Abstract—Herbivore feeding induces plants to emit volatiles that are detectable and reliable cues for foraging parasitoids, which allows them to perform oriented host searching. We investigated whether these plant volatiles play a role in avoiding parasitoid competition by discriminating parasitized from unparasitized hosts in flight. In a wind tunnel set-up, we used mechanically damaged plants treated with regurgitant containing elicitors to simulate and standardize herbivore feeding. The solitary parasitoid *Cotesia rubecula* discriminated among volatile blends from Brussels sprouts plants treated with regurgitant of unparasitized *Pieris rapae* or *P. brassicae* caterpillars over blends emitted by plants treated with regurgitant of parasitized caterpillars. The gregarious *Cotesia glomerata* discriminated between volatiles induced by regurgitant from parasitized and unparasitized caterpillars of its major host species, *P. brassicae*. Gas chromatography-mass spectrometry analysis of headspace odors revealed that cabbage plants treated with regurgitant of parasitized *P. brassicae* caterpillars emitted lower amounts of volatiles than plants treated with unparasitized caterpillars. We demonstrate (1) that parasitoids can detect, in flight, whether their hosts contain competitors, and (2) that plants reduce the production of specific herbivore-induced volatiles after a successful recruitment of their bodyguards. As the induced volatiles bear biosynthetic and ecological costs to plants, down-regulation of their production has adaptive value. These findings add a new level of intricacy to plant–parasitoid interactions.

Key Words—Induced plant defense, headspace analysis, tritrophic interactions, *Pieris*, *Cotesia*.

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INTRODUCTION

Female parasitoids of herbivorous insects have to search for hosts to lay their eggs in or on them. Host searching is guided by volatile synomones that are mostly produced by the host plants after induction caused by herbivore feeding (Dicke, 1999; Shiojiri et al., 2001; Hilker and Meiners, 2002; Turlings et al., 2002; Steidle and van Loon, 2003). The ability to distinguish unparasitized from parasitized hosts and to reject the latter for oviposition (i.e., host discrimination) is widespread among hymenopteran parasitoids (van Lenteren, 1981; Vinson, 1985; van Alphen and Visser, 1990). The reproductive success of endoparasitoids is dependent on the quality and size of the hosts, because hosts provide a limited nutritional resource for their offspring (Godfray, 1994). Oviposition on or into already parasitized hosts by a conspecific parasitoid (i.e., superparasitism) can lead to competition with deleterious consequences such as mortality or reduced fitness. Therefore, avoiding superparasitism can have several advantages, including the optimization of foraging efficiency (van Lenteren, 1981; Godfray, 1994).

So far, host discrimination by parasitoids is known to be mediated by marking pheromones deposited by the ovipositing female either on or in the host, or within the explored patch. In addition, physiological changes in the host hemolymph induced by the parasitoid progeny (van Lenteren, 1981; Nufio and Papaj, 2001) or physical changes of the host surface acting as external markers (Takasu and Hirose, 1988) may provide cues for host discrimination. Marking pheromones are usually perceived through contact chemoreception (van Lenteren, 1981), although some examples of olfactory perception are known as well (Sheehan et al., 1993; van Giessen et al., 1993; van Baaren and Nenon, 1996).

Herbivore-induced volatiles are emitted in considerable quantities by plants, and the blend induced by herbivory differs in a qualitative and/or quantitative sense from the volatile blends of undamaged or mechanically damaged plants (Turlings et al., 1995; Dicke and Vet, 1999; Shiojiri et al., 2000). The emitted blend can be specific for the plant species, the herbivore species that damages the plant, and even for a specific stage of the herbivore (Turlings et al., 1995; Takabayashi and Dicke, 1996; Dicke and Vet, 1999). Based on the high detectability and reliability of herbivore-induced plant volatiles, parasitoids are able to use these cues to locate their hosts from a distance (Vet and Dicke, 1992). However, it is still unknown whether foraging females are able to distinguish unparasitized from parasitized hosts at a distance via olfactory cues produced by their host plant. Yet, this ability would save them foraging time and would reduce risks associated with host defense. We investigated this for a tritrophic system consisting of *Brassica* plants, *Pieris* caterpillars, and *Cotesia* parasitoids.

Brussels sprouts plants are known to emit a blend of volatiles when infested by *Pieris brassicae* L. (Lepidoptera: Pieridae) or *P. rapae* L. caterpillars. These induced volatiles attract *Cotesia rubecula* Marshall (Hymenoptera: Braconidae), a solitary endoparasitoid of the small cabbage white *P. rapae* and *Cotesia glomerata* L., a gregarious parasitoid of both *Pieris* species (Agelopoulos and Keller, 1994; Geervliet et al., 1994, 1998; Mattiacci et al., 1995, 2001). Differences between headspace volatiles collected from undamaged and *Pieris*-infested Brussels sprouts plants are extensively documented (Blaakmeer et al., 1994; Mattiacci et al., 1995, 2001; Smid et al., 2002). Coupled gas chromatography-electroantennography (GC-EAG) analysis has revealed that 20 of the headspace volatiles elicit a response in the antennae of *C. rubecula* and *C. glomerata* (Smid et al., 2002). The application of regurgitant of *P. brassicae* larvae or the enzyme β-glucosidase into a mechanical wound elicits induction of similar volatile blends in cabbage plants as in herbivory, showing that the plants recognize infestation through an elicitor in the oral secretion of *Pieris* caterpillars (Mattiacci et al., 1995).

In the present study, we investigate (1) whether females of the two *Cotesia* species prefer, in flight, induced odors emitted by plants fed upon by unparasitized caterpillars or treated with their regurgitant over odors emitted by plants fed upon by parasitized caterpillars or treated with their regurgitant, and (2) whether the induced plant odor blends differ, thereby providing a chemical basis for this discrimination behavior in the two wasp species.

METHODS AND MATERIALS

Plants and Insects. Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* cv. *Cyrus*) were reared in a greenhouse (18 ± 2°C, 70% RH, 16L:8D). Plants (8 to 12 wk old) having ca. 14–16 leaves were used for the experiments.

P. rapae and *P. brassicae* were reared on Brussels sprouts plants in a climate room (21 ± 1°C, 50–70% RH, 16L:8D). The parasitoid wasps *C. rubecula* and *C. glomerata* were reared on *P. rapae* and *P. brassicae* larvae, respectively, feeding on Brussels sprouts plants under greenhouse conditions (see above).

For bioassay experiments, *C. rubecula* and *C. glomerata* pupae were collected and reared in gauze cages in a climate room (23 ± 1°C, 50–70% RH, 16L: 8D). Once eclosed, the wasps were provided with water and honey. They had no contact with plant material or caterpillars before the initiation of the bioassays. They are referred to as naive wasps.

Regurgitant Collection. Regurgitant was collected from unparasitized and parasitized fifth instar larvae of *P. brassicae*, and from unparasitized and

parasitized fourth instar *P. rapae* larvae 24 hr before the bioassay or headspace collection. Regurgitant droplets were collected with a 5-µl glass capillary tube after gently squeezing the caterpillars with forceps. About 5–10 caterpillars of each group (parasitized and unparasitized larvae) were used to collect 50 µl regurgitant, which was immediately put in separate vials on ice. Larvae that had been exposed to oviposition by female wasps were dissected before the regurgitant was pooled to ensure that they were indeed parasitized.

Plant Treatments. For the bioassay with *P. rapae* caterpillars, 30 first instars were offered to females of *C. rubecula*. Each caterpillar was observed to be stung by a wasp, and subsequently was reared on cabbage. Another 30 unparasitized L1 caterpillars were reared on cabbage under identical circumstances. After 4 d, 15 unparasitized larvae were placed on a Brussels sprouts plant. Another 15 larvae that were supposed to be parasitized were placed on a different plant. After 48 hr, the plants were used in the bioassay to investigate parasitoid odor preference. Parasitized caterpillars were reared until the last instar to examine whether they had indeed been parasitized and to assess the degree of parasitization.

In bioassays during which caterpillar regurgitant was used, each plant was damaged on the fifth fully expanded leaf, and for headspace collection on the fifth and sixth leaves from the top by using a pattern wheel. The wheel was rolled over the leaf surface on each side of the midrib, 10 lines in parallel, and 10 lines perpendicularly to the midrib creating a ca. 8 cm^2 field with punctures being distributed over about 1/3 of the leaf surface. Per artificially damaged leaf, 25 µl of regurgitant, freshly collected from either parasitized or unparasitized caterpillars, were applied with a micropipette and distributed with a brush. Afterwards, the plants were kept in a climate room (23 ± 1°C, 50–70% RH) for 18 to 24 hr until the bioassay or headspace analysis. After the induction period, only the treated leaves were used for both behavioral bioassays and headspace analysis.

Bioassays. Parasitoid odor preference experiments were conducted in a wind tunnel set-up (25 ± 5°C, 50–70% r.h., 0.7 klux) described by Geervliet et al. (1994), with a wind speed of 0.2 m/s. For the bioassays, only the treated leaves that were of the same physiological age and size were used and excised from the induced plants just prior to the experiment. In this way, a highly standardized set of odor sources was offered to the parasitoids in a choice situation (Mattiacci et al., 1994, 1995). The petioles were inserted in vials with water, and the two leaves acting as odor sources were placed at the upwind end of the wind tunnel, ca. 14 cm apart. Experiments were carried out on 9–12 d, spread out over 2–3 mo for each treatment. For each of these days, new plants and new wasps were used. Three different plant treatments were tested in a two-choice flight experiment in the wind tunnel: (1) cabbage leaves infested with either parasitized or unparasitized second instars of *P. rapae*, (2) artificially

damaged cabbage leaves treated with regurgitant collected from either unparasitized (UNPAR) or parasitized (PAR) *P. rapae*, and (3) artificially damaged cabbage leaves treated with regurgitant from either unparasitized or parasitized *P. brassicae* caterpillars. Two to 6-d-old, naive female wasps of each species were used. Each wasp was individually released on a microscope slide with a small part of a leaf from a caterpillar-damaged cabbage plant from which first instars had been removed just prior to the bioassay. For each *Cotesia* species, damage done by the *Pieris* species under test was used. This priming served to increase the responsiveness of the wasps during the bioassay. The microscope slide was placed in the middle of the release cylinder, which was 60 cm downwind from the odor sources.

After release, the behavior of the wasp was observed. Only flights that resulted in the first landing on one of the odor sources were recorded as response. All landings on other parts of the wind tunnel besides the release cylinder or odor source were recorded as no response. If the wasp remained longer than 10 min in or on the release cylinder, it was recorded as no response and discarded. During the bioassay, plant positions were changed from left to right and vice versa after every second wasp. Females of the two wasp species were alternately tested for every treatment with a maximum number of 15 females per species and day. The two species were tested on the same day to expose them to similar conditions. On each observation day, one leaf from one plant of each treatment was tested.

Collection of Headspace Volatiles. Headspace collection was performed at 23 ± 1°C, 50–70% r.h. and approximately 10 klux; nine replicate plants were used per treatment. Volatiles from the treatments to be compared were collected on the same day to minimize variation among plant and caterpillar batches as well as day-to-day variation. Four replicates were obtained from the same batch of 10- to 12-wk-old plants within 9 d in September 2002, and five replicates were obtained from the same batch of ca. 10- to 12-wk-old plants within 11 d in April 2003. First, incoming air was led through a 5-l glass jar for 1 hr, at a flow rate of 300 ml/min to clean the system. Then, two leaves of the same plant induced by regurgitant from unparasitized *P. brassicae* caterpillars, and two leaves from a different plant induced by regurgitant of parasitized caterpillars were excised and immediately placed into separate jars with their petioles in a vial with water. They were purged for a further 1 hr. During the third hour, the headspace was collected using traps (10 cm long), packed with 200 mg Tenax TA (Markes, Pontyclun, UK; 60–80 mesh) connected to the air outlet.

GC-MS Analysis of Headspace Samples. Volatiles were desorbed from the Tenax traps using an automated thermodesorption unit (model Unity, Markes) at 200°C for 10 min (He flow 30 ml/min) and focused in an electrically cooled (−10°C) sorbent trap to allow for narrow starting bands. Volatiles were injected into the GC in splitless mode by ballistic heating of the cold trap for 3 min to

270°C. After separation by capillary gas chromatography (column: 30 m × 0.32 mm ID RTX-5 Silms, film thickness 0.33 μm; temperature program: 40–95°C at 3°C/min, then to 165°C at 2°C/min, and finally to 250°C at 15°C/min), volatiles were introduced into a Finnigan quadrupole mass spectrometer (Model Trace) operating at 70 eV in EI ionization mode. Mass spectra were recorded with full scan mode (33–300 AMU, 3 scans/sec). Compounds were identified using deconvolution software (AMDIS) in combination with Nist 98 and Wiley 7th edition spectral libraries, and by comparing their linear retention indices with those of authentic references from our own library.

Other identifications were made by comparison of mass spectra and linear retention indices (Ruther, 2000; Adams, 2001), by interpolating homologous series, and/or by using reference substances. The identified peaks were integrated by Xcalibur software (Finnigan). To minimize any interference by coeluting compounds, specific ions were selected for each compound of interest. Generally, these ions were identical with the model ions indicated by AMDIS.

Statistical Analysis. The null hypothesis (H_0) was that there is no difference in the probability of wasps flying to the UNPAR treatment or PAR treatment. The alternative hypothesis (H_1) was that the wasps used herbivore-induced plant volatiles in host-discrimination, and thus, that they preferred volatiles from the UNPAR treatment over those from the PAR treatment. The choices of the wasps in the two-choice olfactometer conform to a binomial distribution. Therefore, choices of the wasps between two odor sources within a bioassay were analyzed by using a one-sided binomial test (Siegel, 1956). Responsiveness among the choice tests between the different treatments was analyzed with 2 × 2 contingency tables using chi-square statistics.

Amounts of volatiles trapped were analyzed on the basis of peak area, as determined in the GC-MS analysis. For the headspace analyses, samples of both treatments collected on the same day were analyzed as paired data. The Wilcoxon's matched-pair signed rank test was employed to test whether the peak area per compound differed between leaves treated with regurgitant collected from parasitized and unparasitized caterpillars. A sign test was used to determine whether the number of compounds that were emitted in larger amounts differed from a 50:50 distribution over the two treatments.

RESULTS

Parasitoid Behavior. Females of *C. rubecula* landed more often on leaves infested with unparasitized larvae of *P. rapae* than on leaves infested with parasitized caterpillars (Figure 1a; $P = 0.01$). Only those combinations of leaves were taken into account for which the parasitization rate after dissection was found to be minimally 50%.

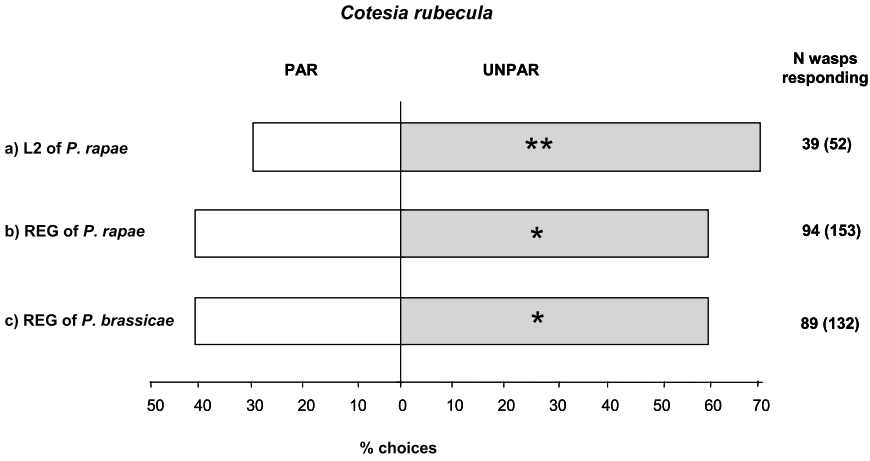

FIG. 1. Response of *C. rubecula* to cabbage leaves (a) exposed to parasitized (PAR) and unparasitized (UNPAR) second instar larvae of *P. rapae*, (b) artificially damaged and treated with regurgitant (REG) of PAR and UNPAR fourth instar *P. rapae* larvae, and (c) artificially damaged and treated with REG from PAR and UNPAR fifth instar *P. brassicae* larvae in a two-choice set-up. Asterisks indicate significant differences within a choice test: $*P < 0.05$, $**P < 0.01$ (binomial test). The bars show the percentages of wasps landing on either odor source. The numbers show the number of wasps responding; the numbers in brackets show the total number of wasps tested. All wasps were released on a leaf damaged by first instar larvae either of UNPAR *P. rapae* (a/b) or UNPAR *P. brassicae* (c).

Because the amount of leaf surface area removed from leaves fed upon by the unparasitized caterpillars was about 20% larger than from leaves fed upon by parasitized caterpillars, and the amount of *ad libitum* feeding could not be manipulated, we conducted subsequent bioassays with leaves to which a standardized amount of artificial damage and regurgitant were applied. In bioassays with regurgitant of *P. rapae* larvae, females of *C. rubecula* preferred to land on leaves treated with regurgitant of unparasitized caterpillars (Figure 1b; $P = 0.039$). Also, when leaves treated with regurgitant of *P. brassicae* were offered, *C. rubecula* females landed more often on the UNPAR treatment than on the PAR treatment (Figure 1c; $P = 0.045$).

C. glomerata wasps did not discriminate between artificially damaged cabbage leaves treated with regurgitant collected from either unparasitized or parasitized *P. rapae* larvae (Figure 2a). However, in tests using leaves treated with regurgitant of *P. brassicae* larvae, *C. glomerata* females showed a clear preference for landing on leaves treated with regurgitant of unparasitized larvae

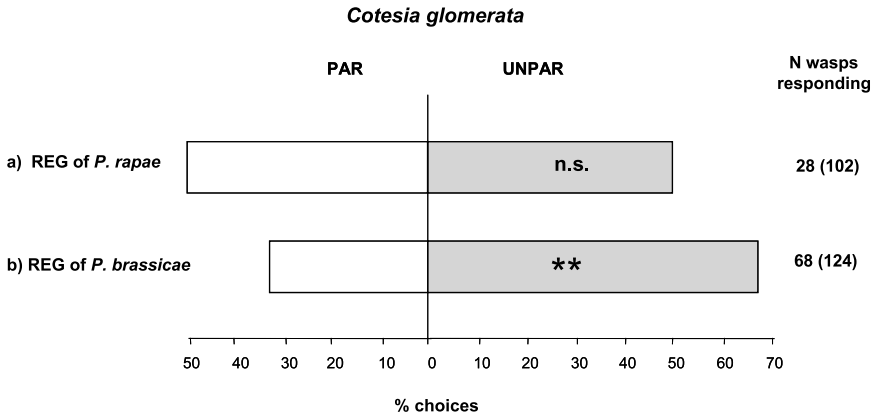

FIG. 2. Response of *C. glomerata* to artificially damaged cabbage leaves treated with regurgitant (REG) from parasitized (PAR) or unparasitized (UNPAR) *Pieris* spp. caterpillars of (a) 4th instar *P. rapae* larvae and (b) fifth instar *P. brassicae* larvae, and in a two-choice set-up. Asterisks indicate significant differences within a choice test: $^{**}P < 0.01$, n.s. not significant (binomial test). The numbers show the number of wasps responding; the numbers in brackets show the total number of wasps tested. All wasps were released on a leaf damaged by first instar larvae either of UNPAR (a) *P. brassicae* or (b) *P. rapae*.

(Figure 2b; $P = 0.002$). Additionally, the wasps' responsiveness was higher in the tests with leaves treated with *P. brassicae* regurgitant than in tests with regurgitant of *P. rapae* larvae ($P < 0.001$, $\chi^2 = 17.2$; $df = 1$).

Headspace Analysis. We focused our headspace analysis on those compounds that elicited electrophysiological responses from antennal olfactory receptors of the two *Cotesia* species (Smid et al., 2002). The plants treated with regurgitant of parasitized or unparasitized *P. brassicae* larvae emitted a volatile blend that consisted of green leaf volatiles, aldehydes, alcohols and esters, terpenes, and methyl salicylate. Averaged over nine replications, 19 out of the 22 compounds were emitted in larger amounts in the UNPAR treatment than in the PAR treatment ($P < 0.001$, sign test). In addition, higher amounts of the major terpenoids limonene ($P = 0.05$), α-thujene ($P = 0.04$), and 1,8-cineole ($P = 0.01$; two-sided Wilcoxon's matched pair signed rank test) were released in the UNPAR treatment (Figure 3). Thus, the quantitative profile of the emitted volatiles changes, and three volatiles are emitted in significantly lower amounts when cabbage plants are induced by regurgitant of parasitized *P. brassicae* compared to cabbage plants induced by regurgitant of unparasitized *P. brassicae*.

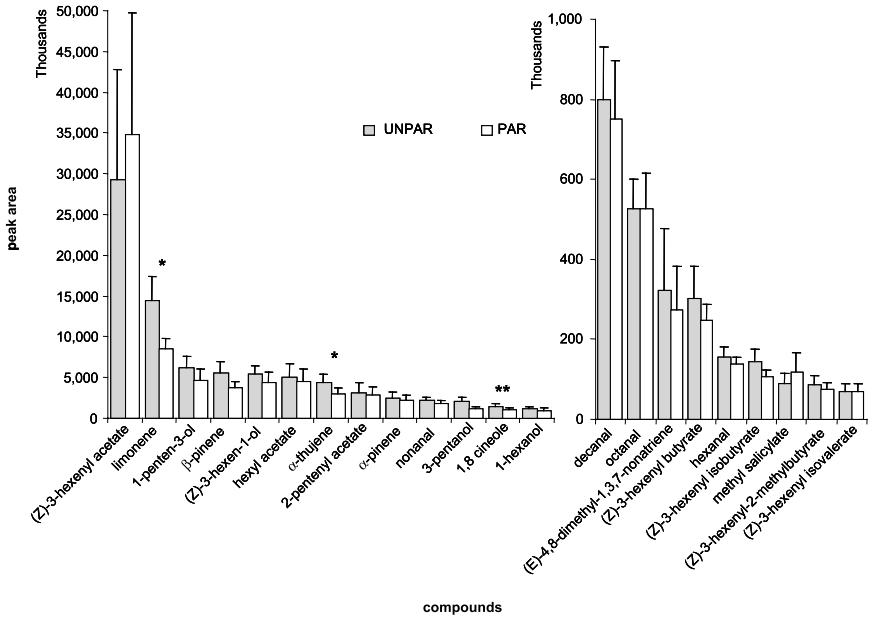

FIG. 3. Comparison of the volatile blends emitted by cabbage plants artificially damaged and treated with regurgitant of unparasitized (UNPAR) or parasitized (PAR) caterpillars of *P. brassicae* for 22 compounds expressed as mean peak area (in arbitrary units) and the standard error. For each compound, the mean of nine peak areas of the UNPAR treatments and nine peak areas of the PAR treatments are shown. Asterisks indicate significant differences: $*P < 0.05$, $**P < 0.01$ (Wilcoxon's matched pairs signed rank test).

DISCUSSION

The results show that *C. rubecula* females without oviposition experience discriminated suitable hosts, i.e., hosts that were not parasitized, from parasitized hosts in flight by using host-induced plant volatiles. Females of *C. glomerata* discriminated between host-induced plant volatiles induced by unparasitized and parasitized larvae in the case of their preferred host *P. brassicae*. As a result, the wasps save time compared to wasps that first have to land and explore a patch to detect nonvolatile marking substances left by previous parasitoid visitors. Additionally, *C. glomerata* females reduce the risk of being bitten or coming in contact with the regurgitant of *P. brassicae* caterpillars. *P. brassicae* caterpillars, unlike *P. rapae* caterpillars, vigilantly defend themselves and can seriously harm their parasitoids (Brodeur et al., 1996).

Especially for a solitary endoparasitoid such as *C. rubecula*, host discrimination is crucial because supernumerary larvae compete to the extent that only a single parasitoid can emerge (Salt, 1961). The older larva usually eliminates the younger. The extent of the disadvantage depends critically on the time that elapses between the two ovipositions. Thus, in cases with a limited number of hosts present, parasitoids should be more apt to attack recently parasitized hosts in which their larvae have a higher probability to survive. Internal markers released by the egg or physiological changes in the host caused by parasitism become more noticeable with time (van Lenteren, 1976; Godfray, 1994 and references therein).

In gregarious parasitoids such as *C. glomerata*, scramble competition may occur, i.e., no aggressive behavior toward other parasitoid larvae or physiological suppression occurs, and the parasitoids develop as long as food is available. Superparasitism can be an adaptive strategy under some conditions, such as when wasps are faced with a limited number of hosts for a longer period. However, an increase in clutch size is often associated with a decrease in size of surviving offspring and a reduced fitness of the emerging adults (e.g., Harvey et al., 1993; Potting et al., 1997).

Naive females of *C. glomerata* discriminate between parasitized and unparasitized hosts in close-range tests (Ikawa and Suzuki, 1982; Le Masurier, 1990). Experienced *C. glomerata* wasps regulate the number of eggs laid in response to the number of available suitable hosts (Ikawa and Suzuki, 1982). We used females experienced with a particular plant–host complex (as described before) to assess whether they discriminated in flight. Therefore, it remains unclear if the response was innate or triggered by the short odor experience just prior to the release in the wind tunnel. Other studies have shown that a single experience with a plant–host complex has no influence on the behavior of either *C. glomerata* or *C. rubecula* toward the offered plant–host complexes in the wind tunnel (Geervliet et al., 1998). However, in our case, the brief experience resulted in a higher response of *C. glomerata* that flew to one of the odor sources in tests with plants treated with regurgitant of *P. brassicae.*

When we offered leaves induced with regurgitant of *P. rapae*, a high proportion of females of our Dutch strain of *C. glomerata* were unresponsive, and those that did fly toward the plants did not discriminate. In the Netherlands, *C. glomerata* specializes on *P. brassicae*, which has been ascribed to the fact that *C. rubecula* outcompetes *C. glomerata* in *P. rapae* larvae (Geervliet et al., 2000). The solitary endoparasitoid *C. rubecula* always wins the competition for the same host against the gregarious *C. glomerata* (Laing and Corrigan, 1987). Our results suggest that the priming of *C. glomerata* wasps with leaf damage of first instar *P. rapae* caterpillars that we practiced just prior to the bioassay induced a low response and a failure to discriminate between plants treated with regurgitant of unparasitized or parasitized *P. rapae* larvae.

To the best of our knowledge, this is the first report in which elicitation of herbivore-induced plant volatile production, by controlling the amount of mechanical leaf damage combined with the application of regurgitant, has been shown to result in a reduced emission of specific plant volatiles when elicitors originate from parasitized caterpillars. Souissi et al. (1998) reported that unparasitized mealybug-infested plants or unparasitized mealybugs are more attractive to walking *Apoanagyrus lopezi* (Hymenoptera: Encyrtidae) endoparasitoids than plants infested with parasitized mealybugs or parasitized mealybugs alone. The amount of herbivore feeding was not controlled in their study, and it was not checked whether the plant was the source of the attractants. Sheehan et al. (1993) showed that females of *Microplitis croceipes* (Hymenoptera: Braconidae) were able to discriminate in flight between previously visited and unvisited sites in a plant patch, even in the absence of the host. In this case, herbivore-induced plant volatiles were not tested, and it was assumed that a chemical marking deposited on the substrate served as the cue for discrimination.

Presently, it is still unknown for any parasitoid species exactly which compounds are involved as synomones (Dicke and van Loon, 2000). Previous work on volatiles emitted by a different Brussels sprouts cultivar (Blaakmeer et al., 1994) showed differences between intact and caterpillar-damaged plants for hexyl acetate, (*Z*)-3-hexenyl acetate, sabinene, and 1,8-cineole. These compounds were also increased in the headspace of plants treated with regurgitant of unparasitized *P. brassicae* caterpillars compared to treatments with regurgitant of parasitized *P. brassicae* caterpillars. The headspace composition documented here also overlaps with that described by Smid et al. (2002), who studied yet another Brussels sprouts cultivar and used feeding first instar caterpillars during headspace collection. An obvious difference is that benzyl cyanide, a breakdown product of glucosinolates (the taxonomically characteristic secondary plant metabolites of Brassicaceous plants), is lacking in our samples. It was recently established (Wittstock et al., 2004) that this compound is released from the feces of *Pieris* caterpillars, which explains its absence from our samples. Limonene elicits an olfactory response in the antennae of both *Cotesia* species (Smid et al., 2002). The fact that the reduced emission of three volatiles correlated with discriminatory behavior that were shown to elicit electrophysiological activity suggests that the quantity of these compounds, possibly relative to those of other compounds, provides information to the parasitoids. We used leaves that were detached just prior to the experiments. For maize, it has been shown that excision of leaves may influence the emission rate of certain components in some treatments at some periods of the day, without affecting the overall effect of treatment (Schmelz et al., 2001). In our experiments, the treatments were maximally standardized with regard to leaf physiological age and size, and leaves from both treatments were excised to investigate locally emitted volatiles only.

Studies on the interrelationship of *Cotesia* larvae with their hosts show drastic effects of parasitism on the caterpillars' physiology such as changes in hemolymph protein titer and composition (reviewed by Beckage, 1993). Application of β-glucosidase, a component of *P. brassicae* regurgitant, to artificially damaged plants resulted in the release of a volatile blend similar to that of leaves treated with regurgitant (Mattiacci et al., 1995). Quantitative differences in headspace composition resulting from treating plants with regurgitant obtained from either parasitized or unparasitized caterpillars might be attributable to a reduced amount of β-glucosidase in regurgitant of parasitized *P. brassicae*.

Parasitization of *P. rapae* by *C. rubecula* has been shown to increase fitness of the brassicaceous plant *Arabidopsis thaliana* (van Loon et al., 2000). In the case of *C. glomerata*, which preferentially parasitizes the gregariously feeding *P. brassicae*, only a suppression of specific synomones, as documented here, would benefit the wasp, because for this parasitoid–host combination an overall reduction in the amount of volatiles released could signal a lower but still sufficient number of available unparasitized hosts rather than parasitized hosts. As we controlled the amount of mechanical damage and the volume of elicitor-containing regurgitant, we can exclude the involvement of other stress factors, such as viral, bacterial, or fungal pathogens, that might have interfered with the feeding performance of the caterpillars and consequently with induced synomone emission by the plants.

The induced volatiles bear biosynthetic and ecological costs (Dicke and Sabelis, 1989; Dicke and van Loon, 2000; Hoballah et al., 2004). Many of the compounds are produced through *de novo* biosynthesis (Paré and Tumlinson, 1997). Ecological costs in terms of attraction of herbivores can be much higher than the biosynthetic costs (Dicke and Vet, 1999). Thus, downregulation of induced volatile production is likely to be advantageous for plants.

In summary, we have shown that parasitoids can use herbivore-induced plant volatiles for host discrimination in flight, a crucial ability to enhance their reproductive success. Besides the fact that plant synomones are reliable and detectable indicators of the presence and identity of herbivores, we document that parasitoids can use them as a source of information on host suitability. Consequently, parasitoids can save energy and time in finding suitable hosts. From the plant's perspective, our study has uncovered an advantage through a reduced production of a subset of herbivore-induced volatiles, after having successfully recruited their bodyguards.

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CHARACTERIZATION OF CONDENSED TANNINS PURIFIED FROM LEGUME FORAGES: CHROMOPHORE PRODUCTION, PROTEIN PRECIPITATION, AND INHIBITORY EFFECTS ON CELLULOSE DIGESTION

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Abstract—To identify simple screening tools for selecting condensed tannin (CT)-containing forages as candidate sources for further study, CT were isolated from nine legumes, and their molecular weights (MW), chromophore production, capacity to precipitate bovine serum albumin (BSA) and Fraction 1 protein (Rubisco) isolated from alfalfa, and inhibition of filter paper digestion were compared. Sources were as follows: leaves of sericea lespedeza (*Lespedeza cuneata* Dum.-Cours.), crown vetch (*Coronilla varia* L.), and sainfoin (*Onobrychis viciifolia* Scop.); stems of hedysarum (*Hedysarum alpinum* L.); seeds of alfalfa (*Medicago sativa* L.); and whole plants of birdsfoot trefoil (*Lotus corniculatus* var. *corniculatus* L.) and three varieties of big trefoil (*Lotus pedunculatus* Cav.), viz., *Lotus uliginosus* Schkuhr, *L. uliginosus* var. *glabriusculus*, and *L. uliginosus* var. *villosus*. Molecular weights and sizes (degrees of polymerization) of the CT varied considerably within and among plant species. Average MW ranged from 3036 Da (crown vetch) to 7143 Da (lespedeza). All CT exhibited greater capacity (w/w basis) to bind alfalfa Rubisco than BSA. Relative astringencies (μg CT required to precipitate 1 mg protein) against BSA ranged from 262.5 for CT from lespedeza to 435.5 for CT from *L. corniculatus*, and against Rubisco,

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from 49.6 (sainfoin) to 108.2 (alfalfa seed). Including CT at 300 μg/ml in cultures of *Fibrobacter succinogenes* reduced digestion of cellulose filter paper by 19.8% (sainfoin) to 92.4% (crown vetch) and increased the specific activity of cell-associated endoglucanase. There were no correlations between inhibitory effects of CT on filter paper digestion and (1) chromophore formation during CT assay by butanol–HCl, vanillin–HCl, or H_2SO_4; (2) precipitation of BSA or alfalfa Rubisco; and (3) MW of CT. The most inhibitory CT for cellulose digestion included those with broad and with narrow MW distributions. Sainfoin was the most desirable source of CT, as it had the highest capacity to bind alfalfa protein and was least inhibitory to cellulose digestion by *F. succinogenes*. This study suggests that these properties are not easily defined via chemical means, and that biological assays using rumen bacteria may help identify those CT with properties of nutritional interest.

Key Words—Condensed tannins, endoglucanase activity, cellulose digestion, legume, ruminant nutrition, *Fibrobacter succinogenes*, ribulose 1,5-bisphosphate carboxylase, Rubisco.

INTRODUCTION

Condensed tannins (CT) in forages are considered antinutritive factors for ruminants, owing to their capacity for binding to macromolecules such as fiber and proteins. At concentrations exceeding 6% of dietary dry matter (DM), CT depress feed intake, reduce the digestibilities of fiber and protein, and decrease the growth rate of ruminant livestock (Leinmuller et al., 1991). Although formation of CT–plant protein complexes occurs at CT concentrations <4% of dietary DM, feed intake is seldom affected (Barry et al., 1986). More recently, forages containing CT have been studied as potentially beneficial agents in ruminant nutrition (Waghorn, 1990; Butter et al., 1999). The reversible and pH-dependent nature of tannin–protein interactions (Perez-Maldonado et al., 1995) enhances the resistance of CT-bound protein to microbial degradation in the rumen, yet it enables dissociation of the complexes in the abomasum, thereby increasing the proportion of plant protein arriving at the small intestine (Barry and Manley, 1984; Mangan, 1988). Moreover, CT act in the rumen to prevent formation of the stable froth (Tanner et al., 1995) that is a prerequisite for development of pasture bloat. Bloat in cattle grazing alfalfa can be prevented by providing CT at 1.7% of dietary DM (Waghorn and Jones, 1989).

The effects of CT on nutrient utilization by ruminants are apparently dependent on the tannin type. For example, the growth rates of sheep fed with *Acacia cyanophylla* or *A. sieberiana* were less than half of those exhibited by sheep consuming *A. seyal*, although the CT concentrations were similar in all three forages (Reed and Soller, 1987). Presumably, these disparate effects arise from variations in the number of reactive phenolic groups present in CT and the

consequent variability in their capacity to bind carbohydrates and proteins (Asquith and Butler, 1986; Hagerman, 1992).

These observations of positive effects of CT on digestion in ruminants have led researchers to propose that pathways for their synthesis be genetically engineered into legume species prone to provoking bloat (Waghorn, 1990; Morris and Robbins, 1997). Before such projects are undertaken, however, it is imperative to identify the properties of CT responsible for these favorable changes in ruminal metabolism. The present study was conducted to isolate and analyze the CT from nine legumes with the objective of determining if correlations exist among their molecular weights, their capacities to precipitate protein, and their inhibitory effects on fiber digestion by the major cellulolytic ruminal bacterium, *Fibrobacter succinogenes*. It was hypothesized that fiber digestion by *F. succinogenes* may serve as a simple bioassay against which to assess the relative inhibitory effects of various sources of CT.

METHODS AND MATERIALS

Sources and Isolation of Condensed Tannins. Condensed tannins were isolated from whole plants of a high tannin cultivar of birdsfoot trefoil, *Lotus corniculatus*, and three varieties of big trefoil, *L. uliginosus*, *L. uliginosus* var. *glabriusculus*, and *L. uliginosus* var. *villosus*; from the leaves of high-tannin sericea lespedeza, *Lespedeza cuneata*, crown vetch, *Coronilla varia*, and sainfoin, *Onobrychis viciifolia*; from the stems of hedysarum, *Hedysarum alpinum*; and from the seeds of alfalfa, *Medicago sativa*. Vegetative plant material was harvested from experimental plots at the Agriculture and Agri-Food Canada Research Centre in Saskatoon, SK, Canada, and alfalfa seed was obtained from a commercial seed producer (Pickseed Canada Inc., Lindsay, ON, Canada).

Condensed tannins were extracted from plant material with aqueous acetone/petroleum ether/ethyl acetate as described by Bae et al. (1993b) and eluted from a 15 × 4 cm i.d. glass column packed with Sephadex LH-20 (Sigma Chemical Co., St. Louis, MO, USA), which excluded low-molecular-weight, non-CT polyphenols from the isolated material. The CT were eluted with 70% aqueous acetone, evaporated to the aqueous phase, lyophilized, and stored at 4°C in a dessicator held in the dark.

Determination of Molecular Weights of Condensed Tannins. Molecular weights of the extracted CT were determined by gel permeation chromatography (GPC) conducted on their peracetate derivatives. The peracetate derivatives were prepared by dissolving the CT in dry pyridine/acetic anhydride (1:1) and recovering the peracetates by filtration after dilution with water as described by Williams et al. (1983). An Ultrastyragel linear (7.8 × 300 mm) and a 10^3-Å

column (Waters, Milford, MA, USA) connected in series were used for GPC, with tetrahydrofuran at 1.1 ml/min (25°C) as the solvent. Relative molecular weights (M_r) were calculated after calibration with polystyrene M_r standards (M_r 687, 2000, 4136, 9000, and 32,660) and phloroglucinol (M_r = 252) and catechin peracetates (M_r = 500, 1000, and 1500 for the monomer, dimer, and trimer, respectively). The degrees of polymerization (DP) of the extracted CT were estimated on the basis of a constituent PA peracetate unit MW of approximately 500 (Williams et al., 1983).

Chromophore Production During Condensed Tannin Assays. The CT isolated from the nine plant sources were assayed in triplicate using four methods: butanol–HCl, vanillin–HCl, Prussian blue, and H_2SO_4 (Bae et al., 1993a). In each case, equal weights of CT were reacted, and results were expressed as a percentage of the value obtained for the sainfoin standard. Subsequently, the H_2SO_4 assay (Bae et al., 1993a) was used to generate calibration curves for each of the nine CT to enable comparison of chromophore production. Briefly, 2 ml of concentrated H_2SO_4 was added to triplicate 0.1-ml aliquots of aqueous stock solutions of each CT (containing 0–20 μg tannin), and absorbance at 580 nm was recorded after 1 hr of incubation at room temperature.

Isolation and Purification of Alfalfa Protein. Soluble forage protein was isolated and purified from leaves of alfalfa, *M. sativa*, by a method based on the procedure outlined by Jones and Mangan (1976). All steps of protein purification were carried out at 4°C to minimize proteolysis. The top 15 cm of herbage was cut from prebloom alfalfa in the afternoon, bagged, and allowed to wilt overnight (to improve protein yield; Mangan, personal communication). The next morning, leaves were stripped from the stems, and 100-g lots were each combined with 10 ml of extraction buffer prepared at 10-fold concentration (0.01 M sodium diethyldithiocarbamate, 0.01 M β-mercaptoethanol, and 10 g/l sodium ascorbate in 0.5 M phosphate buffer, pH 7.4), and the mixture was processed without heating using a commercial centrifugal juice extractor. The juice (plant extract) was combined with 300 ml of single strength extraction buffer (EB; 0.001 M sodium diethyldithiocarbamate, 0.001 M β-mercaptoethanol, and 1 g/l sodium ascorbate in 0.05 M phosphate buffer, pH 7.4). The residues were resuspended in 500 ml of EB, squeezed gently through two layers of Nitex HC3 cloth (53-μm pore size), and the expressed liquid was added to the plant extract.

Soluble protein in the extract was precipitated using 360 g/l $(NH_4)_2SO_4$ (pH 6.2) and collected by centrifugation (500 × *g*, 10 min, 5°C). Precipitated protein was redissolved in EB and purified by gel filtration on two columns of Sephadex G-75 (26 mm × 70 cm; 50 mm × 60 cm; GE Healthcare, formerly Amersham Biosciences, Baie d'Urfe, QC, Canada) and precipitated once again with $(NH_4)_2SO_4$ as described above. The centrifugal pellet was dissolved in EB

and dialyzed (12–14 kDa cutoff) three times in 40 hr against 0.1 g/l sodium azide in distilled water, then twice against distilled water for a further 7 hr to remove residual sodium azide. The tubes were frozen at −40°C, lyophilized, and dried protein was stored in a dessicator at 4°C.

Characterization of Purified Fraction 1 Protein. The UV absorbances of a microfiltered (0.2-μm cellulose acetate filter, Nalgene, Rochester, NY, USA) solution of 2 mg of lyophilized material in 5 ml of 0.05 M phosphate buffer (pH 7.4) were measured at 260 and 280 nm, and protein concentration (mg/ml) was calculated as $1.45A_{280} - 0.74A_{260}$ (Daniels et al., 1994). Soluble protein content of the Fraction 1 material was also determined by the Bradford (1976) microassay using BSA as the standard protein. Purified protein (in a 2-mg/ml solution) was further characterized by SDS-PAGE (Laemmli, 1970) conducted on a Mini-Protean II electrophoresis cell (BioRad Laboratories, Richmond, CA, USA) with 40 g/l acrylamide stacking gels and 120 g/l acrylamide separating gels. Molecular weight standards (10–100 kDa) and ribulose 1,5-bisphosphate carboxylase (Rubisco) EC 4.1.1.39 (Sigma Chemical Co.) were included in each gel.

Precipitation of Proteins by Condensed Tannins. The protein-precipitating capacities of CT from the nine forages were determined using a modification of the method of Martin and Martin (1982). Bovine serum albumin (BSA) and Rubisco from alfalfa were used as model proteins for determining the relative capacities of the extracted CT to bind protein. The BSA was dissolved (2 mg/ml) in 0.2 M acetate buffer (pH 5.0) containing 0.17 M NaCl, and the purified Rubisco was dissolved (2 mg/ml) in 0.1 M 2-[*N*-morpholino]-ethanesulfonic acid (pH 6.1) containing 0.17 M NaCl. One milliliter of either solution was combined with 0.5 ml of aqueous solutions containing 0, 100, 200, 300, 400, or 500 μg of CT from each source, for determinations with BSA, and 0, 20, 40, 60, 80, or 100 μg for determinations with alfalfa Rubisco. Each mixture was vortexed, allowed to stand at room temperature for 30 min, then centrifuged (15,600 × *g*, 5 min). The CT remaining in a 0.5-ml subsample of supernatant were removed by precipitation, accomplished by adding 0.5 ml of 10 mg/ml (aqueous) polyethylene glycol 6000 (Jones and Mangan, 1977). Protein remaining in solution was quantified colorimetrically using a commercial protein assay kit based on the Bradford (1976) assay (BioRad Laboratories, Mississauga, ON, Canada) against a freshly prepared solution of BSA or Fraction 1 protein as a standard. The assay was replicated six times for each dose of CT from each of the nine plant sources.

Effects of Condensed Tannins on Cellulose Digestion. The nine CT were each assessed for their effects on the cellulose-digesting activity of the ruminal bacterium, *F. succinogenes* subsp. *succinogenes* S85 (obtained from the Lethbridge Research Centre culture collection). The bacterium was grown in 10-ml volumes of the liquid medium of Scott and Dehority (1965), modified

by omitting the casein hydrolysate and soluble carbohydrates and adding autoclaved, clarified ruminal fluid (100 ml/l). A 1-cm^2 piece of Whatman No. 1 cellulose filter paper (8.8 ± 0.5 mg) was included in each tube as the sole source of carbohydrate during culture maintenance. Cultures were incubated at 39°C and transferred at 3-d intervals for 15 d prior to experimentation with CT. The anaerobic technique of Hungate (1950), as modified by Bryant and Burkey (1953), was used throughout the experiment.

For assay of the effects of the CT on the cellulose-digesting activity of *F. succinogenes*, each CT was dissolved in anaerobic salt solution (Bryant and Burkey, 1953) and filter sterilized, also under anaerobic conditions. Cells of *F. succinogenes* in 72-hr cultures were harvested by centrifugation (10,000 × *g*; 20 min; 4°C), and the pellets were resuspended in 1 ml of fresh medium. Triplicate tubes containing a preweighed piece of Whatman No. 1 filter paper (approximately 25 mg) in 4 ml of medium were inoculated with 0.1 ml of bacterial suspension and CT solution to yield 0 (control) or 300 μg tannin/ml and incubated at 39°C.

After 3 d of incubation, filter paper residues in the cultures were collected by vacuum filtration onto preweighed Whatman No. 54 filters. Culture filtrates were kept on ice, whereas residues were washed three times with 1 ml of 0.05 M sodium phosphate buffer (PB) at pH 6.5 and once with 1 ml of methanol (washings were discarded). Filter paper residues were dried at 80°C for 24 hr, and then weighed for estimation of extent of digestion (as mg digested/g incubated).

Collected filtrates (1 ml) were centrifuged (15,600 × *g*; 5 min; 4°C) to segregate cell-associated (pellet) and cell-free (supernatant) fractions. Supernatants were kept on ice. Pelleted cells were washed twice by resuspension in 1 ml of PB followed by centrifugation, then were suspended in PB at 4°C and disrupted by 30 sec of pulsed sonication with a Sonic 300 Dismembrator (Artek Systems Corporation, Farmingdale, NY, USA) set at 60% output. Crude cell extract was obtained by centrifuging the disrupted bacteria to remove cell debris.

Cellulase activity in the extracellular fluids and crude cell extracts was determined using the soluble chromogenic substrate ostazin brilliant red (OBR)–hydroxyethylcellulose (Biely et al., 1985) as described by McAllister et al. (1994). Endoglucanase activity was expressed against a commercial cellulase from *Penicillium funiculosum* (EC 3.2.1.4, Sigma Chemical Co.), with one unit of activity defined as the absorbance given by one unit of commercial cellulase per hour. Protein in a crude cell extract was also determined by the method of Bradford (1976) and used for calculation of specific activity of endoglucanase (cellulase).

Statistical Analyses. Data from the protein precipitation titration curves were analyzed using a nonlinear regression procedure (SAS, 1989). Specifically, the curves for each CT were fitted to the Weibull cumulative function for

sigmoid curves: $Y = \mathrm{P1} + \mathrm{P2}\left(1 - e^{-(X/\mathrm{P3})^{\mathrm{P4}}}\right)$ where Y = μg of protein (BSA or Fraction 1) precipitated, X = μg of extracted CT incubated, P1 = lower asymptote (set to 0), P2 = upper asymptote (μg of protein), P3 determines the range of values of X over which the sigmoid curve occurs (1/μg CT), and P4 is the distortion of the curve (Régnière and Beilhartz, 1987). The protein-precipitating capacity of each of the CT was expressed as the amount (μg) of the CT required to precipitate 1 mg of BSA or Fraction 1 protein. Least squares means of the capacities (N = 6) were compared using the PDIFF option of the GLM procedure of SAS (1989). Simple correlations among CT characteristics [mean MW, chromophore production, inhibition of cellulolytic activities (cell-associated and extracellular endoglucanase, and digestion of filter paper by *F. succinogenes*), and protein-precipitating capacities against BSA and Rubisco] were determined.

RESULTS AND DISCUSSION

Condensed Tannin Molecular Weights and Chromophore Production. Molecular weights and degrees of polymerization of the CT from nine sources varied considerably among and within the plants tested (Table 1). On average, CT from *C. varia* (crown vetch) were smallest (M_r = 3036), and those from *L. cuneata* (lespedeza) were largest (M_r = 7143). This inherent variability in CT even within an individual plant sample adds to the difficulty in attributing specific properties to a given polymer source. Other workers have reported MW of CT extracted from leaves (Foo et al., 1982, 1996; Koupai-Abyazani et al., 1993) or whole plants (Jones et al., 1976) from some of the legume species that were examined in leaves or whole plant extracts in the present study (Table 2). Our M_r values for *C. varia* were close to those previously reported, but our estimates of M_r for *Lotus* spp. and *O. viciifolia* were notably higher than those previously reported using GPC. Hedqvist et al. (2000) reported that there was substantial variation in the quantities of high molecular weight tannins in varieties of *L. corniculatus*, and it is possible that such variation in CT in other legume species may contribute to the higher values reported in our study. It is also possible that M_r varies with the portion of the plant from which the CT are isolated or the maturity of the plant at time of harvest.

Similar to MW determinations, variations in chromophore yield among the CT, as determined by butanol–HCl, vanillin–HCl, and H_2SO_4 methods, were also observed (Table 3). The colorimetric assays were each standardized against the production of chromophore by *O. viciifolia* CT. The consistency of ranking of chromophore yield of the sources of CT relative to *O. viciifolia* in the three assays was dependent on the source of CT. For example, *C. varia* exhibited the same rank regardless of the assay employed, whereas the

TABLE 1. MOLECULAR WEIGHT (MW) AND POLYMERIZATION DATA FOR CONDENSED TANNINS ISOLATED FROM NINE FORAGE SOURCES

Source of tannin	MW at max. abs. (Da)	DP[a]	Range of MW (Da)[b]	Range of DP	Peak width[c]
Coronilla varia	3036	6	657–12,775	1–26	12.5
Hedysarum alpinum	3789	8	828–23,071	2–46	22.0
Lespedeza cuneata	7143	14	2003–31,550	4–63	29.5
Lotus corniculatus	4424	9	1825–30,742	4–61	28.5
Lotus uliginosus	3281	7	932–23,172	2–46	22.0
L. uliginosus var. *glabriusculus*	3281	7	828–22,263	2–45	21.5
L. uliginosus var. *villosus*	3929	8	1557–25,695	3–51	24.0
Medicago sativa	3616	7	671–12,096	1–24	11.5
Onobrychis viciifolia	5077	10	2045–25,090	4–50	23.0

[a] Values represent degree of polymerization (DP) rounded to the nearest whole number, estimated as MW/500, on the basis of the MW of a proanthocyanidin peracetate unit of approximately 500 (Williams et al., 1983).
[b] Calculated as the MW at half-peak height on the leading and trailing edges of the peak.
[c] Calculated as $(DP_{max} - DP_{min})/2$. In this calculation, lower numbers represent narrower peaks.

TABLE 2. PUBLISHED VALUES OF MOLECULAR WEIGHTS (MW) OF CONDENSED TANNINS ISOLATED FROM LEAF TISSUE FROM FOUR FORAGE SOURCES AND DETERMINED BY DIFFERENT METHODS OF ANALYSIS

Plant species	Method of analysis[a]	Plant tissue	MW (kDa)	Reference
C. varia	GPC	Leaf	3.3	Foo et al. (1982)
	NMR	Leaf	3.6	Foo et al. (1982)
	Ultracentrifuge	Whole plant	10.1–13.2[b]	Jones et al. (1976)
L. corniculatus	^{13}C-NMR	Leaf	1.8–2.1	Foo et al. (1996)
	GPC	Leaf	2.0	Foo et al. (1982)
	NMR	Leaf	2.8	Foo et al. (1982)
L. pedunculatus	GPC	Leaf	2.9	Foo et al. (1982)
	NMR	Leaf	2.5	Foo et al. (1982)
	Ultracentrifuge	Whole plant	6.8–7.1[b]	Jones et al. (1976)
O. viciifolia	GPC	Leaf	3.3	Foo et al. (1982)
	GPC	Leaf	2.5	Koupai-Abyazani et al. (1993)
	^{1}H-NMR	Leaf	2.4	Koupai-Abyazani et al. (1993)
	HPLC	Leaf	1.8–2.1	Koupai-Abyazani et al. (1993)
	NMR	Leaf	2.9	Foo et al. (1982)
	Ultracentrifuge	Whole plant	17.0–28.1[b]	Jones et al. (1976)

[a] GPC: Gel permeation chromatography; NMR: nuclear magnetic resonance spectroscopy; HPLC: high-pressure liquid chromatography; Ultracentrifuge: ultracentrifugation technique.
[b] Analysis by ultracentrifugation yields measurement of M_z, which is proposed by Foo et al. (1982) to represent approximately three times M_n which is established by the other techniques, e.g., for *L. pedunculatus*, a MW of approximately 2.3 kDa is implied.

TABLE 3. CHROMOPHORE YIELD FROM ISOLATED CONDENSED TANNINS (CT) FROM THREE METHODS OF ANALYSIS

	Method of analysis					
	Butanol–HCl		Vanillin–HCl		H_2SO_4	
Condensed tannin source	Yield[a]	Rank	Yield	Rank	Yield	Rank
O. viciifolia	100	–	100	–	100	–
C. varia	99.1	2	103.9	2	137.5	2
H. alpinum	80.4	8	72.5	5	74.4	7
L. cuneata	101.1	1	77.4	3	84.4	6
L. corniculatus	81.0	6	69.5	7	85.3	5
L. uliginosus	93.2	3	71.7	6	126.5	3
L. uliginosus var. *glabriusculus*	90.6	4	64.3	8	89.0	4
L. uliginosus var. *villosus*	81.0	6	74.4	4	74.2	8
M. sativa	88.7	5	105.4	1	161.7	1

[a] Expressed as a percentage of the yield given by sainfoin, *O. viciifolia*. Equal weights of isolated polymer were reacted in each case. Mean values are presented ($N = 3$).

chromophore yield of *M. sativa* ranked first relative to *O. viciifolia* for the vanillin–HCl and H_2SO_4 assays but fifth in the butanol–HCl procedure. Differences in chromophore yield are a reflection of differences in CT structural properties and the specificity of the chemical reactions that result in the formation of the chromophore in each of the colorimetric procedures employed (Hagerman and Butler, 1989). None of the colorimetric procedures exhibited a strong correlation with the degree to which CT inhibited filter paper digestion by *F. succinogenes* (see below).

Characterization of Fraction 1 Protein (Rubisco). Yield of Fraction 1 protein from alfalfa was approximately 6.7 g/kg wilted weight of herbage. The protein content of a 0.4-mg/ml solution of purified Fraction 1 protein, as determined by UV absorption, ranged from 0.38 to 0.51 mg/ml (mean 0.44 mg/ml) for the five lots of purified Fraction 1 protein. The soluble protein content of the five lots ranged from 649 to 823 mg/g protein (mean 742 mg/g protein), as assayed by the Bradford (1976) method against a BSA standard. Characterization of the Fraction 1 protein by SDS-PAGE revealed two predominant bands, with MW of 55 and 16 kDa. These two bands match the subunits of ribulose-1,5-bisphosphate carboxylase (Rubisco; EC 4.1.1.39).

Whereas Rubisco is the most abundant protein in the world and constitutes the predominant protein in animal forage diets (Mangan, 1982), BSA is likely the most frequently used model protein in tannin–protein assays. Bovine serum albumin is a globular protein tightly cross-linked with disulfide bridges that confer a relatively higher resistance to proteolysis by ruminal microorganisms, compared to casein and Fraction 1 leaf protein (Nugent et al., 1983). In contrast,

Rubisco consists of 16 subunits of two sizes held together by hydrogen bonds, and the subunits possess linear structures lacking disulfide bridges. The isoelectric points of BSA and Rubisco from white clover are pH 4.9 and 5.5, respectively (Perez-Maldonado et al., 1995). Condensed tannins, in general, bind preferentially to hydrophobic and/or proline-rich regions of protein molecules (Hagerman and Butler, 1981) by noncovalent interactions, primarily hydrogen bonds and hydrophobic interactions. Thus, the primary and tertiary structures and isoelectric point will each affect a protein's solubility and tendency to bind with CT.

Precipitation of Proteins by Condensed Tannins. The CT studied in this experiment exhibited greater capacity to bind Rubisco than BSA, on a weight-to-weight basis (Table 4). This is consistent with a report by Martin and Martin (1983), in which tannic acid (a hydrolyzable tannin), quebracho tannin, and oak tannin all precipitated commercially prepared Rubisco to a greater extent than BSA. In that study, 203, 521, and 2801 mg of tannic acid, quebracho CT, and pin oak CT, respectively, were required to precipitate 1 g of BSA (at pH 4.1), compared with 48, 250, and 498 mg for Fraction 1 protein (at pH 6.1). Comparable values were recorded in the present experiment (Table 4) in which protein-precipitating capacities were assayed at pH of approximately 4.9 for BSA and pH 6.1 for Rubisco. In our study, relative protein-precipitating capacities (μg CT per mg protein) against BSA ranged from 262.5 for CT from lespedeza to 435.5 for CT from *L. corniculatus*, and against Rubisco from 49.6 (sainfoin) to 108.2 (alfalfa seed). Thus, both studies illustrate the variation in protein-precipitating capacities among tannins from different sources. The nine CT examined in the present study also differed in the ranking of their protein-precipitating capacities between the two model proteins, indicating that the propensity of a CT and a protein to bind depends on chemical and physical properties of both the target protein and the source of CT.

The number of potential hydrogen bonding sites on both the protein and the CT molecules will determine the extent of their capacity to bind one another. This is in turn influenced by the pH and presence of ions in the solvent in which they are suspended (Perez-Maldonado et al., 1995). The number of potential hydrogen bonding sites on a given molecule of CT is a function of the number and type of monomeric subunits (e.g., flavan-3-ols, flavan-4-ols, or flavan-3,4-diols), their stereochemistry, and the carbon–carbon linkages between them that create linear and/or branching polymers. For example, *L. corniculatus* CT consist of relatively homogeneous polymers of epicatechin-type procyanidin units, whereas *L. pedunculatus* CT contain a more heterogeneous mix of catechin and epicatechin-type prodelphinidin monomers (McNabb et al., 1998). This diversity in chemical structure of CT is a major factor in the differences in binding capacities and nutritional effects among CT from different plant sources.

TABLE 4. PROTEIN-PRECIPITATING CAPACITIES[a] AND WEIBULL CUMULATIVE FUNCTION PARAMETERS (P)[b] OF NINE CONDENSED TANNINS INCUBATED FOR 30 MIN WITH BOVINE SERUM ALBUMIN OR ALFALFA RUBISCO AS MODEL PROTEINS

	Bovine serum albumin						Rubisco					
	P2	P3	P4	Mean PPC	SE	Rank	P2	P3	P4	Mean PPC	SE	Rank
C. varia	1908	381.6	1.9	326.8 cd	6.5	5	1297	61.8	1.8	77.2 bc	1.4	6
H. alpinum	1909	465.8	1.9	389.8 b	2.4	8	2023	101.4	1.7	79.1 b	1.3	7
L. cuneata	1958	316.2	1.8	262.5 f	3.9	1	3267	171.1	1.6	76.6 bc	0.8	5
L. corniculatus	1300	359.6	2.1	435.5 a	6.6	9	1624	81.0	1.8	79.7 b	1.2	8
L. uliginosus	1688	342.7	1.9	322.5 cd	5.0	4	1187	54.0	2.1	72.4 d	1.1	4
L. uliginosus var. *glabriusculus*	1775	383.2	1.8	337.1 cd	15.7	6	2956	121.8	1.6	65.5 e	1.6	3
L. uliginosus var. *villosus*	1686	310.2	1.8	289.2 e	2.7	3	1501	57.9	1.8	60.9 e	1.1	2
M. sativa	1488	330.4	2.2	347.9 c	3.6	7	1459	94.9	1.9	108.2 a	4.7	9
O. viciifolia	1998	323.9	2.0	269.2 f	2.1	2	1645	51.4	1.9	49.6 f	0.8	1

a–f: For each protein (BSA or Rubisco), mean PPC followed by different letters differ significantly ($P < 0.05$).

[a] Expressed as μg CT required to precipitate 1 mg of protein ($N = 6$). PPC: Protein-precipitating capacity.

[b] Data were fitted to the equation: $Y = \mathrm{P1} + \mathrm{P2}(1 - e^{-(X/\mathrm{P3})^{\mathrm{P4}}})$, where Y = μg of protein (BSA or Fraction 1) precipitated; X = μg CT; P1 = lower asymptote (P1 = 0); P2 = upper asymptote (μg of protein); P3 determines the range of values of X over which the sigmoid curve occurs (1/μg CT); and P4 is the distortion of the curve (from Régnierè and Beilhartz, 1987).

Aerts et al. (1999) found significant differences between CT from *L. corniculatus* and from *L. pedunculatus* in their prevention of ruminal hydrolysis of white clover leaf protein, although only minor differences existed in the molecular weights of the CT from the two plant species. However, Osborne and McNeill (2001) concluded that, in general, high MW CT from *Leucaena* spp. precipitate more protein than do the low MW CT. Previous and current observations illustrate clearly that the capacity of CT to bind proteins cannot be predicted solely on the basis of MW.

Bovine serum albumin has been widely used as model protein in tannin–protein precipitation assays. In the present study, however, CT from all nine sources precipitated more Rubisco than BSA (by a ratio of approximately 4.6:1, on average). This may be related to the formation of soluble tannin–BSA complexes (Martin and Martin, 1983; Hagerman, 1992; Haslam, 1998), which could potentially limit the efficacy of BSA precipitation as a model for estimating the propensity of a CT to bind protein. Rubisco is a more logical choice as a model protein, as it is the principal protein in fresh forage and will be a primary focus in the event that genes coding for CT are introduced into legumes as a means of increasing bypass protein and/or preventing pasture bloat (McMahon et al., 2000).

Effects of Condensed Tannins on Cellulose Digestion. Control cultures of *F. succinogenes* (i.e., those containing no CT) digested 514 mg/g of filter paper included as substrate during 3 d of incubation (Table 5). Inhibition of digestion by the nine CT extracts ranged from 19.8% by sainfoin CT (412 mg digested/g incubated) to 92.4% by crown vetch CT (39 mg digested/g incubated).

Including CT in cultures of *F. succinogenes* increased the specific activity of cell-associated endoglucanase, as compared with no CT (Table 5). Declines in the extent of filter paper digestion were strongly correlated ($R = 0.8501$ and 0.8027; Table 6) with the inhibition of endoglucanase activity, which is consistent with our previous study of *F. succinogenes* (Bae et al., 1993b). Variations in the extent of inhibition of cellulose digestion caused by the nine CT may have arisen from different concentrations of CT in the polymer preparations or differences in the number of reactive groups in the CT.

Grouping the GPC spectra of the CT by their relative inhibitory effects on endoglucanase activity (high, moderate, low) revealed by the data in Table 5 yielded no consistent patterns (Figure 1). To further examine possible factors involved in CT-mediated inhibition of cellulolysis, chromophore formation during the three colorimetric assays by each of the CT was plotted against inhibition of filter paper digestion (Figure 2), as was their capacity to precipitate BSA and Rubisco (Figure 3). There was no correlation between inhibitory effects on filter paper digestion and (1) chromophore formation in any of the colorimetric assays, (2) precipitation of BSA or Rubisco ($R^2 \leq 0.255$), or (3) MW (Table 6). Further, MW was not correlated with any of these characteristics

TABLE 5. EFFECT OF CONDENSED TANNINS (CT) ISOLATED FROM NINE FORAGE SOURCES ON DIGESTION OF WHATMAN NO. 1 FILTER PAPER BY *F. succinogenes* subsp. *succinogenes* S85 AND ON CELL-ASSOCIATED AND EXTRACELLULAR ENDOGLUCANASE ACTIVITIES IN 72-HR CULTURES

	Filter paper digested[a]	Inhibition of digestion (%)	Endoglucanase activity[b] (mU/ml)		Cell-associated protein (μg/ml)	Specific activity[c] (mU/mg protein)
			Cell-associated	Extracellular		
Control (no CT included)	514	0	17.5	292.3	35.7	488.7
C. varia	39	92.4	2.9	18.1	4.5	623.1
H. alpinum	257	50.0	4.2	36.2	5.8	716.8
L. cuneata	86	83.2	3.7	17.8	2.7	1059.7
L. corniculatus	336	34.5	15.3	89.0	17.7	862.4
L. uliginosus	389	24.3	11.8	65.0	16.1	732.3
L. uliginosus var. *glabriusculus*	233	54.7	5.1	30.9	8.8	575.3
L. uliginosus var. *villosus*	182	64.6	2.8	16.8	3.8	705.3
M. sativa	98	81.0	4.2	43.0	4.7	890.7
O. viciifolia	412	19.8	18.5	136.7	21.9	841.9

[a] Expressed as mg filter paper digested per g of filter paper incubated as substrate in a 10-ml culture. When included, condensed tannins were present at 300 μg/ml ($N = 3$).
[b] Expressed against a commercial cellulase from *Penicillium funiculosum* (EC 3.2.1.4, Sigma Chemical Co.). See Methods and materials.
[c] Calculated for cell-associated endoglucanase activity.

TABLE 6. CORRELATION COEFFICIENTS (*R*) BETWEEN MEAN MOLECULAR WEIGHT (MW), CHROMOPHORE PRODUCTION, INHIBITION OF *F. succinogenes* CELLULOLYTIC ACTIVITIES, AND PROTEIN PRECIPITATING CAPACITIES AMONG CT ISOLATED FROM NINE LEGUMINOUS FORAGE SOURCES AND ANALYSIS OF REGRESSION OF CHARACTERISTICS AGAINST MW

		Chromophore production			Inhibition of endoglucanase activity		Inhibition of filter paper digestion	PPC		Analysis of regression against MW
Characteristic[a]	Mean MW	Butanol	Vanillin	H_2SO_4	Cell-associated	Extracellular		BSA	Rubisco	
MW										
BuOH	0.3766									$y = 2.5004x + 80.083$
Vanillin	−0.0490	0.4775								$y = -0.6242x + 84.784$
H_2SO_4	−0.3931	0.3648	0.7144							$y = -9.5247x + 143.43$
[i]$CMCase_{CA}$	−0.1375	−0.1162	−0.0045	0.0600						$y = -3.6640x + 71.805$
[i]$CMCase_{EC}$	−0.1204	−0.1269	−0.1761	0.0100	0.9656					$y = -1.2986x + 88.181$
*[i]*FPD	0.0632	0.1741	0.3551	0.2902	0.8501	0.8027				$y = 1.3083x + 50.569$
PPC-BSA	−0.4458	−0.6848	−0.3035	−0.0316	0.1068	−0.0600	0.1744			$y = -19.576x + 413.95$
PPC-Rubisco	−0.1530	−0.2059	0.2718	0.5874	0.3940	0.3493	0.5050	0.4419		$y = -1.9275x + 82.405$

[a] Butanol, vanillin, and H_2SO_4: chromophore production as measured by butanol–HCl, vanillin–HCl, and H_2SO_4 methods, respectively; *[i]*$CMCase_{CA}$ and *[i]*$CMCase_{EC}$: inhibition of endoglucanase (carboxymethylcellulase, CMCase) activities in cell-associated (CA) or extracellular (EC) fractions, respectively, of cultures of *F. succinogenes*; *[i]*FPD: inhibition of digestion of filter paper by *F. succinogenes*; PPC-BSA and PPC-Rubisco: protein-precipitating capacities against bovine serum albumin (BSA) and Fraction 1 protein (Rubisco), respectively.

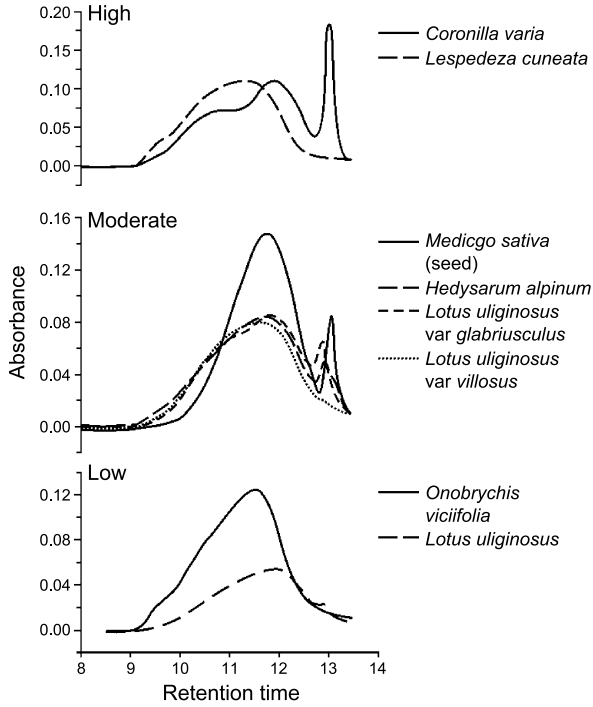

FIG. 1. Gel permeation chromatographic spectra of peracetate derivatives of the condensed tannins (CT) isolated from nine leguminous forage sources. The spectra are grouped to reflect the relative rankings of the effects of the nine CT preparations on cellulose-digesting activity of the ruminal bacterium *Fibrobacter succinogenes* (Table 5).

(Table 6). None of these parameters, therefore, was a reliable indicator for predicting the potential of a particular CT to inhibit cellulose digestion.

It has been suggested that the ability of CT to precipitate proteins is a suitable indicator of their relative astringency (Robbins et al., 1987; Hanley et al., 1992). However, correlation between the ability of the extracted CT to inhibit filter paper digestion and to precipitate BSA (R = 0.1744) or Rubisco (R = 0.5050) was very low (Table 6). Moreover, the most inhibitory CT for cellulose digestion included those with broad and with narrow MW distributions (Tables 1 and 5). In terms of ruminant nutrition, the CT from sainfoin, *O. viciifolia*, appeared to have the most potential of the nine examined, as it caused the least inhibition of cellulose digestion by *F. succinogenes* while exhibiting the greatest capacity to bind Rubisco from alfalfa. This response is consistent with our previous documentation of the bloat-prevention capacity

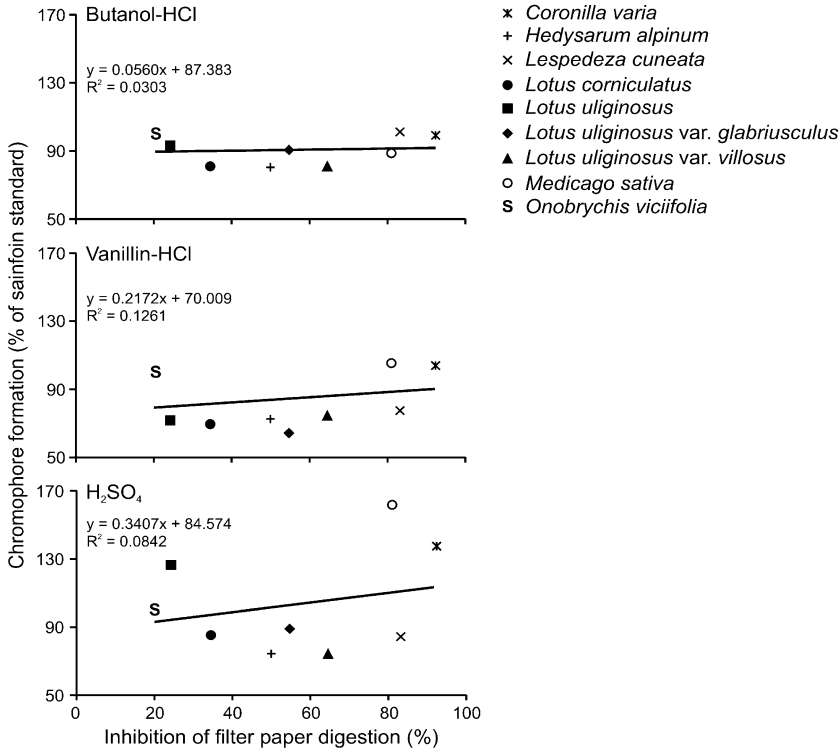

FIG. 2. Relationships between the inhibitory effects of condensed tannins (CT) from nine leguminous forage sources on filter paper digestion by *F. succinogenes* (as compared to digestion with no CT) and their production of chromophore (relative to sainfoin, S) during colorimetric assays (butanol–HCl, vanillin–HCl, or H_2SO_4).

of sainfoin when it is fed in combination with alfalfa to cattle (McMahon et al. 1999).

Characterization of the properties of CT that optimize ruminal escape protein and prevent pasture bloat, without impeding carbohydrate digestion, is pivotal to the success of strategies aiming to achieve these characteristics in alfalfa through the transgenic expression of CT. This study suggests that these properties are not easily defined via chemical means and that biological assays using rumen bacteria may help identify those CT with the most desirable properties. Identification of those CT that result in favorable alterations in protein metabolism without adversely affecting ruminal fiber digestion will be pivotal to the success of using these phenolic compounds to improve ruminant production. Development of similar assays using proteolytic ruminal bacteria could prove useful in achieving this goal.

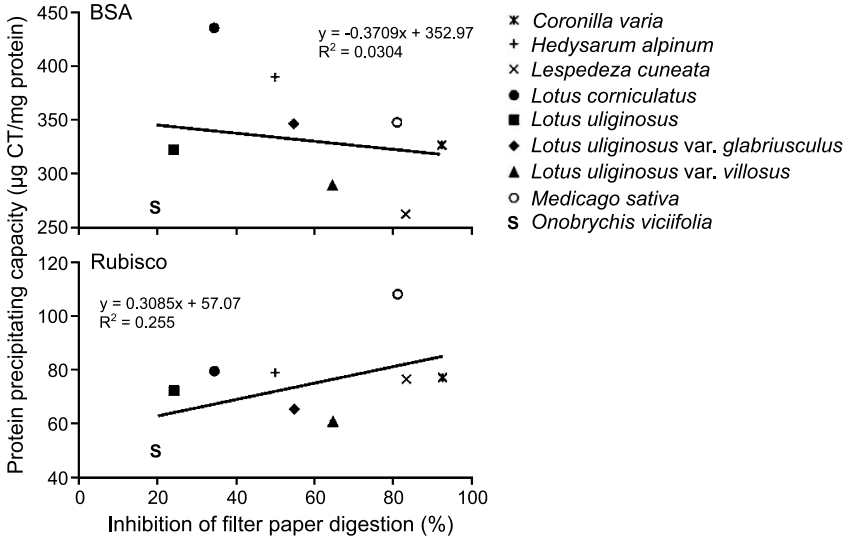

FIG. 3. Relationships between the inhibitory effects of condensed tannins (CT) from nine leguminous forage sources, including sainfoin (S), on filter paper digestion by *F. succinogenes* (as compared to digestion with no CT) and their capacity to precipitate bovine serum albumin (BSA) or Rubisco from alfalfa (µg CT required to precipitate 1 mg of the specified protein).

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THE PLANT STRESS HYPOTHESIS AND VARIABLE RESPONSES BY BLUE GRAMA GRASS (*Bouteloua gracilis*) TO WATER, MINERAL NITROGEN, AND INSECT HERBIVORY

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Abstract—Acting simultaneously or sequentially, plants encounter multiple stresses from combined abiotic and biotic factors that result in decreased growth and internal reallocation of resources. The plant stress hypothesis predicts that environmental stresses on plants decrease plant resistance to insect herbivory by altering biochemical source–sink relationships and foliar chemistry, leading to more palatable food. Such changes in the nutritional landscape for insects may facilitate insect population outbreaks during periods of moderate stress on host plants. We examined the plant stress hypothesis with field experiments in continental grassland (USA) using the C_4 grass *Bouteloua gracilis*. Water, nitrogen fertilizer, and herbivory from the grass-feeding grasshopper *Ageneotettix deorum* were manipulated. Combined stresses from water and mineral-N in the soil decreased plant growth and altered foliar percent total N (TN) and percent total nonstructural carbohydrate (TNC) concentrations in an additive fashion. Grasshopper herbivory affected final biomass only in dry years; plants compensated for tissue loss when rainfall was abundant. Foliar TN and TNC concentrations were dynamic with respect to variable climatic conditions and treatment combinations, showing significant interactions. Grasshopper herbivory had its greatest impact on TN or TNC in dry years, interacting with other forms of stress. Herbivory as a single factor had strong effects on TNC in years with normal precipitation, but not in a dry year. Performance (developmental rate and

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survival) by the grasshoppers *Phoetaliotes nebrascensis* and *A. deorum* were not greatly affected by plant stress in a manner consistent with the plant stress hypothesis.

Key Words—Chewing insects, environmental stress hypothesis, functional-convergence-to-plant-stress hypothesis, grasshopper, insect herbivory, total foliar nitrogen, total nonstructural carbohydrates.

INTRODUCTION

Dynamic biochemical, physiological, and morphological responses by plants to environmental conditions are integrated at organ and whole-plant levels through a variety of source–sink relationships (Mooney and Chiariello, 1984; Bazzaz and Grace, 1997). The plant stress hypothesis states that environmental stresses on plants decrease plant resistance to insect herbivory by altering whole-plant source–sink resource allocation schedules and foliar chemistry, thus changing food palatability (Rhoades, 1983; Mattson and Haack, 1987; Louda and Collinge, 1992; White, 1993; Redak and Capinera, 1994; Koricheva et al., 1998; Huberty and Denno, 2004). Plant resource acquisition (light, water, carbon, elemental nutrients), internal resource allocation among tissues (source–sink relationships, translocation products), and partitioning of resources to different plant functions (growth, maintenance, reproduction, repair, defense, senescence) ultimately prescribe the nature and distribution of nutritional constituents within plants to herbivores (Mooney and Gilman, 1982; Bazzaz et al., 1987; Chapin et al., 1987; Mooney et al., 1991; Aerts and Chapin, 2000)—often considered growth optimization processes (Mooney and Winner, 1991). Variation in water and soil nutrient availability coupled to herbivory may cause unpredictable levels of stress that alters plant metabolism in response to the action of one or all factors with consequences for plant growth (Trlica and Cook, 1971; Bokhari, 1978; Mooney et al., 1991; Louda and Collinge, 1992).

The plant stress hypothesis was proposed as an environmentally determined explanation for outbreaks of insect herbivores operating through plant condition (Rhoades, 1983; Waring and Cobb, 1992; Watt, 1992; Koricheva et al., 1998), in which improved nutritional quality of host plants experiencing intermediate levels of stress resulted in increased demographic performance by herbivores. Rhoades (1983) extended the hypothesis to also include reduced production of chemical defenses under stress conditions in addition to elevated nutritional quality. Experimental tests of the plant stress hypothesis for forest insects provide little general support of the hypothesis (Rhoades, 1983; Waring and Cobb, 1992; Watt, 1992; Koricheva et al., 1998). Although some insect feeding guilds (e.g., boring and sucking feeders) responded as predicted in experimental tests in woody plants, other groups including chewing insects did

not generally respond to plant stress as predicted (Waring and Cobb, 1992; Watt, 1992; Koricheva et al., 1998; Huberty and Denno, 2004). However, about 67% of the examples are consistent with predictions (Waring and Cobb, 1992) in observational studies of trees along environmental stress gradients, although alternate explanations exist (Watt, 1992). Although this system may be prototypical for the action of the plant stress hypothesis, few tests with grasses exist (Waring and Cobb, 1992; Redak and Capinera, 1994).

We seek to clarify the nature of interactions among multiple stresses as they impact growth and variable leaf chemistry in blue grama grass, *Bouteloua gracilis* (H.B.K.) Lag. ex Griffiths, according to predictions of the plant stress hypothesis. *B. gracilis* is a dominant C_4 grass species in western North American (USA) grasslands. Two primary predictions of the plant stress hypothesis were examined in the short grass *B. gracilis* experiencing naturally occurring and variable abiotic conditions: (1) reduced water or soil nitrogen levels coupled to insect herbivory will negatively affect plant growth and increase the palatability of tissues to insect herbivores, (2) chewing insect herbivores will perform better on stressed host plants with higher concentrations of primary nutrients (protein and carbohydrate). In addition, we examined the relative contribution to responses of stresses when combined under field conditions. We examined direct effects and interactions among three common forms of stress to *B. gracilis*: water availability, plant nutrient availability, and grasshopper herbivory within natural levels in the field. Experiments repeated over 3 years included a wide range of weather conditions against which to gauge plant responses. We expected that the imposition of moderate water or nutrient stress should modify plant physiology in such a way that resistance to herbivores decreases, with a concomitant increase in availability of primary nutrients in leaves to herbivores. As food plant palatability increases following moderate stress to *B. gracilis*, performance by the grass-feeding grasshoppers *Ageneotettix deorum* (Scudder) and *Phoetaliotes nebrascensis* Thomas should be enhanced as levels of primary nutrients in leaf tissues, especially protein and carbohydrates, increase. *B. gracilis* does not produce allelochemicals that are expected to influence responses to primary nutrients by herbivores in this experiment (Mole and Joern, 1994), allowing us to restrict our attention to the nutritional component of the problem.

METHODS AND MATERIALS

Study System. We conducted field experiments at Arapaho Prairie (Arthur County, NE, USA), a protected research site in Nebraska sandhills grassland. The site is characterized by upland sandhills grassland composed of large stabilized sand dunes with steep upper ridges that gradually slope into broad flat

valleys. Most plants at Arapaho Prairie experience at least some water and nutrient stress in most years (Barnes, 1985; Mole et al., 1994).

Vegetation at Arapaho Prairie is an open-canopy mixed-prairie, modified by sandy substrate (Barnes, 1985). Grasses contribute 80% to total plant biomass, with long-term NAPP ranging between 75 and 250 g m^{-2} (unpublished data). C_3 and C_4 grass species typical of eastern tallgrass prairie and western shortgrass steppe grasslands intermingle at the site. Dominant plants in this sand dune landscape form loose but recognizable vegetation associations along the existing topographic gradient (Barnes, 1985). The grass canopy is intermingled with extensive bare ground, largely because of extensive disturbance from pocket gophers.

Long-term annual mean precipitation (1951–1980) recorded 15 km from Arapaho Prairie at Arthur County, NE, averaged 47.1 cm (SD = 8.98 cm) from

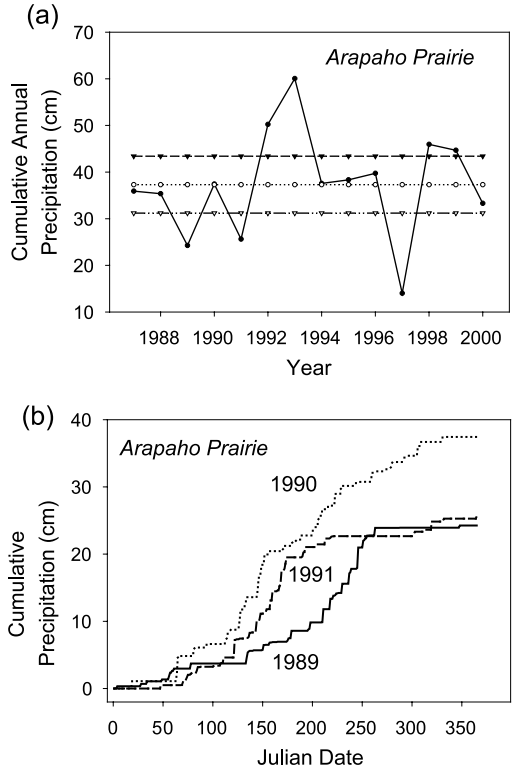

FIG. 1. Precipitation patterns at Arapaho Prairie. (a) Annual rainfall with mean and 95% confidence intervals, 1987–2000. (b) Seasonal pattern of precipitation illustrated by cumulative amount by date for the 3 years of the study.

US Weather Bureau records; the recent 14-year record from Arapaho Prairie (1987–2000) averaged 37.3 cm (SD = 11.4 cm). The amount and timing of precipitation at Arapaho Prairie varies greatly among years (Figure 1). Below-average precipitation was observed in two of the three years of this study (Figure 1a), with rainfall in 1990 equaling the average amount for the site. Perhaps more importantly, the seasonal timing of rainfall over the growing season differs in important ways among years (Figure 1b). Both 1989 and 1991 received approximately the same amount of precipitation, but rain fell early in the season in 1991 compared with late-season rainfall in 1989. In 1990, rainfall occurred throughout the growing season, compared with 1989 and 1991, each of which experienced large periods without significant amounts of rain.

Arapaho Prairie soils contain 80–85% sand with low nutrient concentrations (Barnes et al., 1984). Total nitrogen in soil in the top 10 cm ranges from 0.02 to 0.07% of total soil weight according to landscape position. Valleys exhibit the highest soil total N levels, but all landscape positions are generally low (Alward and Joern, 1993). Nitrate concentrations range from 0.04 to 15 ppm, and ammonium concentrations varied from 0.17 to 3.3 ppm. Light is seldom a major limitation to plant growth because of the open canopy and large proportion of sunny days at this site.

B. gracilis is an often dominant C_4 short-grass species throughout the shortgrass steppe of the Rocky Mountain foothills to the mixed-grass prairies of the central Great Plains of North America. In Nebraska sandhills grasslands, it is commonly found in fine-textured soils typical of dry valleys. At Arapaho Prairie, *B. gracilis* comprises up to 20–30% of the relative cover of valleys and midslope dunes but is nearly absent from dune ridges (Barnes et al., 1984). *B. gracilis* productivity is correlated with soil moisture, and biomass peaks in early August although yearly variability exists. *B. gracilis* is an important dietary component of graminivorous grasshopper species at this site, including *A. deorum* and *P. nebrascensis* (Joern, 1985).

Experimental Design and Statistical Analyses. Overall, two related experiments were run concurrently, one addressing effects of water, N fertilizer, and grasshopper herbivory on plant response, and the other investigating grasshopper performance in response to water and N-fertilizer treatments on plants. Rectangular cages (basal area 0.5 m^2, 80 cm high) were constructed of 0.64-cm mesh and buried 10 cm after severing possible root connections to neighboring ramets. Cages were placed over natural stands of *B. gracilis* "turf" in early June, corresponding to the initiation of growth. Cages housing treatment combinations of both experiments were intermingled randomly within each block, but experiments were analyzed separately.

Plant Responses. We manipulated levels of water, nitrogen fertilizer, and grasshopper herbivory within natural levels to understand variation in plant responses to stress. Biomass accumulation and foliar chemical responses (% total

nitrogen, TN; and % total nonstructural carbohydrates, TNC) by *B. gracilis* to multiple stresses was studied using a 3 × 2 × 2 full-factorial treatment combination (N fertilizer, water availability, and grasshopper herbivory, respectively) experiment in a randomized complete block design, nested within each of 3 years. Six sites (blocks) were arbitrarily selected in a range of natural habitats for *B. gracilis* along a gradient stretching from slope vegetation to valley vegetation. Sites were selected based on the criterion that a sufficient density of *B. gracilis* was available to set up a full set of treatment combinations. Treatment combinations were randomly assigned to predetermined patches of *B. gracilis* within each block.

Grasshopper Performance. Grasshopper performance was evaluated in a field experiment executed in parallel with the plant stress experiment by using a similar experimental design and identical water and mineral-N fertilizer additions using cages as described above. Cages were intermingled randomly with those of the plant stress experiment. The experimental design was a 3 × 2 full-factorial treatment combination experiment (N fertilizer and water availability, respectively) arrayed in a randomized complete block design, nested within each of 3 years. Six blocks were used. A repeated-measures analysis of variance (ANOVA) was used to examine grasshopper survival. Responses of two grasshopper species to plant stress were evaluated in different years (1989, *P. nebrascensis*; 1990, *A. deorum*), but specific responses between species cannot be compared directly because of overall differences in naturally occurring stress between years. Ten fourth instar nymphs were added to each cage in late June or early July to match natural phenological development of each species in the field. The number of survivors and the developmental stage of individuals were determined every 2–3 d from censuses of individuals remaining in each cage.

Statistical Analyses. Statistical analyses were performed using ANOVA, with treatments evaluated as fixed effects in the ANOVA. To normalize data, dependent variables expressed as percent of the total sample weight were transformed by applying arcsine(square root) to original data before statistical analyses. We present and discuss values in the nontransformed state. Treatment variables were treated categorically in analyses.

Manipulations of Plant Stress from Water, Mineral Nitrogen, and Grasshopper Herbivory

(1) *Water*. Two water levels were used: W+, in which water was added weekly for the 10-wk duration of the experiment, and W0, where no additional water beyond ambient rainfall was added. We considered W0 to be more stressful than W+ as water stress is common in grasses (Heinisch, 1981; Barnes, 1985).

In the first 2 wk of the experiment, all plots received water in addition to N fertilizer if scheduled for that cage. After this, W+ cages received 2 l m^{-2} wk^{-1} of supplemental water over the course of the experiment. No attempt was made to standardize the absolute level of plant water stress among years.

(2) *N-Fertilizer*. Soil-nitrogen levels were manipulated using ammonium nitrate (NH_4NO_3). Levels included 0, 3, and 6 g N m^{-2} of N fertilizer (N_0, N_3, and N_6 treatments, respectively). N fertilizer was applied in two half-strength additions over several days in early June in each year.

(3) *Grasshopper Herbivory*. Moderate densities of the *B. gracilis*-feeding grasshopper, *A. deorum*, were added to cages to assess foliar responses to insect herbivory. In the GH+ treatment, we added four adult grasshoppers to each cage in late June. This density corresponded to eight individuals per square meter, about double the long-term average of all grasshoppers at Arapaho Prairie (A. Joern, unpublished data), but about half the economic threshold. Moreover, the densities used in the experiments are routinely observed in some vegetation patches in most years. No grasshoppers were added to cages in the GH0 treatment. Initiation of the grasshopper treatment corresponded to the phenological presence of the adult *A. deorum* in the field. Grasshoppers were replaced weekly to maintain relatively constant levels of herbivory.

Final Biomass Estimates and Chemical Analyses of Leaf Material. Leaf samples of *B. gracilis* were collected at the end of the experiment (mid-August) and prepared for chemical analysis. Initially, a subsample of green leaf material [ca. 2–3 g dry weight (d.w.)] was collected, immediately flash-frozen in liquid nitrogen in the field, and then prepared for chemical analyses. Samples were lyophilized for 48 hr and stored under desiccant in a freezer. Dried leaf material was ground with a Wiley Mill (40-mesh sieve) before chemical analysis. After collecting leaf material for chemical analyses, remaining plant biomass in a cage was clipped, dried (80°C for 24 hr) and weighed.

Total Nitrogen. Total nitrogen was analyzed by using modified micro-Kjehldahl techniques (AOAC, 1984) with a standard digest on 100-mg samples of ground leaf material (2 ml H_2SO_4, a $CuSeO_4$ Kjeltab catalyst tablet). Total N was determined by measuring ammonia generated after adding 100 ml of 5 M NaOH to the digest using a selective ion electrode (Orion). The ammonium probe was calibrated daily with an ammonium sulfate standard.

Total Nonstructural Carbohydrates. Total nonstructural carbohydrates were extracted following the method of (Smith, 1981) except for the use of amylglucosidase (Sigma A-7255) as the enzyme preparation in the digest. These were analyzed by the titrimetric method of Smith (1981) with glucose as a standard without the hydrolysis of sucrose. Sucrose averaged about 0.4–0.5% d.w.

of plant material compared with 17–22% d.w. plant material for TNC as measured and did not vary with TNC concentration (S. Mole, unpublished data).

RESULTS

Total Plant Biomass. On average, total biomass in *B. gracilis* plots at the end of the season (Figure 2) was about 50–100% greater in an average rainfall

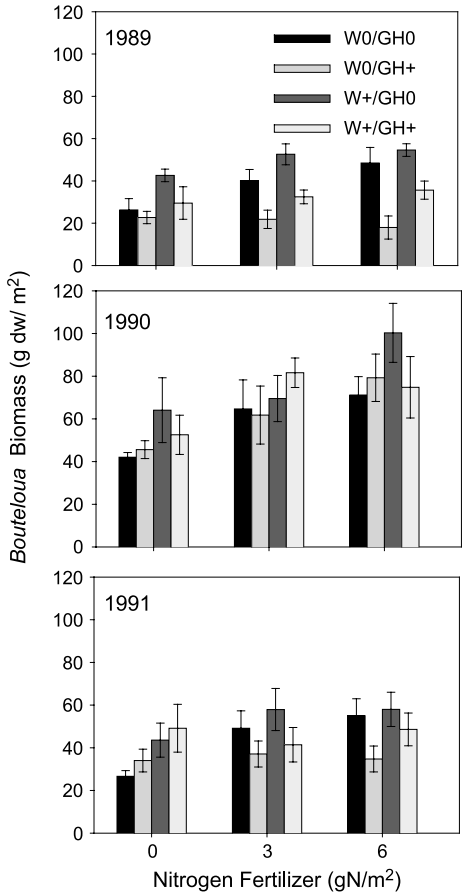

FIG. 2. End of season *B. gracilis* biomass (mean, SE) according to stress treatment conditions [water (W0, W+), N-fertilization (0, 3 and 6 g N m^{-2}), and grasshopper herbivory (GH0, GH+)] for each year of the study.

year (1990) as in dry years (1989, 1991), which were similar. *B. gracilis* biomass was significantly different among experimental treatments depending on the number of stresses applied, indicating that the plants in this study experienced varying degrees of overall stress. Both water (1989: $F_{1,56} = 17.4$, $P < 0.001$; 1990: $F_{1,56} = 6.6$, $P = 0.013$; 1991: $F_{1,56} = 11.3$, $P < 0.001$) and N fertilizer additions (1989: $F_{2,56} = 4.2$, $P < 0.021$; 1990: $F_{2,56} = 11.2$, $P < 0.001$; 1991: $F_{2,56} = 7.1$, $P < 0.001$) resulted in increased biomass in all years as additive, direct effects; no statistical interactions were detected for water and N fertilizer in any year (Figure 2).

Feeding by grasshoppers reduced the final *B. gracilis* biomass in the dry years of 1989 and 1991 (67% in 1989, $F_{1,56} = 34.8$, $P < 0.001$; 32% in 1991, $F_{1,56} = 6.5$, $P = 0.012$), but no effect from grasshopper feeding was detected in 1990, a year of normal rainfall. This indicates that complete compensation for foliage loss was observed in this year with normal rainfall. No statistical interactions among grasshopper herbivory, water availability, and N fertilizer treatments were observed in their combined effect on final *B. gracilis* biomass, but were additive instead. Although biomass estimates do not include the amounts consumed by grasshoppers, these should be similar between years as the grasshopper encounter rate was controlled.

Foliar Total Nitrogen. Foliar TN differed significantly among treatments, year, and block (Figures 3a and 4a, Table 1). TN concentrations were highest for all treatments in 1989, the driest year, a year with almost no precipitation occurring early in the growing period (Figure 1b). TN at the end of the experiments in August 1989 averaged 1.73% total dry weight in all treatment combinations compared with 1.01% (1990) and 1.14% (1991) TN in subsequent years, representing a notable decrease in 1990–1991 compared with 1989.

Foliar TN levels varied in response to both N fertilizer and water treatments in some fashion in all years (Figures 3a and 4a, Table 1), with water addition explaining the most variation in responses (Figure 5). Depending on the year, N fertilizer addition increased foliar TN levels from 5 to 21% dry mass compared with no fertilizer addition treatments. An average 13% increase in foliar TN over the 3-year period was observed. Differences in foliar TN between 3N vs 6N treatments were of smaller magnitude (3–10%), and only significantly different in 1991.

Although the main effects of treatments were pronounced in all cases (Figure 3), treatment interactions that were important and insightful to underlying processes were sometimes detected. W0 treatments resulted in a 10–20% higher level of total foliar-N compared with W+ treatments. The weakest response to water (9.5%) was observed in the driest year (1989), possibly because extreme drought stress in that year was not proportionally offset by the water addition treatment compared to other years. A significant N fertilizer by water interaction existed in 1989 and 1990 but with different

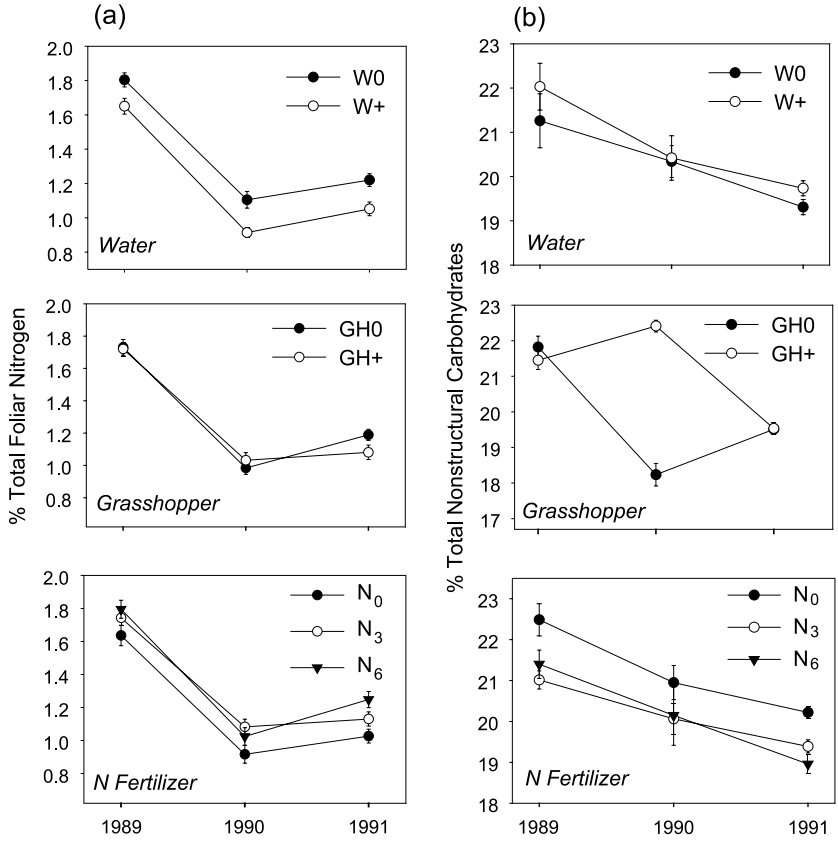

FIG. 3. Responses (mean, SE) in (a) % total N and (b) % TNC to main treatments (water addition, grasshopper herbivory, and N fertilizer) for each year.

responses between the 2 years (Figure 5). In very dry 1989, higher foliar TN levels were seen in W0 only for the N_0 treatment. No differences were seen between W0 and W+ for the N_3 and N_6 fertilization treatment levels. In a year of average rainfall (1990), there was no difference in foliar TN between water treatments at N_0, but significant and about equal increases in total N for N_3 and N_6 treatments in interaction with water availability.

Grasshopper herbivory affected foliar TN levels significantly as a main effect only in 1991. However, grasshopper feeding interacted with other treatments to influence total foliar TN in all years (Figures 4a and 5). In 1989, there was an increase in TN up to the maximum level observed at N_3, a TN level that was reached with N_6 with no grasshoppers. In 1990, grasshopper herbivory interacting with water availability led to higher TN level that was reached in the

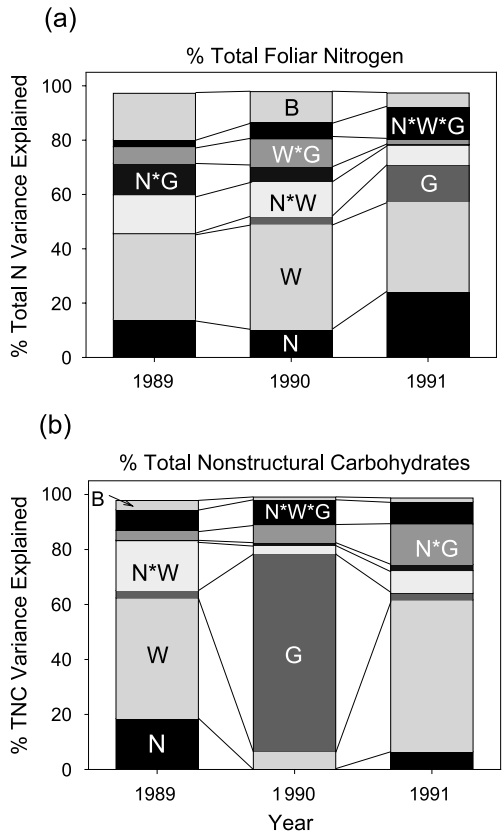

FIG. 4. Percentage of total variance in foliar nutrient responses explained by experimental treatments in each year of study. (a) % Total foliar nitrogen (TN) and (b) % total nonstructural carbohydrates (TNC). Letters refer to main effects (N, nitrogen fertilization; W, water; G, grasshopper herbivory) and statistical interactions (N*W, N*G, W*G) as indicated in the experimental design of Table 1. B is the block (site) effect. Percentage of total variance in response was calculated as the variance associated with the treatment combination compared with the total variance of the experiment.

W0 treatment compared with the W+ treatment for which there was no significant difference between grasshopper treatments.

Foliar Total Nonstructural Carbohydrates. Significant responses in foliar TNC concentrations were also observed (Table 1, Figures 3b, 5b, and 6) in response to combined stresses. Among-year differences averaged 5–10%, with 1989 exhibiting the highest foliar TNC levels. Differences in responses among all treatment combinations showed little variation in 1989 and 1991 compared

t1.1 TABLE 1. ANOVA OF FOLIAR NUTRIENT RESPONSES OF *B. gracilis* LEAF TISSUE AT THE END OF THE SEASON IN RESPONSE TO MULTIPLE STRESSES

t1.2		All years			1989			1990		1991	
t1.3	Source	*df*	*F*	*P*	*df*	*F*	*P*	*F*	*P*	*F*	*P*
t1.4	*Foliar TN (G N/G leaf dry weight)*										
t1.5	Model R^2		57.3			68.7		61.0		61.5	
t1.6	Year	2	142.8	0.001	–	–	–	–	–	–	–
t1.7	Block	5	4.2	0.001	5	7.4	0.001	5.4	0.001	2.2	0.07
t1.8	N-Fertilizer	2	10.9	0.001	2	6.8	0.002	4.9	0.01	8.3	0.001
t1.9	Water	1	17.1	0.001	1	14.9	0.001	18.5	0.001	14.0	0.001
t1.10	Grasshopper	1	15.7	0.001	1	0.01	0.9	1.3	0.26	5.9	0.02
t1.11	N-Fertilizer × Water	2	0.6	0.45	2	4.5	0.02	6.2	0.004	3.1	0.05
t1.12	N-Fertilizer × Grasshopper	2	0.7	0.56	2	5.4	0.007	2.6	0.09	0.2	0.82
t1.13	Water × Grasshopper	1	3.45	0.03	1	3.8	0.05	4.9	0.03	0.9	0.34
t1.14	N-Fertilizer × Water × Grasshopper	2	1.97	0.10	2	1.7	0.18	2.9	0.06	5.0	0.01
t1.15	Error	197			55						
t1.16											
t1.17	*TNCs (g TNC/g leaf dry weight)*										
t1.18	Model R^2		39.8			37.5		91.6		66.0	
t1.19	Year	2	25.8	0.001	–	–	–	–	–	–	–
t1.20	Block	5	0.6	0.9	5	0.8	0.6	3.1	0.02	2.9	0.02
t1.21	N-Fertilizer	2	9.8	0.001	2	7.3	0.002	7.9	0.001	20.8	0.001
t1.22	Water	1	2.9	0.09	1	4.8	0.03	0.02	0.89	6.8	0.0
t1.23	Grasshopper	1	29.4	0.001	1	1.0	0.3	442.0	0.001	0.02	0.89
t1.24	N-Fertilizer × Water	2	0.8	0.5	2	0.1	0.9	21.3	0.001	2.2	0.13
t1.25	N-Fertilizer × Grasshopper	2	1.0	0.35	2	2.6	0.09	21.5	0.001	4.0	0.02
t1.26	Water × Grasshopper	1	0.06	0.8	1	0.1	0.8	11.6	0.001	16.3	0.001
t1.27	N-Fertilizer × Water × Grasshopper	2	0.94	0.39	2	2.2	0.12	18.0	0.001	7.5	0.001
t1.28	Error		197			55					

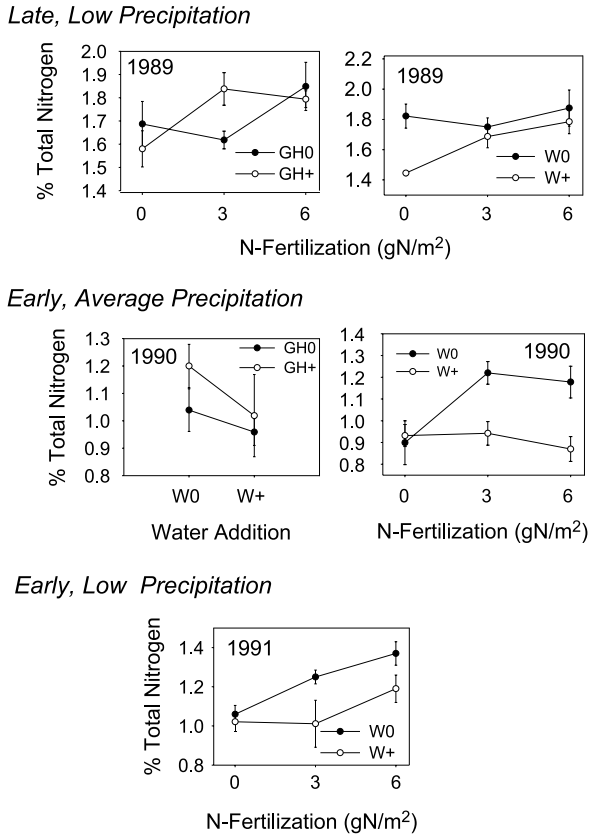

FIG. 5. Responses of significant interactions among treatments for % total foliar N (TN) for each year of study.

with 1990. Total variance in TNC levels among treatments was 1.5–5 times greater in the average rainfall year (1990) than in the other years. Over all 3 years, combined nitrogen fertilizer and grasshopper treatments for all levels were significant as main effects, with no significant statistical interactions. When compared against the N fertilizer treatments, W0/GH0 had the lowest TNC levels and W+/GH+ had the highest levels on average, with each decreasing along the N-fertilization axis. Levels of TN and TNC in leaves were uncorrelated in all years for all treatments combined (1989: $r^2 = 0.014$; 1990: $r^2 = 0.001$; 1991: $r^2 = 0.038$; $P > 0.05$ for all years). However, when years were analyzed separately, interesting differences were observed.

In general, TNC declined 4–6.5% with increased N fertilizer in all years, although no significant differences were observed between the 3 g and 6 g

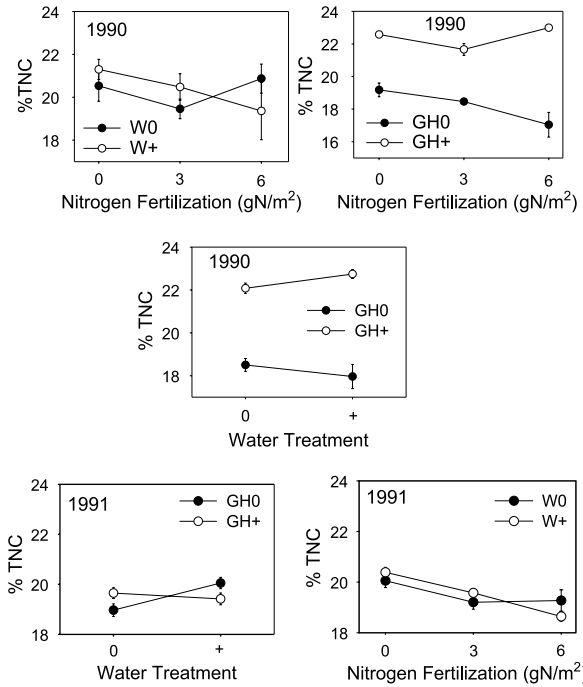

FIG. 6. Responses of significant interactions among treatments for % total nonstructural carbohydrate (TNC) for each year of study.

N fertilizer treatments. When water treatments were significant (1989 and 1991), TNC was greater in W+ compared with the W0 treatments, with differences on the order of about 3–4%. Generally, grasshopper herbivory was a factor when interacting with either N fertilizer or water treatments (Table 1). In 1990, GH+ resulted in a large 23% increase in % foliar TNC, and important interactions with N fertilizer and water were detected.

The nature of interactions among sources of plant stress differed among years. Numerous interactions were observed in both 1990 and 1991 (Figure 6), average and below average rainfall years, respectively. In 1990, all two-way interactions and a three-way interaction were significant. % TNC in the N_6 fertilizer treatment increased in the W0 treatment, but the trend otherwise was for TNC to drop with increased N fertilizer. Grasshopper treatments interacted with both N fertilizer and water in both 1990 and 1991, but the TNC responses were different. In the very dry 1989, no interactions were detected, and all contributions to the variance in TNC content were additive. Inclusion of grasshoppers resulted in increased TNC in high-resource environments (N or

water) compared with the GH0 treatments. In 1991, the opposite response was observed where TNC levels under high-resource conditions were lower if grasshoppers were present.

Grasshopper Performance. P. nebrascensis. This species was studied in a very dry year with late season rainfall. No significant effect of treatment combinations was observed for developmental rate although there is a suggestion that W0/6N develops faster. Repeated-measures ANOVA of the number of

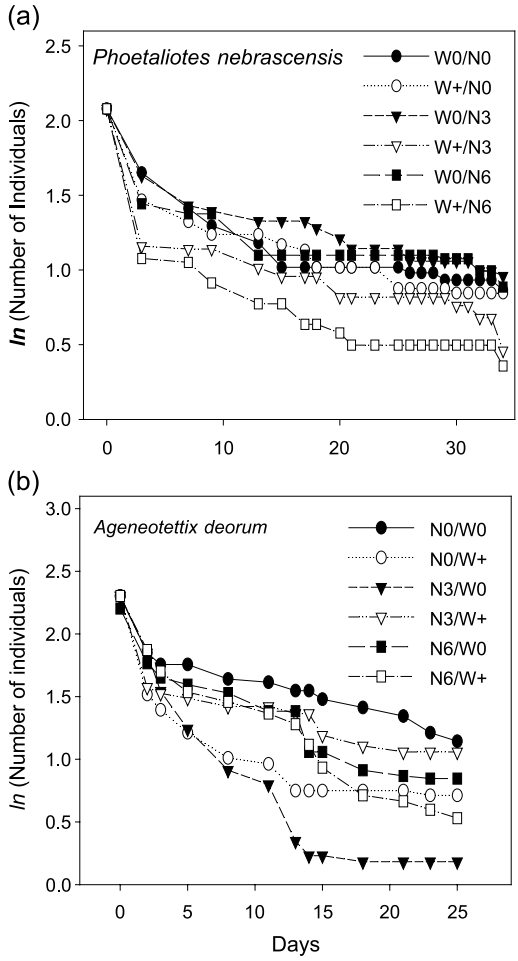

FIG. 7. Mean survival of two grasshoppers in response to plant stress treatments. Experiments were performed in different years as described in the text. Data are transformed as natural log of number alive at each census period.

individuals remaining in cages of *P. nebrascensis* (Figure 7a) was significant (Wilk's $\lambda = 0.10$, $P < 0.001$). However, although observed trends in survival may be suggestive, no significant effect of water and N fertilizer treatments were detected. The significant difference in the repeated-measures ANOVA reflected the decrease in the number of survivors over time, not treatments.

Ageneotettix deorum. This species was studied in a normal rainfall year. No significant effect of water and N fertilizer treatments on developmental rate was detected. *A. deorum* survival (Figure 7b) varied in response to experimental treatments (repeated-measures ANOVA, Wilk's $\lambda = 0.137$, $F_{6,21} = 22.06$, $P < 0.001$). A significant N Fertilizer × Water interaction was detected (repeated-measures ANOVA, $F_{2,6} = 6.3$, $P = 0.006$). In W0 treatments, survivorship was greatest in treatments with no N fertilizer and decreased when N was added. In W+ treatments, survival was highest on fertilized plots, at least for the first half of the trajectory when N0 and N6 N fertilizer treatments converged.

DISCUSSION

This study examines how multiple environmental stresses interact to affect growth and foliar chemistry in the grass *B. gracilis*, and whether plant responses to these stresses increase herbivore performance. Since White's (1993) formulations of the plant stress hypothesis, much effort has been directed at understanding its overall importance and generality to understanding insect herbivore population responses to plant stress (Waring and Cobb, 1992; Koricheva et al., 1998). Testing this hypothesis becomes a greater challenge when multiple stresses operate (Mooney et al., 1991). Soil nutrient stress varies according to substrate type and nutrient cycling characteristics of the site, and ecological processes that deplete nutrient availability such as uptake rates by plants or use by soil microbes (Aerts and Chapin, 2000). Water availability varies at multiple scales within and among years, where plants in natural environments are often water stressed, including *B. gracilis* studied here (Mole et al., 1994). For much vegetation, local light availability is influenced primarily by accumulated total biomass at the site, although intermittent cloud cover can be important. In this study, light was unlikely to limit photosynthesis because of the low stature of vegetation growing in an open habitat under conditions of sunny skies on most days. Tissue loss from herbivory further modifies physiological responses in plants and potentially interacts with other sources of stress in nonlinear ways (Mattson and Haack, 1987) and is the basis of Jones and Coleman's (1991) "phytocentric model" of plant–insect herbivore interactions.

In response to moderate stress to *B. gracilis*, plant growth decreased, concentrations of primary nutrients in leaf tissue increased, and the ability to compensate for tissue loss from herbivory was reduced. Here, foliar chemistry

in response to stress conditions is highly dynamic and effects of combined abiotic stresses are additive, but sometimes appear idiosyncratic when combined with herbivory from grasshoppers. Results observed for *B. gracilis* are consistent with those of other studies. Multiple environmental stresses to plants regularly reduce plant growth compared to the maximum performance possible (Mooney et al., 1991; Louda and Collinge, 1992), and nutrient and water stress or tissue loss often alter tissue palatability to herbivores (Mattson and Haack, 1987; Louda and Collinge, 1992; White, 1993; Redak and Capinera, 1994). Understanding the integrated responses by plants to combined stresses from abiotic conditions and herbivory is limited by our ability to incorporate the consequences of multiple stresses into a predictive framework (Jones and Coleman, 1991; Mooney et al., 1991; Bazzaz and Grace, 1997). Because plants function as integrated units, whole-plant growth responses reflect the underlying coordination and allocation among competing resource sinks (Mooney et al., 1991; Bazzaz and Grace, 1997).

Consequences of Environmental Stresses to B. gracilis. B. gracilis biomass varied significantly with manipulation of water availability, nitrogen fertilizer, and grasshopper herbivory treatments in the field, showing that these factors contribute importantly to plant stress. Responses differed among years as weather conditions varied (hot, dry vs normal precipitation), and both water and nitrogen fertilizer manipulations affected plant growth in each year in an additive fashion. Moreover, plant biomass decreased relative to controls in response to grasshopper herbivory in the driest years (1989, 1991), but herbivory did not affect final biomass accumulation in a year with normal rainfall (1990). These results indicate that *B. gracilis* compensates for tissue losses from herbivory when provided sufficient water and nutrients to support photosynthesis and growth.

Foliar chemistry of *B. gracilis* is highly variable among years; variability in foliar chemical concentrations should increase in response to stress according to variability in plant stress. This is to be expected, as critical soil nutrients, light, and water required for plant growth routinely shift in time and space (Bazzaz and Grace, 1997) under natural conditions. Water stress regularly resulted in increased concentrations of foliar TN in all years and was the most important stress to *B. gracilis*. However, N fertilizer, grasshopper, and year effects contributed greatly to the expression of TN, showing a variety of outcomes among years.

Insect herbivory should affect plants in a manner similar to other environmental stresses in that it alters the capacity for photosynthesis by removing leaf material and changes source–sink relationships to favor regrowth of leaves. Photosynthesis, growth, and foliar nutrients routinely vary in response to the timing and degree of herbivory (Redak and Capinera, 1994). Of greatest interest is the highly variable nature of responses of foliar

chemistry to grasshopper herbivory, especially among years and the large number of interactions that were observed between other plant stresses and grasshopper herbivory.

The Plant Stress Hypothesis. By influencing metabolic activity in general, environmental stresses often alter plant resistance to herbivory, especially because of changes in foliage quality to herbivores (Rhoades, 1983; Bazzaz et al., 1987; Mattson and Haack, 1987; Louda and Collinge, 1992). For example, reduced soil water availability often reduces resistance to herbivory because of increased nutrient concentrations in leaf tissue available to herbivores (McNeil and Southwood, 1978; White, 1993; Redak and Capinera, 1994), decreased or elevated concentrations of defensive compounds (Rhoades, 1983; Gershenzon, 1984; Redak and Capinera, 1994), or some trade-off between nutritional and defensive qualities in leaf tissues that make them more or less palatable to herbivores (Bazzaz et al., 1987). Consequently, increased nutritional quality combined with decreased defensive capability results in improved herbivore performance. Mature grasses contain few chemical defenses compared to other plant taxa (Mole and Joern, 1994), thus simplifying the problem. Primary nutrients are also typically found at much lower concentrations in grasses than are typically observed in forbs and wood plants, decreasing the plant's value as food to herbivores (Bernays and Barbehenn, 1987). Given the expected low concentrations of limiting nutrients relative to consumer needs, small shifts in their availability may provide large fitness consequences to individuals feeding on them (Joern and Behmer, 1997).

B. gracilis responded to multiple stresses more or less as expected, but grasshopper performance was not consistent with predictions of the plant stress hypothesis. No changes in developmental rate were observed in either species, and survival in *P. nebrascensis* showed no significant differences. Survival in *A. deorum* differed among stress treatments, but higher survival was observed in treatments with lower levels of foliar TN, contrary to survivorship patterns expected based on feeding studies with controlled diets (Joern and Behmer, 1997).

Reviews of the plant stress hypothesis on woody plants (Waring and Cobb, 1992; Koricheva et al., 1998; Huberty and Denno, 2004) indicate weak support for the notion at best. No significant concordance with expectations was observed in a meta-analysis of experimental studies (Koricheva et al., 1998; Huberty and Denno, 2004), although comparative studies in the field were reasonably consistent with expectations (Waring and Cobb, 1992; Watt, 1992). Fewer studies are available for nonwoody plants, especially for grasses, and results generally conflict with predictions of the plant stress hypothesis. Redak and Capinera (1994) showed that heavy defoliation of western wheat grass (*Pascopyrum smithii*) either by mechanical means in the laboratory or from herbivory by *P. nebrascensis* in the field altered foliar nutrients and palatability,

but in the opposite direction required for support. Similarly, polyphagous leaf miners on grasses respond positively as expected to foliar nutritional quality, but fertilizer stress has a negative impact on population responses (Scheirs and De Bruyn, 2004).

Without doubt, insect herbivores typically encounter heterogeneous nutritional landscapes while foraging, one largely resulting from the integrated responses of plants to variable environmental conditions (Jones and Coleman, 1991; Louda and Collinge, 1992). In turn, variable nutritional quality is expected to directly influence herbivore fitness and subsequent population fluctuations (Rhoades, 1983; Jones and Coleman, 1991; White, 1993). However, responses by chewing insect herbivores to plant quality are highly variable, with some species responding positively and others negatively or not at all to specific responses (Joern and Behmer, 1997; Fischer and Fielder, 2000). This is especially evident for understanding the dynamic changes in foliar TN and TNC in response to combined stress, which in turn affect subsequent levels of insect herbivory.

Results from our study with grasshoppers are consistent with others that examine free-living, chewing insects. Reviews of the plant stress hypothesis indicate that under continuous stress, most insect herbivore guilds either are negatively impacted or do not respond to plant stress (Watt, 1992; Koricheva et al., 1998). The best current model (Huberty and Denno, 2004) may be the "pulsed plant stress hypothesis" in which foliar nutrients accumulating from stress conditions only become available to herbivores, especially sap feeders, as plants recover from stress. Still, many other insect herbivore guilds are not explained.

Nutritional quality of host plants generally affects performance by grasshoppers, as documented by laboratory and field studies (Joern and Behmer, 1997; Simpson and Raubenheimer, 2001). Why does the plant-stress hypothesis not explain responses by free-living chewing insects, such as grasshoppers, despite the appealing logic inherent in the formulation of plant stress hypothesis? A combination of multiple factors might explain. It may not be possible to directly relate performance to stress treatments because of intervening pathways that interact in unknown ways. (1) Grasshoppers perform best on a diet that is balanced between protein and carbohydrates; as nutritional ratios deviate from the target, performance drops as well. Stressed plants may have elevated foliar protein that is not always in balance with carbohydrates. (2) Grasshoppers can compensate for poor-quality food by altering diets (Simpson and Abisgold, 1985) or by modifying retention time in the gut (Yang and Joern, 1994), a response that could affect nutrient acquisition and insect performance in a nonlinear fashion. Lack of response to stress may actually be "hidden" because of such physiological adjustments. (3) As observed in this study, chemical responses in *B. gracilis* leaves to stress treatment combinations are highly variable, making it particularly difficult to predict performance. Response by grasshoppers may best fit predictions of Price's (1991) "plant vigor hypothesis",

which argues for elevated insect herbivore performance on tissues in actively photosynthesizing tissue; this may allow for a wider range of combinations of protein and carbohydrate. It is noteworthy that TNC and TN concentrations among leaf samples were uncorrelated (unpublished data), making it particularly difficult for grasshoppers to use simple phagostimulatory cues to select balanced diets. (4) Effects of stresses themselves to plants are multifactorial (water, temperature, trampling, N addition, herbivory) such that the expected effects on grasshoppers are not predicted by simple plant stress models; Jones and Coleman (1991) provide a basis for a more comprehensive model. Ultimately, some combination of each of the above and other factors influencing foraging by free-living grasshoppers will determine the relative contribution of plant stress to performance in combination with other factors.

It is important to resolve the ability to relate insect herbivore response to plant stress. Historically, forecasts of insect pest outbreaks including grasshoppers often assume that insect populations do better under hot/dry weather conditions because of better food quality (Rhoades, 1983; Mattson and Haack, 1987). This weather-induced link between plant stress and insect performance is often circumstantial at best (Huberty and Denno, 2004) and remains to be vetted carefully before it is recognized as a key mechanism underlying insect outbreaks. However, it is also true that many experimental studies, including those with grasshoppers, implicate elevated host plant quality as an important determinant of insect performance and population responses in the field. Alternate explanations that include multiple factors (Belovsky and Joern, 1995) or incorporate more sophisticated views of nutritional contributions and environmental influences on observed population variability (Simpson and Raubenheimer, 2001) must be developed to fill the void left by the inability of the plant stress hypothesis to explain natural patterns.

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THE EFFECTS OF ANTS ON THE ENTOMOPHAGOUS BUTTERFLY CATERPILLAR *Feniseca tarquinius*, AND THE PUTATIVE ROLE OF CHEMICAL CAMOUFLAGE IN THE *Feniseca*–ANT INTERACTION

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Abstract—Butterfly caterpillars in the lycaenid subfamily Miletinae are predators of ant-tended Homoptera, yet they lack specialized secretory and call-production organs crucial to ant association in other lycaenids. Here, we address the question of how miletine caterpillars have invaded the ant–Homoptera symbiosis through a study of the only New World miletine, *Feniseca tarquinius*, a predator of the wooly aphid *Prociphilus tesselatus*. Previous interpretations have suggested that *F. tarquinius* and other miletine caterpillars avoid ant aggression by concealing themselves under silken webs. In contrast, our field data indicate that *F. tarquinius* caterpillars are less likely to be concealed in the presence of the ants *Camponotus pennsylvanicus* and *Formica obscuriventris* than in the absence of ants, although caterpillar and ant behaviors vary between years. Chemical analysis and behavioral assays suggest that chemical camouflage, not physical concealment, is responsible for the ants' failure to detect and remove *F. tarquinius* caterpillars from aphid colonies. Analyses by gas chromatography indicate that the cuticular lipid composition of caterpillars are similar to that of their aphid prey, although it varies with prey species. Behavioral assays confirm that solvent extracts of *F. tarquinius* caterpillars and *P. tesselatus* aphids evoke similar behavioral responses in *C. pennsylvanicus* ants. Chemical camouflage is well known in social parasites

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of ants, but the present study represents one of a few documented cases where chemical deceit is important to interactions with ants outside the nest.

Key Words—Lycaenidae, Miletinae, *Feniseca tarquinius*, chemical camouflage, cuticular hydrocarbons, lycaenid–ant interactions, carnivorous caterpillars, *Camponotus pennsylvanicus*, Eriosomatidae, *Prociphilus tesselatus*.

INTRODUCTION

Caterpillars in the butterfly family Lycaenidae are unique in their propensity to form symbioses with ants. An estimated 6000 species of lycaenids account for nearly 50% of all butterflies, and about 75% of documented lycaenid caterpillars are associated with ants (DeVries, 2001; Pierce et al., 2002). Many lycaenid–ant associations are deemed mutualistic; here, ants protect caterpillars from predators and parasitoids in exchange for food secretions, and caterpillars mediate these symbioses through a suite of secretory and call production organs (Malicky, 1970; Atstatt, 1981; Cottrell, 1984; DeVries, 1988, 1990; Cushman et al., 1994; Pierce et al., 2002). Other lycaenid caterpillars are social parasites that infiltrate ant nests and consume ant brood or food regurgitations (Cottrell, 1984; Pierce, 1995). These caterpillars appear to employ one of two strategies. Some are recognized as intruders but are heavily armored to survive attack; others mimic the cuticular hydrocarbons by which ants recognize their brood and are accepted into the ant nest where they are tended and sometimes fed (Cottrell, 1984; Akino et al., 1999).

In marked contrast to other lycaenid groups, the predominantly Old World subfamily Miletinae has a unique relationship with Homoptera and ants. Miletine adults feed on the honeydew of homopterans, and the caterpillars are predators of the same homopterans, which are also tended by ants for their honeydew excretions (Clark, 1926; Cottrell, 1984). Thus, unlike other lycaenids, miletines compete with ants for homopteran resources (Cottrell, 1984; Maschwitz et al., 1988). All miletine caterpillars lack the secretory organs considered crucial to myrmecophily, and they are not exceptionally well armored (Cottrell, 1984). This raises the question of how miletines have invaded the ant–homopteran symbiosis, and how ants affect miletine fitness.

Our understanding of miletine–ant interactions is drawn from largely anecdotal evidence. Miletine caterpillars are often concealed under silken webs thought to protect them from aggressive ants (Edwards, 1886; Scudder, 1889; Atstatt, 1981; Cottrell, 1984). It appears that some miletine caterpillars are attractive or neutral to ants or are bitten only in moments of stress; others are known to survive within ant nests, perhaps as social parasites (Cottrell, 1984; Kitching, 1987; Maschwitz et al., 1988; Pierce, 1995). Further evidence is needed to clarify the nature of miletine–ant interactions. Here, we address the

behavioral and chemical ecology of the miletine caterpillar *Feniseca tarquinius* (Fabricius) and the ants that tend its aphid prey.

F. tarquinius is the only known miletine in the New World and is the sole representative of the genus. It is widespread in eastern North America and feeds predominantly on wooly alder aphids, *Prociphilus tesselatus* (Fitch), that are facultatively tended by various formicine, dolichoderine, and myrmicine ants (Scudder, 1889; Clark, 1926; Holldobler and Wilson, 1990; Youngsteadt, personal observations). Like other miletine caterpillars, *F. tarquinius* often conceals itself under a silken web covered with carcasses and "wool" of its aphid prey. A series of field and garden observations in the late 1800s gave rise to the prevailing hypothesis that physical concealment is crucial to survival of *F. tarquinius* caterpillars among ants (Edwards, 1886; Scudder, 1889).

Observations presented here suggest that physical concealment is not a defense against ants, but support the alternative hypothesis that *F. tarquinius* caterpillars are chemically concealed among their prey. Chemical mimicry and camouflage have been documented among taxonomically diverse nest parasites of social insects. Such parasites infiltrate their host's nestmate recognition system by bearing cuticular hydrocarbons similar to those of the host (reviewed in Holldobler and Wilson, 1990; see also Howard et al., 1990; Akino et al., 1996, 1999; Allan et al., 2002). Chemical similarity is deemed mimicry if a parasite synthesizes host-like hydrocarbons or camouflage if the parasite incorporates host-synthesized hydrocarbons into its own cuticle (Howard et al., 1990; but see alternative definitions in Dettner and Liepert, 1994). The mechanism by which camouflaged insects incorporate host hydrocarbons into their cuticles probably varies among species. One mechanism, implied or demonstrated in various insect–insect interactions, is passive transfer of hydrocarbons by physical contact (e.g., Vander Meer and Wojcik, 1982; Akino et al., 1996; Liang and Silverman, 2000).

Ants may also defend resources outside the nest, such as homopterans and extrafloral nectaries (Way, 1963; Holldobler and Wilson, 1990; Huxley and Cutler, 1991). Other insects invade these symbioses as competitors and circumvent ant defenses by various means (e.g., Eisner et al., 1978; DeVries and Baker, 1989; Liepert and Dettner, 1993, 1996). However, with the exception of one aphid parasitoid that evades ant aggression by chemical resemblance of its host aphids (Liepert and Dettner, 1993, 1996), the role of cuticular hydrocarbons in competitive interactions with ants outside the nest has received little attention. The present study of *Feniseca*–ant interactions offers further evidence for the role of chemical deceit in the exploitation of ant-tended resources outside the nest.

Here, we examine aspects of the *Feniseca*–ant interaction through field observations, chemical analyses, and behavioral assays that address two questions. First, is caterpillar physical concealment a defense against ants?

Second, is chemical camouflage responsible for the ants' apparent inability to perceive *Feniseca* caterpillars?

METHODS AND MATERIALS

Field Observations. To characterize the *Feniseca*–ant interaction, field observations were made on *F. tarquinius* caterpillars during August and September 2001, and July through September 2002, at two study areas in southeast Wisconsin (USA). At one site, the carpenter ant *Camponotus pennsylvanicus* (DeGeer) was the predominant species tending *P. tesselatus* aphids; at the other, *Formica montana* Emery and *F. obscuriventris* Mayr dominated. Ants were identified by using Creighton (1950), and voucher specimens are housed in Youngsteadt's collection.

In 2001, caterpillars were censused daily on all known aphid colonies and the following data were recorded: species and number of ants present on the host aphid colony, dimensions of the aphid colony, caterpillar instar, and presence or absence of a silken web over the caterpillar. Direct caterpillar–ant interactions and caterpillar disappearance were also recorded. Caterpillars develop over four instars, each 2–5 days in duration, before dropping from the aphid colony and wandering to a pupation site on other vegetation (Scudder, 1889; Youngsteadt, personal observations). Therefore, disappearance of a caterpillar prior to the second day of its fourth instar was attributed to death; later disappearance was attributed to pupation.

In 2002, censuses were conducted weekly, so fates of individual caterpillars were not followed; all other variables were recorded as in 2001. Survey intervals made it unlikely that an individual caterpillar was recorded repeatedly and certainly never more than once in a given instar.

Dependence of concealment behavior on presence and species of ants was evaluated by using a series of Fisher's exact tests (Sokal and Rohlf, 1981), in which, for each caterpillar instar, behavior in the presence of each ant species was compared with behavior in the absence of ants. Hence, up to three comparisons were performed per instar. Resultant P values were subjected to Bonferroni corrections to reflect family-wise type II error rates within each instar. Site effects were not considered in the analysis of concealment behavior because the entire study area was composed of similar habitat, caterpillar behavior varied between ant species within a site, and caterpillars at nontended colonies behaved the same way at both sites. Pupation rate was also tested for ant dependence with a Fisher's exact test (Sokal and Rohlf, 1981). A Mann–Whitney U-test compared ant density on aphid colonies between 2001 and 2002 (Sokal and Rohlf, 1981).

Chemical Analysis. Cuticular lipid composition of *F. tarquinius* caterpillars was compared to that of ants and aphids to evaluate whether chemical similarity to another species played a role in ants accepting caterpillars on aphid colonies. When results indicated that caterpillar surface lipid composition was similar to that of *P. tesselatus*, caterpillars were also reared on a novel aphid host to test whether caterpillars were true *P. tesselatus* mimics or whether they were camouflaged with surface lipids acquired from the host. Live specimens of *P. tesselatus*, *F. tarquinius*, *F. montana*, and *F. obscuriventris* were collected, frozen, and immediately stored at −80°C until analysis.

For the mimicry vs. camouflage experiment, five *F. tarquinius* caterpillars were reared from the second instar on an unidentified dogwood aphid species (Aphididae). Well into the fourth instar, caterpillars and their dogwood aphid hosts were killed and stored as above.

To extract cuticular lipids, insects (1 caterpillar, 5 ants, 7 *P. tesselatus*, or 60 dogwood aphids per extract) were placed in a 10 ml borosilicate conical bottomed screw-cap centrifuge tube and immersed in two sequential 3 ml hexane washes for 2.5 min each. The two washes were combined and spiked with 7.5 μg each of *n*-tricosane ($C_{23}H_{48}$) and *n*-dotriacontane ($C_{32}H_{66}$) as internal standards. The extract was concentrated to dryness under a gentle stream of N_2 and resuspended in 30 μl hexane. Periodically, a hexane blank including internal standards was prepared by the same method to confirm absence of contamination.

Extracts were analyzed by gas–liquid chromatography (GC). The gas chromatograph was a Hewlett-Packard 5890-II equipped with flame ionization detector and HP 3396-II integrator. Injection was split, with a split ratio of 129:1; injections were manual, 1.5 μl. The column was a fused silica capillary column (30 m × 0.32 mm) with a 1-μm DB-1 stationary phase. Helium was the carrier gas at a flow of 0.5 ml/min. Programmed conditions were as follows: injection port 280°C, FID 320°C, oven at 80°C 1 min, ramp at 10°C/min, 310°C for 30 min.

Components were distinguished by retention times. Bray–Curtis similarities, computed with fourth root transformed relative abundances of 42 components, were used to compare lipid profiles. The similarity matrix of pairwise comparisons among all samples was represented in a nonmetric multidimensional scaling ordination plot and analyzed in the statistical package Primer Version 5. Clarke (1993) and Elmes et al. (2002) discussed these methods. Included in the analysis were 5 extracts each of *P. tesselatus* and *F. tarquinius*, 3 extracts each of *F. montana*, dogwood aphids, and *F. tarquinius* reared on dogwood aphids, and 2 extracts of *F. obscuriventris*. Total cuticular lipids were considered, and these may include, in addition to hydrocarbons, other lipid classes not specifically implicated in ant recognition systems (Singer, 1998; Lahav et al., 1999).

To describe robust patterns of peak presence and absence underlying the mathematical similarities between caterpillars and aphids, we prepared a reduced dataset that included only those components that were present in all individuals of at least two species, or that were present in all individuals of at least one species but absent in all individuals of at least one other species. Excluded from this dataset were components that were variable in all species, present in one species but variable in all others, or absent in one or more species but variable in others. The remaining "indicator peaks" form the basis for descriptive statements about patterns of peak presence and absence.

Behavioral Assays. To test for behavioral relevance of the hexane extracts analyzed by GC, comparable extracts were offered to workers from three different *C. pennsylvanicus* colonies in the context of *P. tesselatus* colonies during September 2002. Extracts and blanks were prepared as described above, excluding the internal standards. Except for ants and aphids (as above), each extract was of a single insect. Solvent blanks ($N = 5$) and extracts of the following insects were offered: *Formica ulkei* Emery ($N = 7$ extracts); *P. tesselatus* ($N = 6$); adults of the aphid predator *Harmonia axyridis* (Pallas) (Coleoptera: Coccinelidae) ($N = 4$); and fourth instar *F. tarquinius* caterpillars ($N = 6$).

In the field, filter paper disks (5-mm diam) were treated with 30-μl insect extract (i.e., equivalent of 1 caterpillar, 1 beetle, 5 ants, or 7 aphids) or pure hexane, allowed to dry, then pinned onto the alder adjacent to the tended aphid colony. A single experimental set consisted of a blank and a series of the 4 extracts, or a subset thereof, in variable haphazard order. If conditions allowed for a second set to be presented immediately to the same ants, it was presented in reverse order to the previous series. Ant response to each extract was recorded for 20 min. Total number of ants present was recorded, along with the number of each of the following behaviors: lunge bite, bite, open-jawed lunge, open jawed inspection, closed-jawed inspection, ignore. Repeated instances of the same behavior were recorded when an ant discontinued, then reestablished physical contact with the disk. An encounter was scored as "ignore" if an ant made antennal contact with the disk in passing, but did not maintain contact or show observable change in behavior or orientation.

For analysis, behaviors were pooled as either aggressive or nonaggressive, and ant response to each extract or blank was quantified in two ways: proportion of aggressive acts out of total number of encounters and number of aggressive acts per ant per minute. Ant response to blanks and extracts was compared using two Kruskal–Wallis tests—one for proportional aggression and one for absolute aggression (Sokal and Rohlf, 1981). Transformation of proportion data was not necessary in this case (Zar, 1999). To test for qualitative differences in ant aggression toward different extracts and blanks, aggression was subdivided into its four component categories. Using a logistic regression model in the SAS

system proc genmod, extracts and blanks were tested for differences in the likelihood of eliciting each category of aggressive behavior.

Caterpillar Morphology. To determine whether the setae of *F. tarquinius* caterpillars are modified in such a way as to enhance collection or dissemination of semiochemicals, we preserved field-collected fourth instar *F. tarquinius* in 80% EtOH and examined them under a scanning electron microscope.

RESULTS

Field Observations. Table 1 presents frequencies of caterpillar concealment in 2001 and 2002. In 2001, behavior of second through fourth instar *F. tarquinius* caterpillars appeared to depend on the presence and species of ant: caterpillars were concealed less often in the presence of *C. pennsylvanicus* and *F. obscuriventris* than *F. montana* or no ants. First instar caterpillars showed a similar trend. In 2002, this effect was marginally significant only for third instars. Other caterpillars were predominantly concealed regardless of ants. However, density of attendant *C. pennsylvanicus* differed significantly among years (Figure 1). Ants tended *P. tesselatus* colonies at a consistently higher density in 2001, but this was lower and more sporadic in 2002.

Caterpillar survival data were available only for one site in 2001. Here, 54% of caterpillars on nontended aphid colonies disappeared prior to pupation, whereas only 35% of those on *C. pennsylvanicus*-tended colonies disappeared. Pupation among treatments did not differ significantly ($P = 0.472$, $N = 13$ for nontended colonies, 20 for tended colonies).

Few direct caterpillar–ant interactions were observed: ants generally ignored caterpillars or tapped them with their antennae before moving away. On one occasion, when a tended aphid colony was disrupted by the observer bumping a branch, a *C. pennsylvanicus* bit a third instar caterpillar and dropped it from the colony. However, the caterpillar remained suspended on a thread and returned to the colony unharmed and without further incident. Once a *C. pennsylvanicus* ant was observed to kill and consume a freshly pupated *F. tarquinius* that had anchored itself on a vine entwined with a tended aphid-infested alder.

Chemical Analysis. Representative chromatograms are shown in Figure 2. Mean Bray–Curtis pairwise similarities (Table 2) indicate that cuticular lipid similarity was higher within species (77–90%) than between any pair of species (41–76%). Of the between-species comparisons, two similarities were unusually large: that between *P. tesselatus* and "normal" *F. tarquinius* that had eaten *P. tesselatus* (76%) and that between "normal" *F. tarquinius* and *F. tarquinius* that ate dogwood aphids (71%). All other between-species comparisons,

TABLE 1. FREQUENCY OF CATERPILLAR CONCEALMENT ON APHID COLONIES IN THE ABSENCE OR PRESENCE OF EACH OF THREE ANT SPECIES

Ant species	*N*	Percentage of caterpillars concealed (%)	*P* (two-tailed)
Year: 2001 (caterpillars censused on 29 aphid colonies)			
Fourth instar			
C. pennsylvanicus	12	0	<0.001
F. obscuriventris	12	0	<0.001
F. montana	11	73	ns
No ants	13	85	
Third instar			
C. pennsylvanicus	12	17	<0.001
F. obscuriventris	4	0	<0.005
F. montana	11	91	ns
No ants	10	100	
Second instar			
C. pennsylvanicus	15	60	<0.05
F. obscuriventris	3	67	ns
F. montana	11	91	ns
No ants	19	100	
First instar	15		
C. pennsylvanicus	4	75	ns
F. montana	3	100	ns
No ants	8	100	
Year: 2002 (caterpillars censused on 34 aphid colonies)			
Fourth instar			
C. pennsylvanicus	19	79	ns
F. montana	6	67	ns
No ants	23	87	
Third instar			
C. pennsylvanicus	17	76	0.07
F. montana	7	100	ns
No ants	20	100	
Second instar	33		
C. pennsylvanicus	14	100	ns
No ants	19	100	
First instar	6		
C. pennsylvanicus	3	100	ns
No ants	3	100	

Probabilities are shown for pairwise comparison of each ant species to "no ants" (Fisher's exact test with Bonferroni correction). No data are presented for *F. obscuriventris* in 2002 because no aphids established near the one known *F. obscuriventris* nest.
ns = Not significant.

including those between caterpillars and ants, yielded similarities ≤57%. The nonmetric MDS ordination plot (Figure 3) discriminates among species. Analysis of similarities (ANOSIM) is typically used to test for significance of MDS groupings (Clarke, 1993). The global ANOSIM test was significant for this

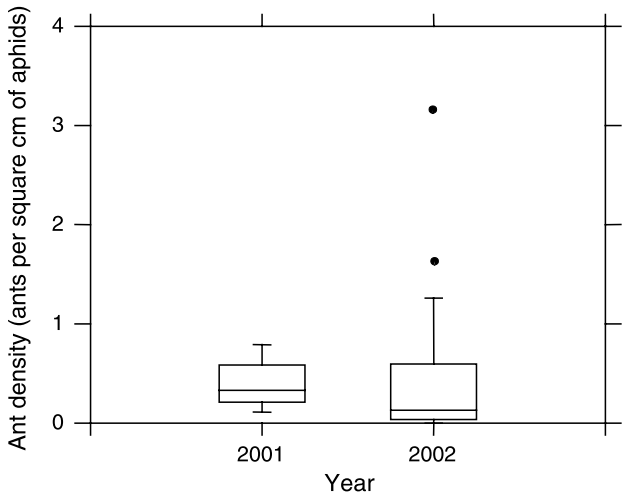

FIG. 1. Density of attendant *C. pennsylvanicus* ants on *P. tesselatus* aphid colonies in 2001 and 2002 based on 32 and 40 measurements, respectively. Mann–Whitney U = 859.0, P = 0.013. Each box encompasses the midrange; central horizontal line is the median; whiskers extend to furthest data points within 1.5 midranges of q1 and q3. Data points further dispersed are indicated by dots.

dataset (R = 0.97, P = 0.001), but sample sizes were too small for valid pairwise comparisons between species.

The "indicator peaks" found in *P. tesselatus*, "normal" *F. tarquinius*, dogwood aphids, and *F. tarquinius* that ate dogwood aphids are presented in Table 3. *P. tesselatus* and "normal" *F. tarquinius* have 15 indicator peaks in common, five of which are unique to this pair; they differ by only three. Of these three, two are present in *F. tarquinius* but not in *P. tesselatus*, whereas one is present in *P. tesselatus* but not in *F. tarquinius*.

All peaks present on *F. tarquinius* caterpillars that ate dogwood aphids were a subset of those found on "normal" *F. tarquinius*—including the two peaks absent in *P. tesselatus* aphids. On the other hand, "normal" *F. tarquinius* had five additional peaks not found in *F. tarquinius* that ate dogwood aphids, and all of these were shared with *P. tesselatus*. Peak 15 was notably more abundant in both caterpillar groups than in either aphid species.

Dogwood aphids had a simple lipid profile that included 10 indicator peaks. Two of these were absent from *F. tarquinius* that ate dogwood aphids. Six peaks were common to dogwood aphids and caterpillars that ate them, but were not unique to this pair, and three of these differed markedly and consistently in abundance. Finally, *Feniseca* that ate dogwood aphids had three

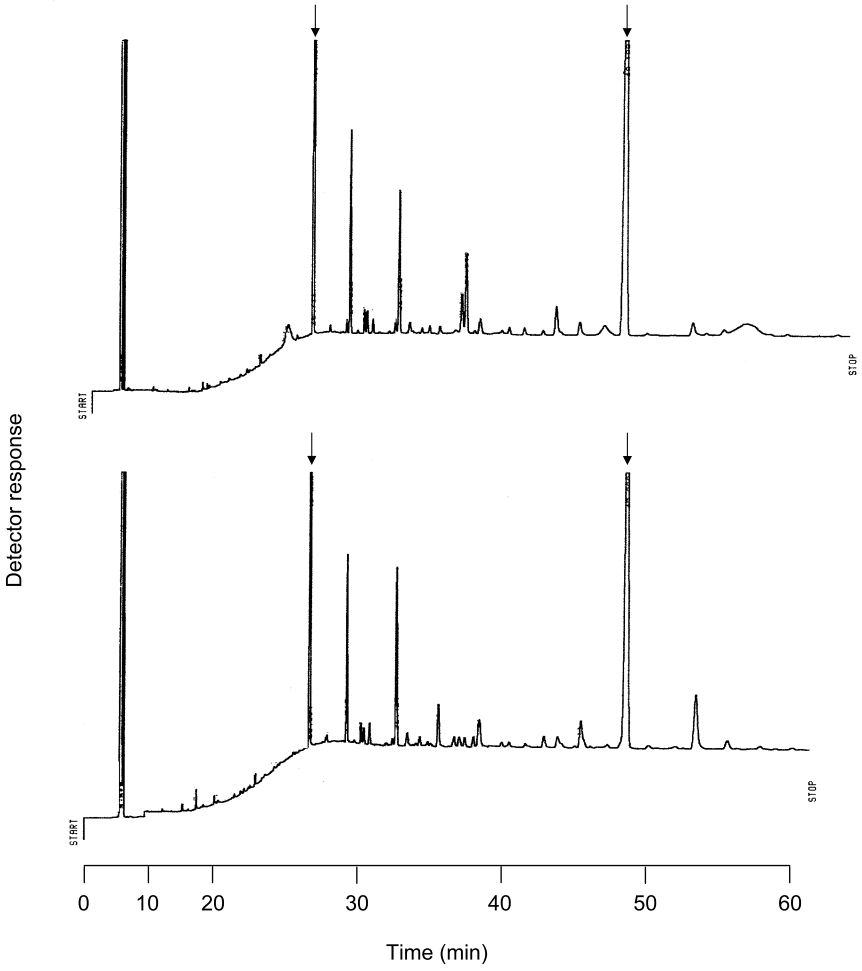

FIG. 2. Gas chromatograms of hexane extract of cuticles of fourth instar *F. tarquinius* caterpillar (top) and adult *P. tesselatus* aphids (bottom). Internal standards n-C_{23} and n-C_{32} are indicated by arrows.

peaks that were absent from those aphids, but which also occurred in normal *F. tarquinius*.

Although the *F. tarquinius* lipid profile varied with diet, the amount of lipids extracted did not differ between the two groups of caterpillars. Total cuticular lipids recovered per caterpillar were 4.73 ± 1.058 μg (mean ± SD) for normal caterpillars and 5.45 ± 0.433 μg for caterpillars reared on dogwood aphids (N = 8 and 5, respectively).

TABLE 2. MEAN BRAY–CURTIS PERCENT SIMILARITIES ± SDS WITHIN AND BETWEEN SPECIES IN THE *Feniseca* SYSTEM BASED ON 42 CUTICULAR LIPID COMPONENTS

	Pt	Ft	FD	DA	Fm	Fo
Pt	89.5 ± 4.9 (10)					
Ft	76.1 ± 4.3 (25)	86.9 ± 4.8 (10)				
FD	53.4 ± 4.3 (15)	70.5 ± 3.2 (15)	76.9 ± 9.5 (3)			
DA	52.8 ± 3.6 (15)	54.6 ± 6.9 (15)	57.2 ± 6.2 (9)	85.0 ± 2.5 (3)		
Fm	40.8 ± 3.0 (15)	53.3 ± 2.6 (15)	54.5 ± 4.4 (9)	43.1 ± 5.8 (9)	85.5 ± 4.8 (3)	
Fo	51.8 ± 3.0 (10)	51.8 ± 2.2 (10)	52.4 ± 3.1 (6)	49.3 ± 2.4 (6)	41.6 ± 5.5 (6)	77.8 (1)

Numbers of pairs are reported in parentheses.
Pt, *P. tesselatus* aphids; Ft, *F. tarquinius* caterpillars reared on *P. tesselatus*; FD, *F. tarquinius* reared on dogwood aphids; DA, dogwood aphids; Fm, *F. montana* ants; Fo, *F. obscuriventris* ants.

Behavioral Assays. Ant response to filter papers treated with solvent and insect extracts is shown in Figure 4, as proportion of aggressive acts. The absolute aggression measure yielded an identical pattern (Kruskal–Wallis test statistic = 18.89, $P < 0.001$; Bonferroni–Dunn *post hoc* test results were also exactly as described for relative aggression in Figure 4). *C. pennsylvanicus*

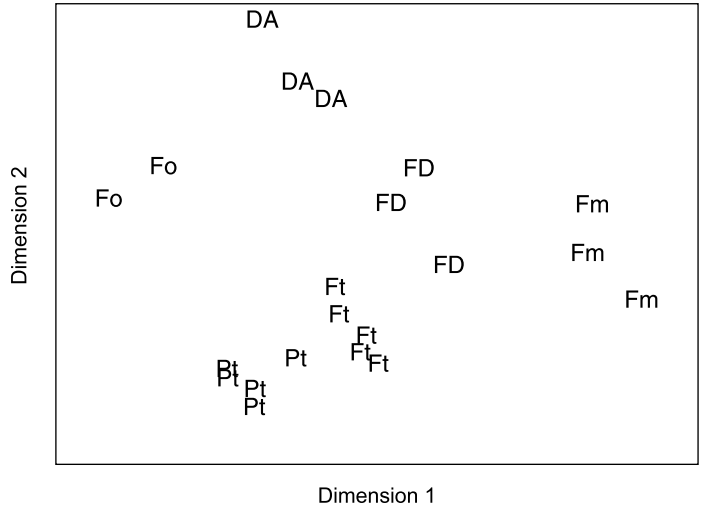

FIG. 3. Two-dimensional nonmetric multidimensional scaling ordination of 21 samples, derived from Bray–Curtis similarities between each pair of samples (stress = 0.13). Species are *P. tesselatus* aphids (Pt), *F. tarquinius* caterpillars reared on *P. tesselatus* (Ft), *F. tarquinius* reared on dogwood aphids (FD), dogwood aphids (DA), *F. montana* ants (Fm), and *F. obscuriventris* ants (Fo).

TABLE 3. PRESENCE AND ABSENCE OF "INDICATOR PEAKS" IN *F. tarquinius* CATERPILLARS AND APHIDS

Peak	Retention time (min)	Pt	Ft	DA	FD
1	28.06	x	x	0	x
2	29.15	0	x	0	x
3	29.38	x	x	x	x
4	30.34	x	x	0	0
5	30.52	x	x	(v)	(v)
6	30.91	x	x	x	x
7	32.42	(v)	x	(v)	x
8	32.67	x	x	x	x
9	33.41	x	x	0	0
10	34.47	0	x	0	x
11	34.97	(v)	x	(v)	x
12	35.46	x	(v)	0	(v)
13	36.51	x	0	0	0
14	36.83	x	x	x	x
15	37.20	x	x	x	x
16	38.18	x	x	0	0
17	41.17	x	x	x	x
18	42.43	x	x	0	0
19	43.32	x	x	x	(v)
20	44.89	x	x	0	0
21	49.35	x	(v)	x	0
22	52.51	x	x	x	(v)
23	56.31	0	0	x	0

Pt, *P. tesselatus* aphids; Ft, *F. tarquinius* caterpillars reared on *P. tesselatus*; DA, dogwood aphids; FD, *F. tarquinius* reared on dogwood aphids. x indicates presence of a peak in all individuals of a "species", 0 indicates absence of a peak in all individuals of a species, and (v) indicates variability: the peak is present in some but not all individuals of the species.

workers were continuously hostile toward extracts of other ants and the aphid predator *H. axyridis*. Ants were initially aggressive toward caterpillar and aphid extracts and blanks—it was impossible to introduce samples onto aphid colonies without ants responding to the intrusion—but they quickly accepted the samples and did not display further aggression.

Ant response did not depend on the order in which extracts were offered. A Kruskal–Wallis test with position, rather than extract identity, as the grouping variable was not significant, and we observed that ant response to a given extract was consistent regardless of when it was presented relative to other extracts. Differences in aggression toward extracts and blanks were quantitative, not qualitative. Extracts and blanks did not differ in the likelihood of eliciting the four categories of aggressive behaviors (uncorrected P values ≥ 0.7 for all 10 comparisons between extracts and blanks).

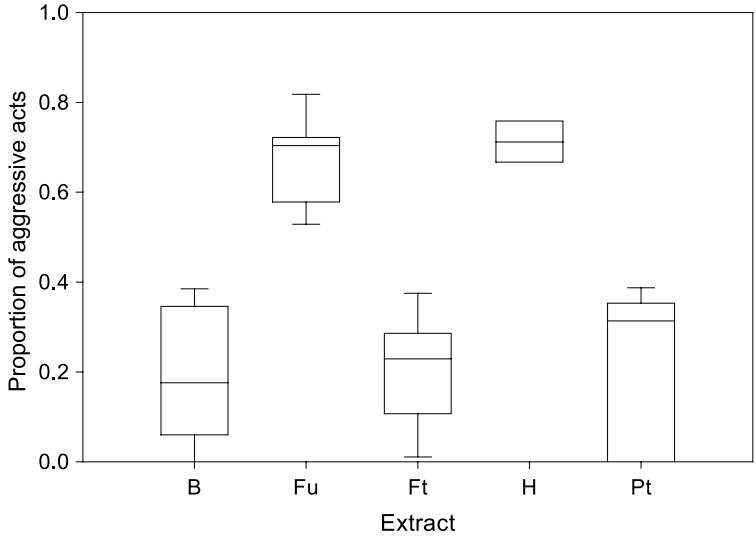

FIG. 4. *C. pennsylvanicus* response to filter paper dummies introduced to tended *P. tesselatus* aphid colonies. *Y*-axis indicates proportion of aggressive acts out of total number of encounters. Dummies were treated with solvent blank (B) or extract of one of the following insects: *F. ulkei* ants (Fu), *F. tarquinius* caterpillars reared on *P. tesselatus* aphids (Ft), *H. axyridis* beetles (H), or *P. tesselatus* (Pt). Median, box, and whiskers are as in Figure 1. Kruskal–Wallis test statistic = 19.623, $P < 0.001$. Bonferroni–Dunn *post hoc* comparisons confirmed that ant response to both *F. ulkei* and *H. axyridis* differed from response to each of the other three extracts ($P < 0.001$ for those six comparisons, ns for all others).

Caterpillar Morphology. Typical body setae of a fourth instar *F. tarquinius* caterpillar are shown in Figure 5. These setae are simple, resembling ordinary tactile and defensive body setae found in a wide range of nonmyrmecophilous lycaenid caterpillars (DeVries 1997, unpublished data).

DISCUSSION

Effects of Ants on Caterpillars. Given the prevailing notion that ants select for concealment behavior in *F. tarquinius* caterpillars (Edwards, 1886; Scudder, 1889; Atstatt, 1981; Cottrell, 1984), it was surprising to observe the reverse pattern in the field in 2001 (Table 1). It is tempting to speculate that concealment protects caterpillars from natural enemies, a function performed by ants when they are present. It is unclear why *F. montana* had no effect on

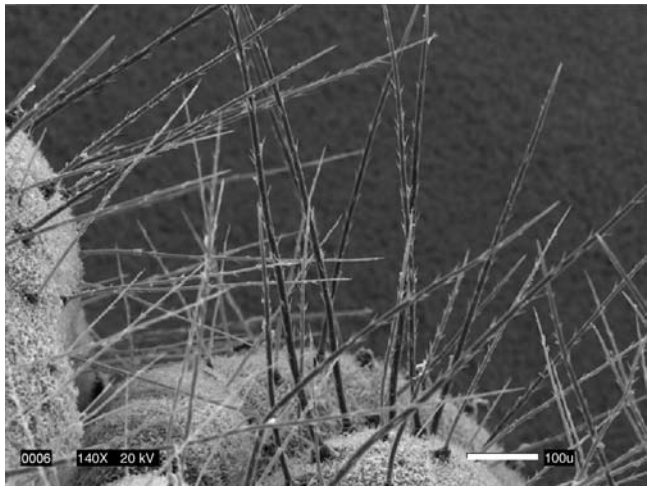

FIG. 5. Scanning electron micrograph of dorsolateral setae on the third thoracic (left) and first abdominal (right) segments of a fourth instar *F. tarquinius* caterpillar.

caterpillar concealment, but it may relate to interspecific differences in ant behavior. Although ant behavior was not quantified, it appeared that, relative to the other two species, *F. montana* had a higher rate of worker turnover on aphid colonies, was less likely to assume defensive posture, and more likely to move away or drop off the plant when disturbed. If these characteristics made *F. montana* less likely to deter intruders from aphid colonies, caterpillar behavior was consistent with the interpretation that caterpillars are concealed when not defended, albeit inadvertently, by ants. Similarly, low and sporadic density of *C. pennsylvanicus* in 2002 may have decreased its protective value and contributed to more universal caterpillar concealment that year.

In 2002, we reproduced the alder–wooly aphid system in a screened experimental garden. Alders were paired, and *C. pennsylvanicus* ants were allowed to tend aphids on one tree in each pair. Paired caterpillars were introduced to aphid colonies on paired trees. In this experiment, there was no effect of *C. pennsylvanicus* on *F. tarquinius* caterpillar growth rate, survival, or behavior. Thus, we found no evidence, in the field or garden, that ants were a threat to *F. tarquinius* caterpillars. Our suggestion that aggressive ants tending *P. tesselatus* colonies also defend resident caterpillars is speculative, but suggests testable hypotheses about interactions in the *Feniseca* system. Although the causes and cues for caterpillar concealment remain unknown, our observations indicate that ants are not aggressive toward *F. tarquinius* caterpillars and suggest that caterpillar concealment is not a defense against ants.

Chemical Resemblance Between Caterpillars and Aphids. Cuticular lipid analysis suggests a mechanism by which *F. tarquinius* invades the ant–aphid mutualism. The 76% similarity in lipid composition between *F. tarquinius* and *P. tesselatus* (Table 2) is comparable to the hydrocarbon similarity between another well-documented chemical mimicry pair. The cuticular hydrocarbon composition of the socially parasitic lycaenid caterpillar *Maculinea rebeli* is 72% similar to that of its host ant *Myrmica schencki* when similarity is calculated by the same methods used here; this level of similarity is adequate to maintain peaceful host–parasite relations within the host ant colony (Elmes et al., 2002). It seems plausible that *Feniseca–Prociphilus* similarity is also adequate to deceive ants. It is important to note that, although only hydrocarbons are specifically implicated in ant recognition systems (Singer, 1998; Lahav et al., 1999), and only hydrocarbons were considered in the case of *Maculinea*, the present study considers total cuticular lipids. Separation and identification of the hydrocarbon fraction from the total lipid extract remain for future studies of the *Feniseca* system.

Caterpillars could invade aphid resources by resembling ants, rather than aphids. However, our data do not support this scenario. Resemblance between *F. tarquinius* caterpillars and attendant ant species was ≤55%, compared to 76% similarity between caterpillars and *P. tesselatus*. Resemblance to a specific ant species would limit caterpillars to aphid colonies tended exclusively by that species. On the other hand, resemblance to the host aphids should allow caterpillars to feed on aphid colonies tended by any ant species. The latter ant–generalist strategy could explain why *F. tarquinius* coexists with many ant species across its geographic range and also with different ant species that tend the same aphid colonies at different times of day.

Behavioral assays confirmed that cuticular extracts contained behaviorally relevant information for *C. pennsylvanicus* tending *P. tesselatus* colonies. Ants responded to extracts of potential competitors for aphid resources, *F. ulkei* and *H. axyridis*, with high levels of both absolute and relative aggression (Figure 4). They responded to blanks and extracts of *P. tesselatus* with significantly lower levels of aggression. Notably, ants responded to *F. tarquinius* extract as they did to the chemically similar aphid extract, not as they did to the extracts of other competitors, suggesting that ants did not recognize *F. tarquinius* caterpillars as aphid predators. Although trace volatiles may have been present in cuticular extracts (as in Allan et al., 2002), our results support the idea that chemicals present on the caterpillar cuticle can account for low levels of ant aggression toward *F. tarquinius* compared to other insects that might "trespass" on an aphid colony.

Camouflage vs. Mimicry. Rearing experiments suggested that the *Feniseca–Prociphilus* resemblance was due to chemical camouflage, not mimicry. In this study, caterpillars reared on dogwood aphids lost their resemblance

to *Prociphilus*, a result consistent with camouflage. They did not, however, acquire a resemblance to dogwood aphids. Because caterpillars might acquire camouflage when aphid lipids rub off on them through physical contact, we suspect that this result was because of aphid size. Dogwood aphids are minute relative to *Prociphilus*, and feeding caterpillars had relatively little bodily contact with them, whereas caterpillars feeding on *Prociphilus* had constant and abundant bodily contact with their prey. Thus, dogwood aphid lipids would be unlikely to rub off on feeding caterpillars, which would, therefore, display only *Feniseca*'s native lipids. Caterpillars feeding on larger *Prociphilus* would still produce their native lipids, but additional lipids acquired from prey would alter the total lipid profile.

This interpretation is consistent with the pattern of similarities among caterpillars and aphids (Table 2 and Figure 3) where *Prociphilus*-reared caterpillars appeared intermediate between the lipid profile of their prey and that of putatively unaltered caterpillars (i.e., those reared on dogwood aphids). This pattern is also reflected in the presence and absence of "indicator peaks" (Table 3), which provide no evidence that *Feniseca* that fed on dogwood aphids (henceforth "dogwood *Feniseca*") acquired lipids from its prey. Dogwood aphids had few unique components that could serve as "labels" for tracing lipid transfer. However, the many disparities between the simple lipid profiles of dogwood aphids and dogwood *Feniseca* do not suggest hydrocarbon acquisition. The indicator peaks of normal *Feniseca* are the sum of those present on dogwood *Feniseca* and *Prociphilus*, also consistent with our suggestion that the lipid profile of normal *Feniseca* is the result of both biosynthesis and acquisition. However, to appreciate the significance of specific differences between species, chemical identifications are necessary. For instance, it is important to know whether the caterpillar-specific peaks (peaks 2 and 10, Table 3) were nonhydrocarbons or other chemicals unlikely to influence ant behavior.

Our results support the camouflage hypothesis and a hydrocarbon rub-off mechanism, but cannot exclude the possibility that observed differences in caterpillar lipid profiles were due to diet-induced shifts in biosynthesis. We can conceive of three approaches that might distinguish between these alternatives. *In vitro* hydrocarbon synthesis experiments could clarify which components are biosynthesized by caterpillars. Alternatively, aphids could be treated with a synthetic hydrocarbon normally absent from the system. If this hydrocarbon appeared unaltered on caterpillar cuticle, the rub-off mechanism would be implicated. Finally, caterpillar internal and external hydrocarbon composition could be compared. Insect cuticular and internal hydrocarbons are generally the same, so if caterpillars had cuticular hydrocarbons that were absent internally, such hydrocarbons were likely acquired by rub-off.

Some myrmecophilous butterfly caterpillars possess ant organs that appear to release semiochemicals that modify ant behavior (DeVries 1988, 1997;

Pierce et al., 2002), and when examined under high magnification, the setae associated with these organs may show flaps and convolutions that could act to disseminate volatile chemistry (DeVries 1997). Our data suggest that chemical camouflage is important to *F. tarquinius* caterpillars, but their setae showed no evidence of increased surface area or abrasive qualities that might aid in acquisition or dispersal of aphid chemistry (Figure 5). That *F. tarquinius* setae resemble the ordinary tactile and defensive body setae of nonmyrmecophilous lycaenid caterpillars is consistent with the mechanism of chemical camouflage by passive, nonspecialized lipid acquisition.

In summary, although the role of physical concealment in *F. tarquinius* caterpillars remains unclear, all evidence from this study points away from ant avoidance. Instead, acquisition of chemical resemblance to the aphid prey appears adequate to defuse ant aggression. Such a relationship admits the possibility that ants could indirectly benefit caterpillars, especially when ants are abundant, pugnacious, and effective at excluding caterpillar enemies from aphid colonies. This study points to the possibility that chemical camouflage occurs among Old World miletines and other aphid predators and may be as common among users of ant-tended resources as it is among social parasites of ant nests.

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VOLATILE COMPOUNDS FROM ANAL GLANDS OF THE WOLVERINE, *Gulo gulo*

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Abstract—Dichloromethane extracts of wolverine (*Gulo gulo*, Mustelinae, Mustelidae) anal gland secretion were examined by gas chromatography–mass spectrometry. The secretion composition was complex and variable for the six samples examined: 123 compounds were detected in total, with the number per animal ranging from 45 to 71 compounds. Only six compounds were common to all extracts: 3-methylbutanoic acid, 2-methylbutanoic acid, phenylacetic acid, α-tocopherol, cholesterol, and a compound tentatively identified as 2-methyldecanoic acid. The highly odoriferous thietanes and dithiolanes found in anal gland secretions of some members of the Mustelinae [ferrets, mink, stoats, and weasels (*Mustela* spp.) and zorillas (*Ictonyx* spp.)] were not observed. The composition of the wolverine's anal gland secretion is similar to that of two other members of the Mustelinae, the pine and beech marten (*Martes* spp.).

Key Words—Wolverine, *Gulo gulo*, Mustelinae, Mustelidae, scent marking, fear-defense mechanism, short-chain carboxylic acids.

INTRODUCTION

The wolverine (*Gulo gulo*) is the largest terrestrial member of the Mustelidae and is part of the subfamily, Mustelinae, which includes ferrets, fishers, martens, mink, stoats, weasels, and zorillas. Historically, two species of wolverine have been described, one from North America and the other from

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Eurasia, but today only one species is recognized (Pasitschniak-Arts and Larivière, 1995). The wolverine, as with most mustelids, possesses paired glands that open on both sides of the anus, which produce a tannish-yellow, highly odoriferous secretion (Hash, 1987). Wolverine anal musking has been described by several authors (Haglund, 1966; Pulliainen and Ovaskainen, 1975; Koehler et al., 1980; Magoun, 1985) as a method of scent marking in addition to marking with feces or urine (Jackson, 1961). Others have suggested that anal gland secretions function primarily as a fear-defense mechanism (Krott, 1959; Ewer, 1973; Long, 1987).

The composition of the anal gland secretion from some members of the subfamily Mustelinae was described in a recent review (Burger, 2005). Odoriferous cyclic sulfides (thietanes and dithiolanes) and in some cases, sulfides, disulfides, and other organosulfur compounds, were found in the secretion from smaller members of this subfamily: American mink, *Mustela vison* (Brinck et al., 1978, 1983; Schildknecht et al., 1976, 1981); ferret, *Mustela putorius* (Crump, 1980b; Crump and Moors, 1985; Schildknecht et al., 1976; Schildknecht and Birkner, 1983); Siberian weasel, *Mustela sibirica* (Zhang et al., 2002, 2003); steppe polecat, *Mustela eversmanni* (Zhang et al., 2002, 2003); stoat, *Mustela erminea* (Brinck et al., 1983; Crump, 1978, 1980a; Crump and Moors, 1985; Schildknecht and Birkner, 1983); weasel, *Mustela nivalis* (Brinck et al., 1983; Buglass et al., 1991; Schildknecht and Birkner, 1983) and zorilla, *Ictonyx striatus* (Apps et al., 1988; Wheeler et al., 1997). These sulfur compounds are absent in anal gland secretion of the two other members of the Mustelinae that have been investigated, the pine marten (*Martes martes*) (Brinck et al., 1983; Schildknecht and Birkner, 1983) and the beech marten (*Martes foina*) (Schildknecht and Birkner, 1983). Schildknecht and Birkner (1983) reported short-chain carboxylic acids from these two species, whereas Brinck et al. (1983) identified benzaldehyde from the pine marten. We investigated the anal gland secretion of the wolverine and report the analysis of volatile components in the secretion.

METHODS AND MATERIALS

Anal glands from four individuals (sex not recorded) were obtained incidental to fur trapping during the winter of 2002–2003 from the Brooks Range in Alaska. These glands were excised from the trapped animals and immediately frozen at −20°C until they could be analyzed. Upon thawing, a 0.25-g sample of the secretion from both glands for each individual was pooled and extracted with 2 ml of CH_2Cl_2. Two additional pooled samples of anal gland secretion from a male and a female were obtained from Glacier National Park, Montana. These samples were kept at −20°C until they were extracted

with CH_2Cl_2 as had been done with the previous samples. Control samples of CH_2Cl_2 were analyzed to identify contaminants in the solvent.

Extracts were analyzed by gas chromatography–mass spectrometry (GC-MS) in splitless mode (0.5 min), using a Hewlett-Packard GCD Plus fitted with a 30 × 0.25-mm HP-5MS column. The GC was programmed from 40°C/4 min, then 30°C/min to 325°C/2 min. Mass spectral fragments below *m*/*z* 39 were not recorded. The relative amount of each component is reported as the percent of the total ion current (TIC). Minor components less than 0.1% of the TIC were not investigated and impurities found in the solvent were subtracted from the analyses.

Compounds were initially identified by comparison of mass spectra to those in the NIST 1998 mass spectral database. All compounds that did not match spectra in the NIST library were examined to see if they matched published spectra of thietanes, dithiolanes, and other organosulfur compounds reported from some members of the Mustelinae (Brinck et al., 1983; Crump, 1980a,b; Crump and Moors, 1985; Schildknecht et al., 1981; Schildknecht and Birkner, 1983; Zhang et al., 2002, 2003). Authentic samples were used to confirm identifications by comparisons of spectra and retention times, except for compounds that were not commercially available or were prepared synthetically (see Table 1). The chirality of compounds containing stereogenic centers was not determined. Reference samples were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA) and Fisher Scientific (Acros, Pittsburgh, PA, USA), except the following, which were synthesized. Isoamyl laurate was prepared by acid-catalyzed reaction of the corresponding alcohol and carboxylic acid. 3-Methyl-dihydro-2(3*H*)furanone was prepared from 3-methyl-2(5*H*)furanone by catalytic hydrogenation with platinum oxide and H_2. 2-Methylpropanamide and 3-methylbutanamide were prepared from 2-methylpropanoic and 3-methylbutanoic acids by treatment with thionyl chloride to form the acid halides and subsequent reaction with ammonia.

RESULTS AND DISCUSSION

The CH_2Cl_2 extracts of the anal gland secretion from the six wolverines showed great variation between individuals. In all, 123 peaks were seen in the chromatograms, with the number per animal ranging from 45 to 71 (Table 1). We identified 49 of the 123 compounds in the extracts by comparison of their retention times and mass spectra with those of authentic samples. The tentative identification of ten additional compounds could not be confirmed because authentic standards were not available.

The secretions had few of these 59 compounds in common—26 of these compounds were observed in only one sample. Only six of the identified

TABLE 1. VOLATILE COMPOUNDS IDENTIFIED AND TENTATIVELY IDENTIFIED IN THE ANAL GLAND SECRETION OF WOLVERINE (% TOTAL ION CURRENT)

Compound	Anal gland samples					
	1[a]	2[a]	3[a]	4[a]	5[b]	6[c]
Carboxylic acids						
2-Methylpropanoic acid						7.3
3-Methylbutanoic acid	3.6	2.9	3.2	14.8	1.6	47.2
2-Methylbutanoic acid	0.5	0.7	0.7	1.4	0.2	9.2
Pentanoic acid						0.3
2-Methylpentanoic acid	0.2			1.6		0.3
Hexanoic acid				0.3	0.4	
2-Methylhexanoic acid				0.2		
Heptanoic acid		0.1			0.7	
Octanoic acid	0.3	0.6			3.2	
2-Octenoic acid		0.4				
7-Octenoic acid[d]	0.2					
2-Methyloctanoic acid				3.4		
4-Methyloctanoic acid[d]					0.7	
Phenylacetic acid	0.5	0.2	0.2	2.0	0.9	3.0
Nonanoic acid		0.1			1.1	
3-Phenylpropanoic acid			8			
Decanoic acid		0.1			2.2	
2-Methyldecanoic acid[d]	0.8	2.5	3.4	5.5	3	0.7
2-Decenoic acid		0.7				
Dodecanoic acid				0.2	0.4	
Tetradecanoic acid	1.4	1.4		0.4		
Pentadecanoic acid	2.6	2.4		0.5	2.1	
(*Z*)-9-Hexadecenoic acid	2.5	1.7	0.3		1.9	
Hexadecanoic acid	5.9	6	1	1.3	5	
Linoleic acid	15.5	12.2	3.3	5.2	10.5	
Oleic acid	0.1				4.3	
Stearic acid	3.4	4.5	1	1.6	0.3	
Alcohols						
3-Methyl-1-butanol	0.1					0.4
1-Octen-3-ol			0.2			
Benzyl alcohol		0.1			0.1	1.0
Linalool	0.1		0.6			
2,3-Dimethylcyclohexanol[d]		0.1				0.2
2-Phenylethanol			0.1			
1,2-Hexadecanediol[d]	7	9.4	3.3		6.1	3.8
α-Tocopherol	1	1	8.1	11.2	0.9	0.1
Cholesterol	3.2	3.6	11.1	11.5	2.4	3.3
Hydrocarbons						
Nonane		0.2				
α-Pinene					0.1	
β-Pinene					0.1	
3-Methylnonane[d]		0.1				

TABLE 1. CONTINUED

Compound	Anal gland samples					
	1[a]	2[a]	3[a]	4[a]	5[b]	6[c]
Decane		0.8	0.2			
Undecane		0.4				
Nitrogen compounds						
2-Methylpropanamide						0.2
3-Methylbutanamide						0.6
2-Pyrrolidinone						0.8
2,5-Pyrrolidindione						0.8
2-Piperidinone		0.6	0.2	0.4		2.1
Methylpyrrolidinone[d]		0.2				
Indole						0.3
Other compounds						
Dimethyl disulfide	0.1			0.2		0.1
Dimethyl trisulfide	0.2				0.1	0.1
3-Methyl-dihydro-2(3*H*)furanone						0.3
4-Methyl-dihydro-2(3*H*)furanone[d]						1.7
Benzaldehyde					0.2	
3-Octanone			0.1			
2-Pentylfuran[d]	0.1				0.1	
4-Hydroxybenzaldehyde						0.8
Isoamyl laurate		0.1				
5,6,7,7-Tetrahydro-2(4*H*)benzofuranone[d]	0.3	0.4	0.2		0.5	0.4
Subtotal (% TIC)	49.6	53.5	45.2	61.7	49.1	85.0
No. identified compounds	18	23	16	17	22	20
No. tentatively identified compounds	5	6	3	1	5	5
No. unidentified compounds	45	42	37	29	44	21
Subtotal (% TIC)	51.4	46.5	54.8	38.3	51.9	15.0

[a] From Alaska.
[b] Male from Montana.
[c] Female from Montana.
[d] Identified only by NIST Library match.

compounds were common to all extracts: 3-methylbutanoic acid, 2-methylbutanoic acid, phenylacetic acid, 2-methyldecanoic acid (tentative identification only), α-tocopherol, and cholesterol. Hexadecanoic, linoleic, and stearic acids, and two tentatively identified compounds [5,6,7,7-tetrahydro-2(4*H*) benzofuran and 1,2-hexadecanediol] were common to five of the extracts. Only pentadecanoic and (*Z*)-9-hexadecenoic acids were common to four of the six samples.

The odoriferous thietanes or dithiolanes found in some other mustelines (*Mustela* spp.) were not observed in wolverine anal gland secretion. The fetid nature of the secretion is due in part to the short-chain carboxylic acids and phenylacetic acid found in all of the samples. Four of the samples contained

small amounts of dimethyl disulfide and/or dimethyl trisulfide, compounds that would also contribute to the odor, and one contained 8.0% of pungent 3-phenylpropanoic acid. All samples contained a large number of relatively nonvolatile components, including free fatty acids and cholesterol. Thus, the chemical composition of the wolverine's anal gland secretion is similar to that of the pine and beech marten (*Martes* spp.). The anal gland secretions from these species were reported to contain short-chain acids and benzaldehyde (Brinck et al., 1983; Schildknecht and Birkner, 1983), but not the cyclic sulfur compounds found in other members of the Mustelinae (ferrets, mink, stoats, weasels, and zorillas). These chemical differences are consistent with recent mitochondrial DNA (Dragoo and Honeycutt, 1997) and nuclear DNA studies (Koepfli and Wayne, 2003) that indicate the wolverine and martens are more closely related to each other than either is to mustelines of the genera *Mustela* or *Ictonyx*.

Mustelinae anal gland composition has been reported as variable, relating to differences in age, sex, and estrous condition (Crump, 1980a,b; Zhang et al., 2002, 2003), and also as relatively stable, not varying for individuals during the year, by sex, or during the reproductive period (Brinck et al., 1978). Similarities in chemical products within species have led to hypotheses regarding species and individual recognition, but the semiochemical function of anal sac secretions remains unclear (Albone, 1984). Future investigations would be benefited by an increased sample of anal gland material from wolverines of known sex, age, and season.

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IDENTIFICATION OF TERMITE SPECIES BY THE HYDROCARBONS IN THEIR FECES

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Abstract—Blends of abundant cuticular hydrocarbons are species-specific for termites (Isoptera) and can be used to identify a given taxon without the diagnostic castes, soldiers or adults. We demonstrate that hydrocarbon extracts of termite fecal pellets from damaged wood can also be characterized and used to identify termites responsible for damage, even though termites are no longer present or easily recovered. In structures infested by drywood termites, it is common to find fecal pellets, but difficult to extract termites from the finished wood in service. Nine species belonging to two families (Kalotermitidae and Termopsidae) were examined to compare the hydrocarbon composition of termites and their fecal pellets. Diversity was extensive: at least one half of the amount of the hydrocarbons from *Neotermes connexus*, *Incisitermes immigrans*, *Cryptotermes brevis*, *Cryptotermes cynocephalus*, *Procryptotermes corniceps*, and *Zootermopsis nevadensis nuttingi* was olefins. *Incisitermes minor* and *Pterotermes occidentis* incorporated only small amounts of olefins in cuticular hydrocarbons; *Marginitermes hubbardi* had no detectable olefins. Hydrocarbons extracted from fecal pellets were qualitatively and quantitatively similar to cuticular extracts and can be used to determine the termite species responsible without the termites present.

Key Words—Chemotaxonomy, cuticular hydrocarbons, dampwood termites, drywood termites, fecal pellets, frass, Kalotermitidae, Isoptera, species identification, Termopsidae.

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INTRODUCTION

The cryptic nature of drywood and dampwood termites often precludes their collection for purposes of identification. Because they make their homes within wood, it is often not possible to collect them without destroying the wooden structure. A commonly utilized sign for determining the presence of wood-inhabiting termites is the associated occurrence of fecal pellets, which are ejected from the galleries inside the wood. These pellets are diagnostic for drywood and dampwood termites and can be used to distinguish their damage from other wood-destroying insects (Ebeling, 1975; Moore, 1992).

Drywood and dampwood termites excrete feces in the form of hard, evenly shaped fecal pellets. While collecting drywood termites in a survey of the termites of the Hawaiian Islands (Haverty et al., 2000; unpublished data), we gathered fecal pellets while removing termites from wood. Rather than simply signaling the general presence of termites, we wondered whether these pellets could be used for more precise diagnosis of the species of termite inhabiting the wood. We hypothesized that termite fecal pellets contain the same mixture of hydrocarbons as the insects that produced them, and because cuticular hydrocarbons are species-specific in termites (Page et al., 2002), we further hypothesized that the species can be identified by these chemicals in their feces.

The cuticle covering the outside surface of the termite also lines the inside of the rectum and likely also has a covering of wax composed mostly of hydrocarbons. Fecal pellets are formed in the elongate, bulbous rectum that is used for the temporary storage of undigested food particles (Child, 1934). Pellets are elongate and have six flattened sides that coincide with the rectal papillae. The six surfaces are a result of pressure applied by the six plates within the rectal epithelium. The hindgut is lined with chitinous intima that is continuous through the anus with the chitinous exoskeleton; the epithelial layer of the rectum is thickened and contains a heavily chitinous intima (Child, 1934).

Fecal pellets must be dehydrated before expulsion; resorption of water represents an essential ecological adaptation for xeric conditions (Noirot and Noirot-Timothée, 1969). The function of the rectum is to press out and conserve the water content of material that enters from the colon. Water is absorbed through the rectal wall. During compression for removal of water, it is likely that pellets acquire a hydrocarbon coating before extrusion. This may facilitate discharge of pellets.

Characterization of the cuticular hydrocarbons of termite species supports the concept of species specificity of hydrocarbon mixtures (Haverty et al., 1988, 1996a, 1997, 1999, 2000; Watson et al., 1989; Kaib et al., 1991; Brown et al.,

1994, 1996; Haverty and Nelson, 1997; Takematsu and Yamaoka, 1999; Clément et al., 2001; Nelson et al., 2001; Page et al., 2002). Owing to the ontogenic origin of the insect hindgut (Snodgrass, 1935; Romoser and Stoffolano, 1988), i.e., invaginated ectoderm, we hypothesized that external (cuticular) hydrocarbons are also present internally and are picked up by expelled fecal material.

METHODS AND MATERIALS

Collection of Termites. Termites were gathered from the British Virgin Islands (Haverty et al., 1997) and the Hawaiian Islands (Haverty et al., 2000). Collections of *Pterotermes occidentis* (Walker) and *Marginitermes hubbardi* (Banks) were made in the vicinity of Tucson, AZ, USA, and collections of *Zootermopsis nevadensis nuttingi* Haverty and Thorne were made near Mt. Shasta, CA, USA. In total, nine species belonging to two families were collected. Representatives of the Kalotermitidae included *Neotermes connexus* Snyder, *Incisitermes immigrans* Snyder, *Cryptotermes brevis* (Walker), *Cryptotermes cynocephalus* Light, *Incisitermes minor* (Hagen), *Procryptotermes corniceps* (Snyder), *P. occidentis*, and *M. hubbardi*; *Z. nevadensis nuttingi* was the sole representative of the Termopsidae.

Naturally infested wood samples were bagged and brought to the laboratory, where termites and fecal pellets were separated from wood and other debris. Samples of 15–20 pseudergates or nymphs were placed in separate vials, frozen, and dried (Haverty et al., 1997, 2000). As soon as the termites were completely dry, specimens were placed in vials that were tightly capped. Fecal pellets were separated from debris by sequentially sifting them through successively smaller sieves. Pellets were separated from fine debris by removing them with forceps or with an aspirator until 3–10 ml were obtained. Concurrently, fresh (i.e., not dried) termite voucher samples were preserved in 85% ethanol and deposited in the National Museum of Natural History, Smithsonian Institution, Washington, DC (Haverty et al., 1997) or in the University of Hawaii, Manoa Campus collection (Haverty et al., 2000).

Extraction Procedure and Characterization of Hydrocarbons. Hydrocarbons from the termites and fecal pellets were extracted, characterized, and quantified as reported in Haverty et al. (1997). Termites, as a group, or 3–10 ml of fecal pellets were extracted by immersion in 10 ml of *n*-hexane for 10 min. After extraction, hydrocarbons were separated from other compounds by pipetting the extract through 4 cm of activated Sigma silica gel (70–230 mesh) in Pasteur pipet minicolumns. An additional 5 ml of clean *n*-hexane were

dripped through the silica gel. The resulting extracts were evaporated to dryness under a stream of nitrogen and redissolved in 60 µl of *n*-hexane for gas chromatography-mass spectrometry (GC-MS) analyses. A 3-µl aliquot was injected into the GS-MS.

GC-MS analyses were performed on a Hewlett-Packard (HP) 5890 gas chromatograph equipped with an HP 5970B Mass Selective Detector interfaced with HP Chemstation data analysis software (HP59974J Rev. 3.1.2). The GC-MS was equipped with an HP-1 fused silica capillary column (25 m × 0.2 mm ID) and operated in split mode (with a split ratio of 8:1). Each mixture was analyzed by a temperature program ranging from 200 to 320°C at 3°C/min with a final hold of 11 or 16 min. Electron impact (EI) mass spectra were obtained at 70 eV.

n-Alkanes were identified by their mass spectra. Mass spectra of methyl-branched alkanes were interpreted as described by Blomquist et al. (1987) to identify methyl branch locations. Mass spectra of di- and trimethylalkanes were interpreted as described by Page et al. (1990a,b) and Pomonis et al. (1978). Alkenes were identified by their mass spectra, but double bond positions were not determined (see Haverty et al., 1997). Equivalent chain lengths (ECL) were calculated for some of the unsaturated compounds to distinguish the various isomers.

In the text and tables, we use shorthand nomenclature to identify individual hydrocarbons or mixtures of hydrocarbons. This shorthand uses a descriptor for the location of methyl groups (X-me), the total number of carbons (CXX) in the hydrocarbon component excluding the methyl branch(es), and the number of double bonds following a colon (CXX:Y). Thus, heptacosane becomes *n*-C27; 2-methylheptacosane becomes 2-meC27; 13,15-dimethylheptacosane becomes 13,15-dimeC27; and heptacosadiene becomes C27:2. Hydrocarbons are presented in the tables for each species and its feces in the order of elution on our GC/MS system.

Integration of the total ion chromatogram was performed using the HP Chemstation data analysis software. GC-MS peak areas were converted to percentages of the total hydrocarbon fraction. A summary of the relative amounts of each peak for termites and fecal pellets using a representative chromatogram of each species was prepared.

Cluster Analysis of Hydrocarbon Mixtures from Termites and Fecal Pellets. The percentage of each hydrocarbon was used as the response variable. The presence of coeluting compounds precluded exact quantification of many individual hydrocarbons; the quantity of each hydrocarbon in each such peak was equal to $1/n$ of the value for the peak, with n = the number of hydrocarbons in the peak. The Euclidean distance for all 18 combinations of termite species by insect or fecal pellets was calculated using all hydrocarbons (R Statistical Language, 2004).

RESULTS AND DISCUSSION

The cuticular hydrocarbons for five species [*N. connexus*, *C. brevis*, *C. cynocephalus*, *I. immigrans*, and *I. minor*] from Hawaii (Haverty et al., 2000), and one additional species [*P. corniceps*] from the British Virgin Islands (Haverty et al., 1997) were previously characterized. The composition of the hydrocarbon mixtures for these six species and the hydrocarbons from their fecal pellets are displayed in Figures 1–6 and summarized in Tables 1–6.

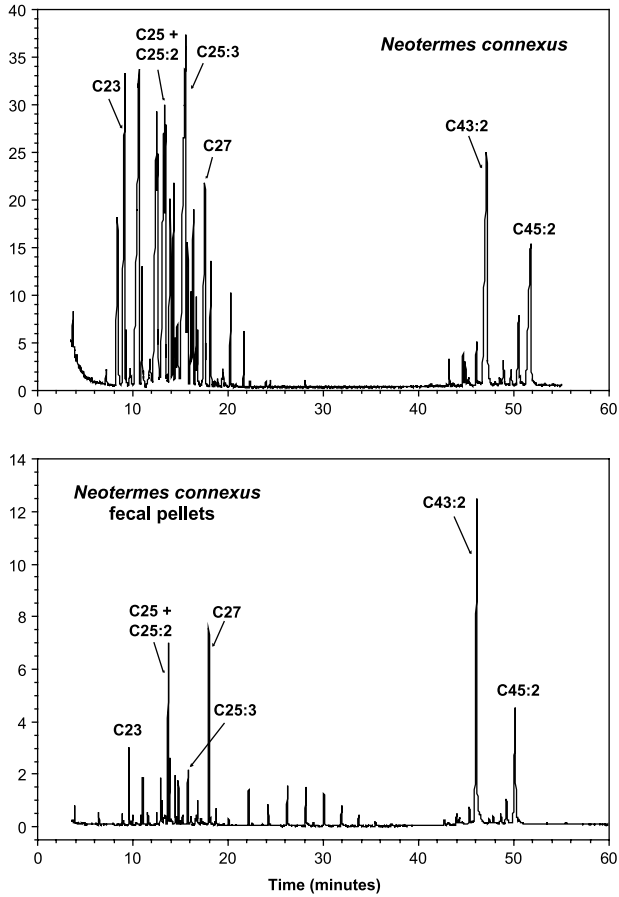

FIG. 1. Total ion chromatogram of the cuticular hydrocarbons from nymphs of *Neotermes connexus* Snyder from Iao Valley Lookout, Maui, HI, and from fecal pellets of *N. connexus* from junctions of Hwy 550 and 552, Kauai, HI.

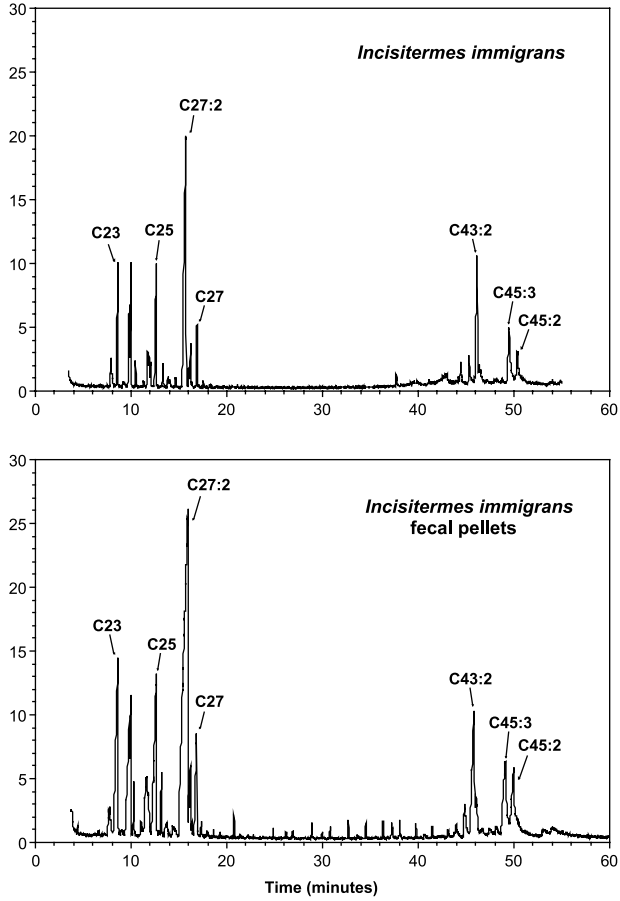

FIG. 2. Total ion chromatogram of cuticular hydrocarbons from pseudergates of *Incisitermes immigrans* Snyder from Poipu Beach, Kauai, HI, and from fecal pellets of *I. immigrans* from Kualoa Beach, Oahu, HI.

Detail for the cuticular hydrocarbon mixtures of two additional drywood termite species, *P. occidentis* and *M. hubbardi*, from Arizona, as well as the hydrocarbons from their fecal pellets, that have not been previously reported are shown (Figures 7 and 8, Tables 7 and 8). The composition of the cuticular hydrocarbons for frozen samples of *Z. nevadensis nuttingi* (*Zootermopsis* Phenoytpe III) has been described previously (Haverty et al., 1988). Here, we identify additional hydrocarbons not previously detected, as well as hydrocarbons from fecal pellets (Figure 9, Table 9). The results of the method used

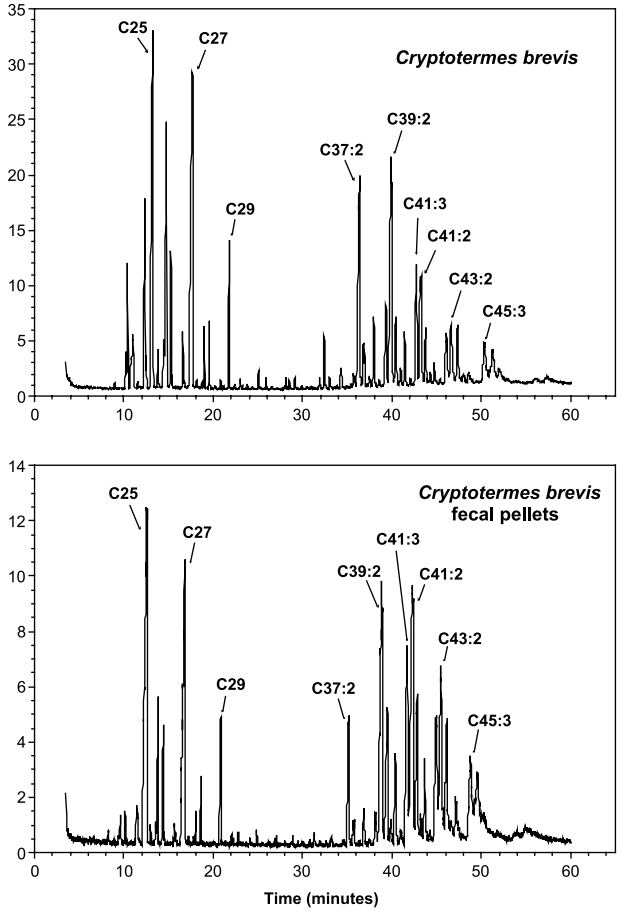

FIG. 3. Total ion chromatogram of the cuticular hydrocarbons from pseudergates of *Cryptotermes brevis* (Walker) from Honolulu, Oahu, HI, and from fecal pellets of *C. brevis* from NOAA laboratory, Kewalo Basin, Oahu, HI.

here are not equivalent to extracting live or freshly frozen termites, and demonstrate that there are quantitative differences in the composition of cuticular hydrocarbons extracted from dried specimens (Haverty et al., 1996b).

The cuticular hydrocarbon mixture of most drywood termite species examined thus far from the West Indies and Hawaii (Haverty et al., 1997, 2000) reflected a general pattern. Cuticular hydrocarbons occurred in two distinct groups: early eluting compounds (23 to 29 or 31 carbons in the parent chain) and late-eluting compounds (37 to 45 carbons in the parent chain). The mixtures

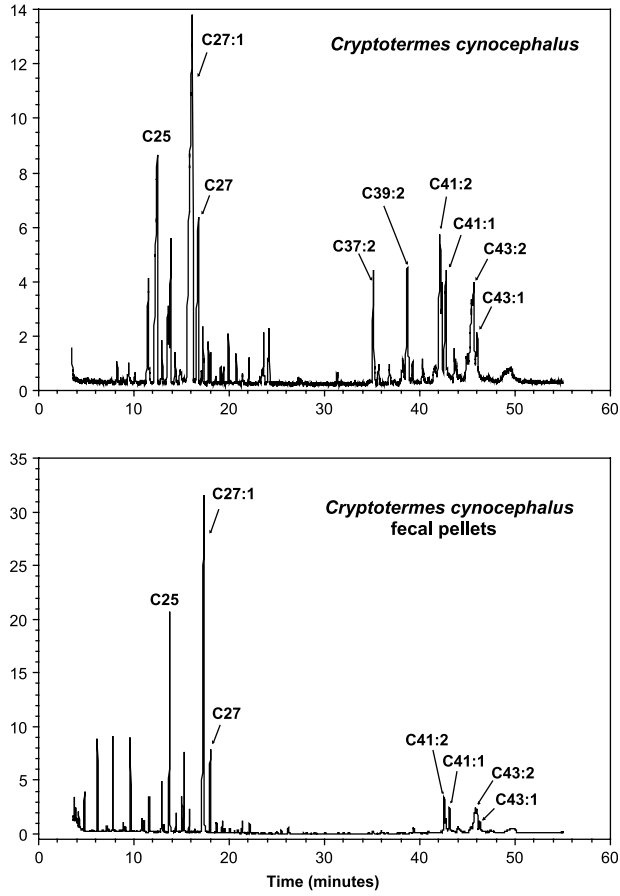

FIG. 4. Total ion chromatogram of the cuticular hydrocarbons from pseudergates of *Cryptotermes cynocephalus* Light from Waiahole Valley Road, Kamehameha Highway, Oahu, HI, and from fecal pellets from the same collection.

of *I. minor* and *M. hubbardi* were different in that hydrocarbons occurred continuously from the early eluting hydrocarbons with 23 carbons in the parent chain to the late eluting hydrocarbons with 43 carbons in the parent chain (Figures 5 and 8). *Z. nevadensis nuttingi* displayed a pattern similar to the general pattern for drywood termites with the addition of a few hydrocarbons with 31 carbons in the parent chain (Figure 9).

Hydrocarbons from Termites. N. connexus is generally found in rotting wood or living trees in moist, forested areas in Hawaii between 200 and 2000 m in elevation (Zimmerman, 1948). The chromatograms of cuticular hydrocarbons

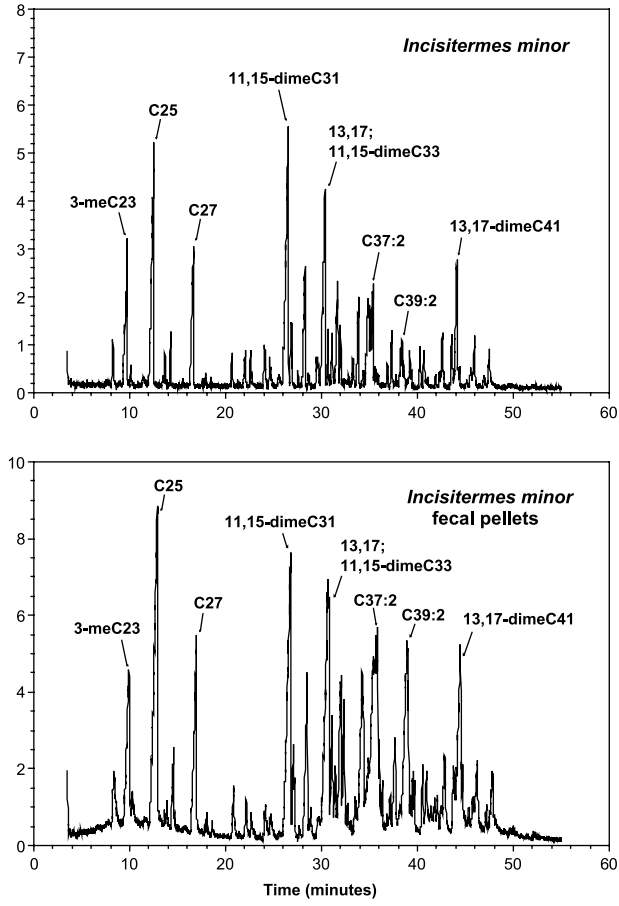

FIG. 5. Total ion chromatogram of cuticular hydrocarbons from pseudergates of *Incisitermes minor* (Hagen) from Fresno, CA, and from fecal pellets from the same collection.

reflected the general pattern of drywood termites (Figure 1, Table 1). *n*-Alkanes comprised 21.8% of the total hydrocarbon; *n*-C23, *n*-C25, and *n*-C27 were the most abundant. Olefins were, by far, the most predominant, representing over 52% of the total hydrocarbon; dienes and trienes were the most abundant olefins. Terminally branched monomethylalkanes, mainly 2-; 3-meC23; and 2-meC24, accounted for 23.6% of the total hydrocarbon. Internally branched monomethylalkanes were rare; neither dimethylalkanes nor trimethylalkanes were detected.

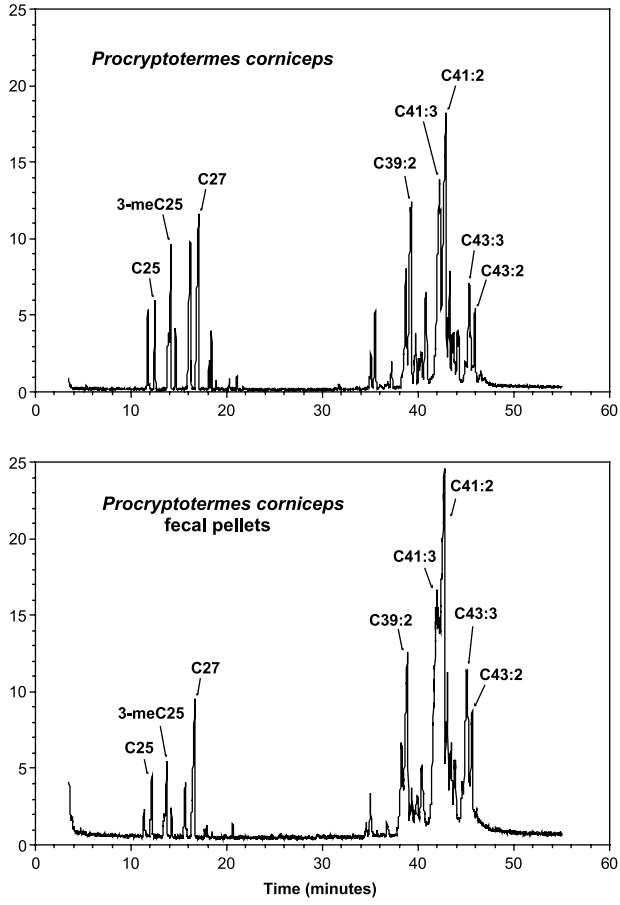

FIG. 6. Total ion chromatogram of the cuticular hydrocarbons from pseudergates of *Procryptotermes corniceps* (Snyder) from Guana Island, British Virgin Islands, and fecal pellets from the same collection.

I. immigrans is the predominant drywood termite in lowland xeric and coastal littoral forests, where it infests living and dead, standing trees, exclusive of man-made structures (Zimmerman, 1948). It is displaced by *N. connexus* at higher elevations, and the two species coexist at low elevations on mesic sites. The general appearance of the chromatograms of *I. immigrans* (Figure 2) was similar to those of *N. connexus* (Figure 1); over 75% of the hydrocarbons had 23 to 29 carbons (Table 2). *n*-Alkanes, primarily *n*-C25 and *n*-C27, comprised 24.8% of the total hydrocarbon. The unsaturated components constituted 59.4%

TABLE 1. RELATIVE ABUNDANCE[a] OF HYDROCARBONS FROM NYMPHS AND FECAL PELLETS OF *Neotermes connexus* SNYDER

Hydrocarbons	ECL[b]	Termites	Fecal pellets
n-C22		0.21	0.10
2-meC22		3.36	0.38
C23:1	22.70	0.00	0.19
n-C23		7.26	2.59
C23:2	23.01	0.37	0.00
Unknown		0.00	0.38
9-meC23		0.34	0.12
2-; 3-meC23[c]		9.75	2.11
n-C24		1.23	0.47
C24:2	24.01	0.20	0.00
3,7-dimeC23		0.00	0.17
C25:2	24.50	0.69	0.54
2-meC24		8.00	2.07
C25:1	24.70	1.67	1.03
C25:3 + Unknown[c]	24.80	0.41	0.00
C25:1	24.80	0.00	1.29
n-C25		8.40	7.26
C25:2	25.01	2.17	2.42
Unknown		0.00	0.42
C25:3	25.20	0.10	0.00
C25:2	25.30	3.11	1.99
C25:2	25.35	0.81	0.37
C25:2	25.50	2.91	1.79
2-meC25		0.63	0.20
3-meC25		1.25	0.54
C25:3	25.98	15.38	2.95
C25:3	26.07	1.29	0.58
C25:3	26.10	0.89	0.00
C26:2; C27:2; C25:3[c]	26.30	1.07	0.54
C25:3	26.47	2.56	1.17
2-meC26; C27:1[c]	26.70	1.02	0.34
C27:1	26.85	0.53	0.18
n-C27		4.09	8.16
C27:2	27.01	0.00	0.33
C27:2; 13-; 11-meC27[c]	27.35	1.79	0.89
C27:2	27.50	0.05	0.00
C28:2	27.60	0.09	0.00
3-meC27		0.08	0.00
C27:3	27.85	0.15	0.00
n-C28		0.12	0.31
C29:2	28.50	0.94	0.00
n-C29		0.47	1.61
n-C30		0.00	0.99
n-C31		0.00	1.85
n-C32		0.00	1.82

TABLE 1. CONTINUED

Hydrocarbons	ECL[b]	Termites	Fecal pellets
n-C33		0.00	1.66
n-C34		0.00	1.10
n-C35		0.00	0.67
n-C36		0.00	0.32
n-C37		0.00	0.11
C41:2		0.27	0.35
C41:1		0.00	0.28
15-meC41		0.38	0.79
C42:2		0.33	0.54
C43:3		0.61	1.20
C43:2		8.08	27.95
15-meC43		0.12	0.39
C44:2		0.42	0.64
C45:4		0.25	0.86
C45:3		1.46	2.43
C45:2		4.70	12.55

[a] Percent of total hydrocarbon composition.
[b] Equivalent chain length.
[c] An isomeric mixture or two or more compounds coelute in this peak.

of the total hydrocarbon; olefins with 27 or 43 carbons predominate. The terminally branched monomethylalkanes comprised 14.4% of the total hydrocarbon. Small amounts of internally branched monomethylalkanes were detected; di- and trimethylalkanes were absent.

C. brevis is the common drywood termite found in structures in Hawaii; its distribution is pantropical. It is almost exclusively found in buildings and furnishings. The hydrocarbon mixture of *C. brevis* resembles the general pattern of drywood termites living in xeric, tropical habitats (Haverty et al., 1997). Hydrocarbons occurred in two groups: the early eluting compounds consisted almost exclusively of *n*-alkanes and terminally branched monomethyl alkanes, whereas late-eluting compounds were chiefly olefins (Figure 3, Table 3). *n*-Alkanes, primarily *n*-C25 and *n*-C27, comprised ca. 29.4% of the total hydrocarbon. Alkenes, alkadienes, and alkatrienes were the predominant class, representing over 50% of the total hydrocarbon. The terminally branched monomethylalkanes amounted to 16% of the total hydrocarbon. Internally branched monomethylalkanes and dimethylalkanes occurred in small quantities.

C. cynocephalus was recently discovered in native vegetation in Hawaii (Haverty et al., 2000; Scheffrahn et al., 2000; Grace et al., 2002), and is found in structures in the Philippine Islands (Scheffrahn et al., 2000). This species also reflects the general pattern of hydrocarbon mixtures of drywood termites living in xeric, tropical habitats (Haverty et al., 1997) and is similar to *C. brevis*.

TABLE 2. RELATIVE ABUNDANCE[a] OF HYDROCARBONS FROM PSEUDERGATES AND FECAL PELLETS OF *Incisitermes immigrans* SNYDER

Hydrocarbons	ECL[b]	Termites	Fecal pellets
2-meC22		1.65	1.37
n-C23		4.73	8.63
2-meC23		6.36	5.00
3-meC23		2.95	2.99
n-C24		0.77	0.82
C25:2	24.30	0.29	0.48
C25:2; 2-meC24[c]	24.60	4.04	3.59
C25:1	24.70	1.42	1.20
n-C25		8.29	8.56
C26:2	25.30	1.32	1.41
2-meC25		0.24	0.28
C26:2; 3-meC25[c]	25.65	1.00	0.54
n-C26		1.52	0.76
C27:2	26.30	27.79	31.63
C27:2; C27:1; 2-meC26[c]	26.60	0.78	0.79
C27:1	26.70	3.30	1.20
Unknown		0.92	0.20
n-C27		6.69	3.58
11-meC27; C28:2[c]	27.30	0.36	0.34
2-meC27		0.18	0.14
3-meC27		0.11	0.10
n-C28		0.31	0.12
C29:2	28.30	0.20	0.12
n-C29		2.53	0.32
n-C31		0.00	0.19
n-C33		0.00	0.24
n-C34		0.00	0.18
n-C35		0.00	0.30
n-C36		0.00	0.30
n-C37		0.00	0.31
15,19-; 13,17-dimeC37[c]		0.00	0.48
n-C38		0.00	0.36
C39:2		0.05	0.00
n-C39		0.00	0.35
n-C40		0.00	0.25
C41:2		0.12	0.23
13,23-dimeC41		0.00	0.58
C42:2		0.23	0.00
C43:3		1.74	1.53
C43:2		8.23	7.10
C43:1		1.52	1.79
C44:3		0.25	0.43
C44:2		0.38	0.49
C45:4		0.64	0.42
C45:3		5.10	5.78
C45:2		4.00	4.51

[a] Percent of total hydrocarbon composition.
[b] Equivalent chain length.
[c] An isomeric mixture or two or more compounds coelute in this peak.

TABLE 3. RELATIVE ABUNDANCE[a] OF HYDROCARBONS FROM PSEUDERGATES AND FECAL PELLETS OF *Cryptotermes brevis* (WALKER)

Hydrocarbons	Termites	Fecal pellets
n-C23	0.08	0.17
2-meC23	0.58	0.12
3-meC23	1.87	0.46
n-C24	0.66	0.59
Unknown	1.42	0.00
Unknown	0.13	0.00
2-meC24	4.20	1.05
3-meC24	0.52	0.07
n-C25	12.48	11.76
13-; 11-; 9-meC25[b]	0.64	0.24
2-meC25	1.00	0.34
3-meC25	6.47	2.19
n-C26	1.76	1.65
3,13-; 3,15-dimeC25[b]	0.14	0.10
2-meC26	0.87	0.35
3-meC26	0.15	0.00
n-C27	11.19	10.18
13-; 11-meC27[b]	0.15	0.08
2-meC27	0.10	0.06
3-meC27	0.69	0.28
n-C28	0.78	0.69
C29:1	0.12	0.00
C29:1	0.06	0.00
n-C29	2.34	1.93
13-meC29	0.06	0.00
2-meC29	0.15	0.13
C31:1	0.27	0.11
n-C31	0.13	0.13
3,7-dimeC31	0.14	0.06
C33:2	0.13	0.00
C33:1	0.16	0.09
C35:3	0.19	0.00
C35:2	1.02	0.22
C35:1	0.23	0.00
C36:2	0.60	0.00
C37:3	0.59	0.00
C37:2	7.13	2.85
C37:1	1.35	0.64
C38:3	0.28	0.00
C38:2	1.39	0.77
C38:1	0.22	0.00
C39:3	2.28	0.93
C39:2	9.14	9.78
C39:1	2.24	3.56
C40:3	0.53	0.58

TABLE 3. CONTINUED

Hydrocarbons	Termites	Fecal pellets
C40:2; 15-; 13-meC39[b]	1.13	2.06
C40:1	0.21	0.40
C41:3	3.60	6.18
C41:2	4.29	10.93
C41:1	1.43	3.98
C42:3	0.35	0.71
C42:2; 15-; 13-meC41[b]	0.56	1.65
C42:1	0.18	0.17
C43:3	2.03	4.45
C43:2	2.32	6.44
C43:1	1.90	3.62
C44:2; 15-; 13-meC43[b]	0.27	1.38
C45:3	2.22	3.33
C45:2	2.06	2.51
C45:1	0.80	0.00

[a] Percent of total hydrocarbon composition.
[b] An isomeric mixture or two or more compounds coelute in this peak.

Hydrocarbons occurred in two groups: the early eluting compounds were dominated by one olefin, C27:1; late-eluting compounds were predominantly olefins (Figure 4, Table 4). *n*-Alkanes comprised 16% of the total hydrocarbon. Alkenes, alkadienes, and alkatrienes were the predominant class of cuticular hydrocarbons, representing 70.3% of the total hydrocarbon; C27:1 accounts for 28% of the total hydrocarbon. Terminally branched monomethylalkanes amounted to 11.1% and internally branched monomethylalkanes 2.8% of the total hydrocarbon. No di- or trimethylalkanes were detected.

I. minor has an extensive geographical and ecological range. It is common in structures and in trees, especially at pruning scars, in the southwestern, coastal counties of California. Its distribution extends north to Mendocino County, south into Baja California and along the coast of western Mexico, and east into eastern Arizona and Utah (Weesner, 1970). *I. minor* has been introduced, and possibly established, in two separate locations on the island of Oahu (Grace et al., 2002). The general pattern of the chromatograms of *I. minor* (Figure 5) was more similar to that of *M. hubbardi* (see below) than that of *I. immigrans* (Figure 2), or other drywood termites from the tropics (Haverty et al., 1997, 2000). Hydrocarbons occurred in a continuous "series" from *n*-C23 to 13,17-dime C43 without the unoccupied region from C29 to C33 (Table 5). *n*-Alkanes comprised 17.7% of the total hydrocarbon. The unsaturated components constituted only 19.7% of the total hydrocarbon, a proportion much lower than that of tropical kalotermitids. Late-eluting olefins predominated in

TABLE 4. RELATIVE ABUNDANCE[a] OF HYDROCARBONS FROM PSEUDERGATES AND FECAL PELLETS OF *Cryptotermes cynocephalus* LIGHT

Hydrocarbons	ECL[b]	Termites	Fecal pellets
n-C20		0.00	1.15
n-C21		0.00	3.24
2-meC21		0.00	0.23
n-C22		0.00	3.68
2-meC22		0.00	0.45
3-meC22		0.00	0.31
n-C23		0.39	3.95
2-meC23		0.40	0.61
3-meC23		0.00	0.46
n-C24		0.20	1.58
2-meC24		3.65	2.70
3-meC24		0.00	0.42
n-C25		9.17	13.91
13-; 11-meC25[c]		0.93	1.04
2-meC25; C26:1[c]	25.70	2.81	2.02
3-meC25		3.47	3.93
n-C26		0.70	1.12
C27:1	26.30	0.61	0.31
C27:1	26.70	27.41	32.94
n-C27		4.91	4.13
C28:1	27.30	0.20	0.09
13-; 11-; 9-meC27[c]		1.23	0.89
2-meC27; C28:1[c]	27.70	0.96	0.73
3-meC27		0.46	0.26
n-C28		0.11	0.30
C29:1	28.30	0.32	0.15
C29:2	28.50	0.36	0.18
C29:1	28.70	0.92	0.67
n-C29		0.44	0.51
C29:2	29.30	0.24	0.06
2-meC29		0.39	0.11
C31:2	30.50	1.21	0.20
C31:1	30.70	0.37	0.00
2-meC30		0.88	0.22
n-C31		0.09	0.36
C35:2	34.50	0.22	0.00
C37:2	36.50	3.82	0.30
C37:1	36.70	0.58	0.00
C38:2		0.42	0.00
C39:3		0.85	0.00
C39:2		4.69	0.62
C39:1		0.53	0.00
C40:2		0.48	0.17
C41:3		0.91	0.09
C41:2		8.14	4.31
C41:1		4.04	2.09
15-; 13-meC41; C42:2[c]		1.29	0.95
C43:3		1.22	0.92
C43:2		8.15	6.35
C43:1		1.82	1.28

[a] Percent of total hydrocarbon composition.
[b] Equivalent chain length.
[c] An isomeric mixture or two or more compounds coelute in this peak.

TABLE 5. RELATIVE ABUNDANCE[a] OF HYDROCARBONS FROM PSEUDERGATES AND FECAL PELLETS OF *Incisitermes minor* (HAGEN)

Hydrocarbons	Termites	Fecal pellets
n-C23	1.37	1.55
2-meC23	3.38	2.91
3-meC23	2.22	1.39
n-C24	0.42	0.78
n-C25	9.20	8.91
2-meC25	0.22	0.18
3-meC25	0.60	0.36
n-C26	1.07	0.97
n-C27	4.86	4.35
3-meC27	0.25	0.22
n-C29	0.80	0.76
9,13-dimeC29	0.94	0.55
9,13,17-trimeC29	0.84	0.25
10,14-dimeC30	1.37	0.69
10,14,18-trimeC30	1.05	0.49
15-; 13-; 11-; 9-meC31[b]	0.32	0.00
13,17-; 11,15-dimeC31[b]	12.99	8.17
11,15,19-; 9,13,17-trimeC31[b]	1.68	1.13
7,11,17-trimeC31	0.31	0.27
14-; 12-meC32[b]	0.30	0.18
12,16-dimeC32	3.54	2.98
10,14,18-; 8,X,X-trimeC32[b,c]	0.46	0.49
15-; 13-meC33	1.28	0.53
13,17-; 11,15-; 9,13-dimeC33[b]	8.66	8.80
9,13,17-; 7,X,X-trimeC33[b,c]	1.28	1.97
C35:3	1.45	1.08
C35:2	3.60	3.32
12,16-dimeC34	1.30	2.03
15-; 13-; 11-meC35[b]	0.82	0.77
13,17-dimeC35	2.67	4.68
9,13,17-trimeC35	0.50	1.91
C37:3	4.30	5.37
C37:3	1.73	2.56
C37:2	2.30	1.88
12,16-dimeC36	0.32	0.80
C37:1	0.28	0.52
10,14,18-trimeC36	0.28	0.41
13; 11-meC37[b]	0.67	0.59
13,17-; 11,15-dimeC37[b]	1.75	1.92
9,13,17-trimeC37	0.27	0.58
C39:3	1.76	5.43
C39:3; C39:2; 12,16-dimeC38[b]	0.73	1.16
C39:1	1.13	1.16
13-; 11-meC39[b]	0.96	0.89
13,17-; 11,15-dimeC39[b]	0.99	0.97

TABLE 5. CONTINUED

Hydrocarbons	Termites	Fecal pellets
12-; 10-meC40[b]	0.30	0.44
12,16-dimeC40	0.40	0.40
C41:1	1.51	1.65
13-; 11-meC41[b]	1.71	1.28
13,17-; 11,15-dimeC41[b]	4.46	4.69
9,13,17-trimeC41	0.36	0.74
12-; 10-meC42[b]	0.25	0.35
12,16-dimeC42	0.63	0.62
C43:1	1.62	1.36
15-; 13-meC43[b]	0.30	0.32
13,17-dimeC43[b]	1.21	1.24

[a] Percent of total hydrocarbon composition.
[b] An isomeric mixture or two or more compounds coelute in this peak.
[c] The exact methyl branch positions are uncertain.

this component. The terminally branched monomethylalkanes amounted to 6.7% of the total hydrocarbon, whereas internally branched monomethylalkanes totaled 6.9%. Dimethylalkanes were the predominant class of compounds, making up 42% of the total hydrocarbon. *I. minor* also made a homologous series of trimethylalkanes representing 7.0% of the total hydrocarbon.

P. corniceps is found throughout most of the islands of the West Indies (Scheffrahn et al., 1994; Haverty et al., 1997). It can be found in dead wood of native vegetation, including fence posts, but has not yet been recorded from structures (Collins et al., 1997). The cuticular hydrocarbon mixture of *P. corniceps* (Figure 6) was similar in gross comparison to that of *C. brevis* (Figure 3). The early eluting components were almost exclusively *n*-alkanes and terminally branched monomethylalkanes, and the late eluting compounds were primarily olefins (Table 6). *n*-Alkanes comprised 8.7% of the total hydrocarbon. As with *C. brevis*, the alkenes, alkadienes, and alkatrienes were the predominant class of cuticular hydrocarbons, totalling over 76% of the total hydrocarbon. The terminally branched monomethylalkanes made up 12.2% of the total hydrocarbon.

P. occidentis occurs primarily within the general limits of the Sonoran Desert in southern Arizona and Baja California. *P. occidentis* has been found in few tree species, most commonly in blue paloverde, *Parkinsonia florida* (Bentham ex A. Gray) S. Watson (Nutting, 1966). The cuticular hydrocarbon mixture of *P. occidentis* has never been published, therefore, we provide a detailed description here. Once again, cuticular hydrocarbons occurred in two distinct groups: early eluting (25 to 31 carbons in the parent chain) and late-eluting compounds (41 to 43 carbons in the parent chain). The early eluting

TABLE 6. RELATIVE ABUNDANCE[a] OF HYDROCARBONS FROM PSEUDERGATES AND FECAL PELLETS OF *Procryptotermes corniceps* (SNYDER)

Hydrocarbons	Termites	Fecal pellets
2-meC24	1.52	0.68
3-meC24	0.06	0.00
n-C25	1.62	1.37
2-meC25	1.87	0.55
3-meC25	3.10	1.58
n-C26	0.87	0.47
2-meC26	4.18	1.55
3-meC26	0.27	0.00
n-C27	5.95	4.43
2-meC27	0.36	0.12
3-meC27	0.70	0.15
n-C28	0.11	0.09
2-meC28	0.17	0.27
n-C29	0.18	0.00
C35:2	0.09	0.00
C37:3	0.84	0.41
C37:2	1.65	1.02
C37:1	0.08	0.06
C38:3	0.14	0.00
C38:2	0.65	0.43
C39:3	4.88	4.04
C39:2	8.10	8.63
C39:1	13.63	1.62
C40:X[c]	0.58	0.48
C40:3	1.32	1.53
C40:2; 15-; 13-meC39[b]	3.53	3.31
C41:3	13.35	22.83
C41:2	15.11	20.27
C41:1	0.96	1.23
C41:1	2.65	3.62
C42:X[c]	0.97	1.39
C42:3	1.46	2.24
15-; 13-meC41[b]	1.26	1.68
C42:2	0.82	1.03
C43:4	0.80	1.47
C43:3	4.04	8.08
C43:2	1.79	3.09
C43:1	0.37	0.29

[a] Percent of total hydrocarbon composition.
[b] An isomeric mixture or two or more compounds coelute in this peak.
[c] This peak appears to be a mixture of unsaturated compounds, their exact identities are undetermined.

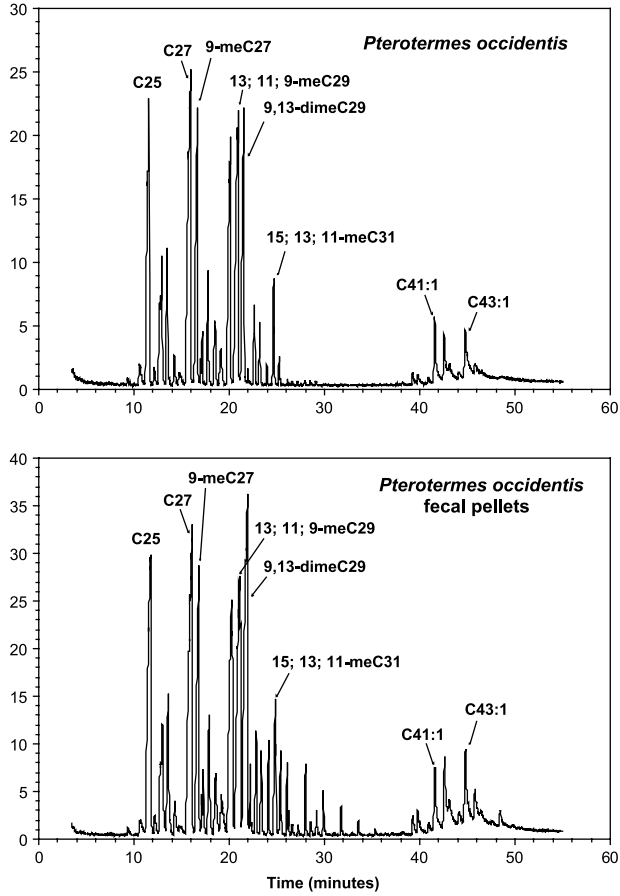

FIG. 7. Total ion chromatogram of the cuticular hydrocarbons from pseudergates of *Pterotermes occidentis* (Walker) from the Santa Rita Experimental Range near Tucson, AZ, and fecal pellets from the same collection.

compounds predominated, representing over 92 percent of the total hydrocarbon (Figure 7, Table 7).

n-Alkanes were, by far, the most abundant hydrocarbons representing over 40% of the total (Table 7). *n*-C25, *n*-C27, and *n*-C29 comprised ca. 10.5%, 14.7%, and 9.1% of the total hydrocarbon, respectively. The other *n*-alkanes accounted for 6.6% of the total (Table 7). In contrast to the tropical drywood termite species, olefins were not the predominant hydrocarbons, representing only 5.0% of the total (Table 7). Two monoenes, C41:1 and C43:1, made up the majority of these hydrocarbons.

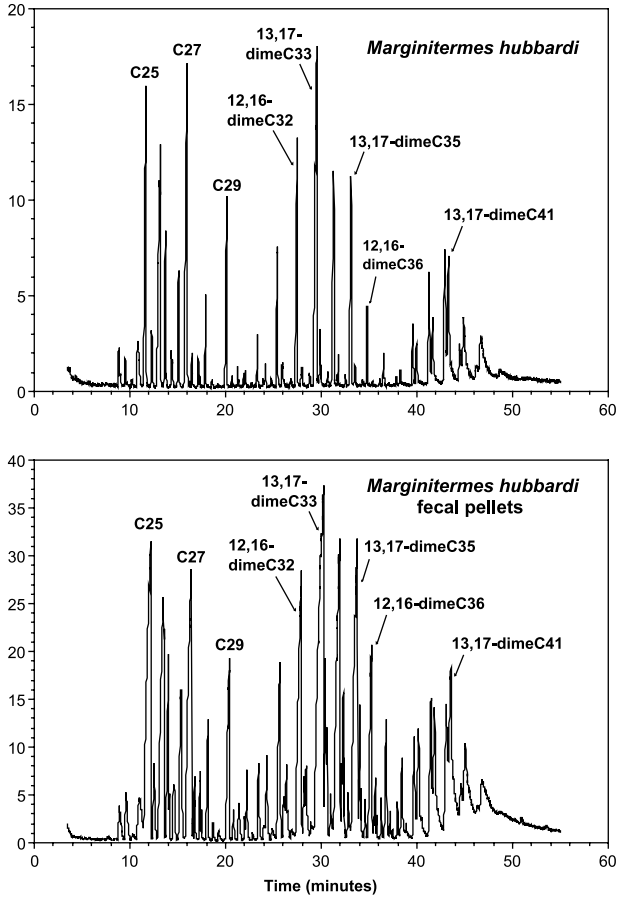

FIG. 8. Total ion chromatogram of cuticular hydrocarbons from pseudergates of *Marginitermes hubbardi* (Banks) from the Saguaro National Monument southeast of Tucson, AZ, and fecal pellets from the same collection.

Terminally branched monomethylalkanes were identified for C24 to C27 (Table 7). 2-MeC25 and 3-meC25 accounted for 2.8% and 2.6% of the total hydrocarbon, respectively. The remainder of these terminally branched monomethylalkanes comprised only 2.2% of the total. Internally branched monomethylalkanes comprised 33.0% of the total hydrocarbon (Table 7). The homologous series of internally branched monomethylalkanes, C27, C29, C31, and C41 constituted 26.9% of the total hydrocarbon; none of the other internally branched monomethylalkanes represented more than 0.85% of the total. Dimethylalkanes comprised 13.4% of the total hydrocarbon; 9,13-dimeC29

TABLE 7. RELATIVE ABUNDANCE[a] OF HYDROCARBONS FROM PSEUDERGATES AND FECAL PELLETS OF *Pterotermes occidentis* (WALKER)

Hydrocarbons	Termites	Fecal pellets
n-C24	0.16	0.16
2-meC24	0.75	0.43
n-C25	10.52	9.17
13-; 11-; 9-meC25[b]	0.51	0.38
2-meC25	2.79	1.00
3-meC25	2.62	2.57
n-C26	3.39	2.90
9-; 8-meC26[b]	0.85	0.72
2-meC26	0.51	0.37
n-C27	14.71	11.97
13-; 11-; 9-meC27[b]	9.59	7.39
9,13-dime; 2-meC27[b]	0.47	0.41
3-meC27	0.73	0.73
n-C28	2.32	2.26
12-; 10-; 9; 8-meC28[b]	2.03	1.54
9,13-; 8,12-; 8,14-dimeC28[b]	1.40	1.32
n-C29	9.13	9.37
15-; 13-; 11-meC29[b]	13.17	10.55
9,13-dimeC29	9.63	12.61
n-C30	0.19	0.56
14-; 12-; 10-meC30[b]	1.81	1.84
C31:1; 10,14-dimeC30[b]	1.25	1.28
n-C31	0.33	1.01
15-; 13-; 11-; 9-meC31[b]	2.52	2.81
9,13-dimeC31	0.37	0.89
7,13-dimeC31	0.11	0.15
n-C32	0.07	0.74
3,7-dimeC31	0.00	0.21
14-; 12-; 10-meC32[b]	0.08	0.19
9,13-dimeC32	0.06	0.18
n-C33	0.12	0.84
13-; 11-; 9-meC33[b]	0.09	0.23
9,13-dimeC33	0.06	0.33
n-C34	0.00	0.49
n-C35	0.00	0.35
n-C36	0.00	0.17
n-C37	0.00	0.11
13-; 11-meC39[b]	0.35	0.42
9,13-dimeC39	0.33	0.64
14-; 13-; 12-meC40[b]	0.14	0.28
C41:1	2.13	1.84
15-; 13-; 11-meC41[b]	1.57	2.00
9,13-dimeC41	0.63	1.34
14-; 13-; 12-; 11-meC42[b]	0.17	0.71
C43:1	2.20	3.03
15-; 13-; 11-meC43[b]	0.17	1.51

[a] Percent of total hydrocarbon composition.
[b] An isomeric mixture or two or more compounds coelute in this peak.

TABLE 8. RELATIVE ABUNDANCE[a] OF HYDROCARBONS FROM PSEUDERGATES AND FECAL PELLETS OF *Marginitermes hubbardi* (BANKS)

Hydrocarbons	Termites	Fecal Pellets
n-C24	0.16	0.16
2-meC24	0.75	0.43
n-C25	10.52	9.17
13-; 11-; 9-meC25[b]	0.51	0.38
2-meC25	2.79	1.00
3-meC25	2.62	2.57
n-C26	3.39	2.90
9-; 8-meC26[b]	0.85	0.72
2-meC26	0.51	0.37
n-C27	14.71	11.97
13-; 11-; 9-meC27[b]	9.59	7.39
9,13-dime; 2-meC27[b]	0.47	0.41
3-meC27	0.73	0.73
n-C28	2.32	2.26
12-; 10-; 9-; 8-meC28[b]	2.03	1.54
9,13-; 8,12-; 8,14-dimeC28[b]	1.40	1.32
n-C29	9.13	9.37
15-; 13-; 11-meC29[b]	13.17	10.55
9,13-dimeC29	9.63	12.61
n-C30	0.19	0.56
14-; 12-; 10-meC30[b]	1.81	1.84
C31:1; 10,14-dimeC30[b]	1.25	1.28
n-C31	0.33	1.01
15-; 13-; 11-; 9-meC31[b]	2.52	2.81
9,13-dimeC31	0.37	0.89
7,13-dimeC31	0.11	0.15
n-C32	0.07	0.74
3,7-dimeC31	0.00	0.21
14-; 12-; 10-meC32[b]	0.08	0.19
9,13-dimeC32	0.06	0.18
n-C33	0.12	0.84
13-; 11-; 9-meC33[b]	0.09	0.23
9,13-dimeC33	0.06	0.33
n-C34	0.00	0.49
n-C35	0.00	0.35
n-C36	0.00	0.17
n-C37	0.00	0.11
13-; 11-meC39[b]	0.35	0.42
9,13-dimeC39	0.33	0.64
14-; 13-; 12-meC40[b]	0.14	0.28
C41:1	2.13	1.84
15-; 13-; 11-meC41[b]	1.57	2.00
9,13-dimeC41	0.63	1.34
14-; 13-; 12-; 11-meC42[b]	0.17	0.71
C43:1	2.20	3.03
15-; 13-; 11-meC43[b]	0.17	1.51

[a] Percent of total hydrocarbon composition.
[b] An isomeric mixture or two or more compounds coelute in this peak.

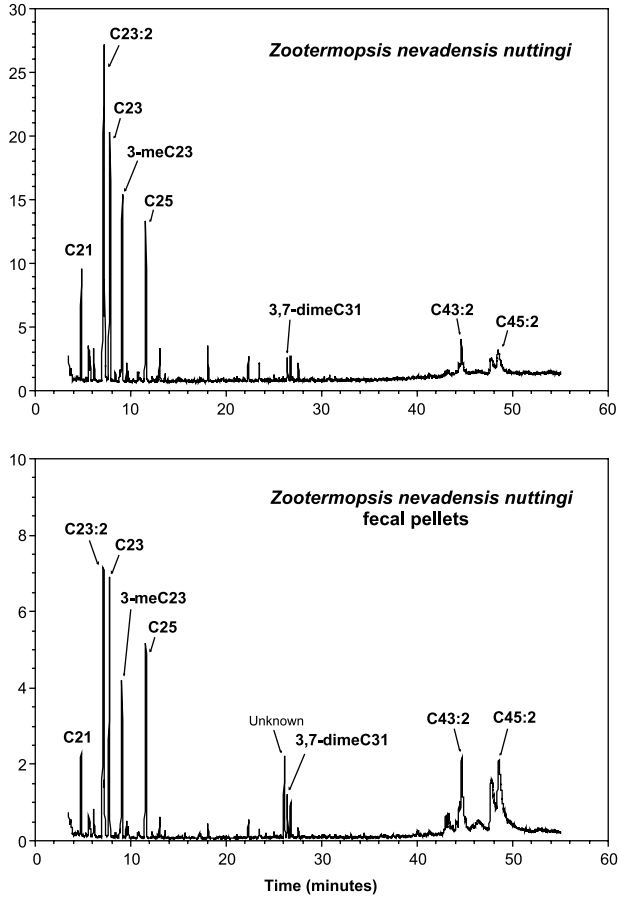

FIG. 9. Total ion chromatogram of cuticular hydrocarbons from pseudergates of *Zootermopsis nevadensis nuttingi* Haverty and Thorne from the Klamath National Forest northeast of Mount Shasta, CA, and fecal pellets from the same collection.

was the foremost member of this class with 9.6% of the total (Table 7). No trimethylalkanes were detected (Table 7).

M. hubbardi is one of the most common drywood termites in the Sonoran Desert. It is often found in the skeletal remains of the saguaro cactus, *Cereus giganteus* Englm., and is also commonly found in wood of structures. *M. hubbardi* replaces, or is more frequently encountered than, *I. minor* at lower elevations and drier regions of the Sonoran Desert (Weesner, 1970). As observed for *P. occidentis*, the cuticular hydrocarbon mixture of *M. hubbardi* has not been published, therefore, we provide a detailed description here.

TABLE 9. RELATIVE ABUNDANCE[a] OF HYDROCARBONS FROM PSEUDERGATES AND FECAL PELLETS OF *Zootermopsis nevadensis nuttingi* HAVERTY AND THORNE

Hydrocarbons	ECL[b]	Termites	Fecal pellets
n-C21		5.15	3.75
2-meC21		1.80	0.92
3-meC21		1.32	0.64
n-C22		1.71	1.15
C23:2	22.50	29.21	16.10
C23:1	22.70	3.21	1.87
n-C23		15.34	12.58
11-meC23		0.60	0.49
2-meC23		0.73	0.41
3-meC23		10.47	6.82
n-C24		0.75	0.69
3,7-dimeC23		0.64	0.49
C25:1	24.70	0.69	0.32
n-C25		9.06	9.10
11-meC25		0.00	0.28
3-meC25		1.62	0.89
n-C26		0.24	0.36
n-C27		0.00	0.19
3,7-dimeC27		1.70	0.57
3,7-dimeC29		1.37	0.81
C31:2	30.50	0.86	0.45
C31:1	30.70	0.00	0.23
7-meC31		0.00	0.24
Unknown		0.00	4.10
3,7-dimeC31		1.32	1.78
C33:3		1.31	1.32
C33:2		0.89	0.51
C43:X[c]		0.00	1.11
C43:X[c]		0.00	2.21
C43:X[c]		0.00	0.42
C43:X[c]		0.00	1.01
C43:3		0.67	2.06
C43:3		4.66	7.18
C45:X[c]		1.63	9.20
C45:3		3.04	9.74

[a] Percent of total hydrocarbon composition.
[b] Equivalent chain length.
[c] This peak appears to be a mixture of unsaturated compounds, their exact identities are undetermined.

The general pattern of the chromatograms of *M. hubbardi* (Figure 8) was more similar to that of *I. minor* (Figure 5) than that of other drywood termites from Hawaii or the dry tropics (Figures 1–4, 6). Hydrocarbons occurred continuously from *n*-C25 to dimeC43 without the unoccupied region from C29 to C33. *n*-Alkanes present were *n*-C24, *n*-C25, *n*-C26, *n*-C27, *n*-C28, *n*-C29 *n*-C30, and *n*-C31 (Table 8). *n*-C25 and *n*-C27 were the most abundant, comprising 7.8% and 9.7% of the total hydrocarbon, respectively (Figure 8). The remaining *n*-alkanes amounted to 8.1% of the total hydrocarbon. There were no unsaturated components detected.

Terminally branched monomethylalkanes were identified for C23 to C27; 3-meC25, representing 2.3% of the hydrocarbons, was the most abundant. Terminally branched monomethylalkanes constituted only 3.6% of the total hydrocarbon. Internally branched monomethylalkanes were the third most abundant class in *M. hubbardi*. Most were among the later-eluting compounds with carbon numbers in the parent chain ranging from 29 through 43 (Table 8). The isomeric mixture of 11-; 13-; 15-meC41 was the largest peak in this class accounting for 3.7% of the total (Figure 8).

In *M. hubbardi*, as in *I. minor*, dimethylalkanes were the predominant class of compounds, accounting for 53.9% of the hydrocarbon. The isomeric mixture of 11,15-; 13,17-dimeC33 was the most abundant peak accounting for 10.8% of the total hydrocarbon. *M. hubbardi* made a homologous series of internally branched dimethylalkanes (9,13-; 11,15-; 12,16-; 13,17-) from C24 to C43 (Figure 8; Table 8). *M. hubbardi* also made numerous trimethylalkanes with 25 to 39 carbons in the parent chain; these trimethylalkanes accounted for 3.9% of the total hydrocarbon (Table 8).

Z. nevadensis nuttingi is the common dampwood termite found in the coastal forests of California, Oregon, and Washington west of the Cascade Range (Thorne et al., 1993). *Zootermopsis* species infest standing dead trees, stumps, and logs in contact with the soil. Like drywood termites, *Zootermopsis* species produce pelletized feces that are stored in chambers within their workings. *Z. nevadensis nuttingi* was originally designated Phenotype III of the North American *Zootermopsis* (Haverty et al., 1988). It was later designated as a subspecies of *Z. nevadensis* on the basis of its cuticular hydrocarbon mixture and its ability to recognize the other phenotype or subspecies, *Z. nevadensis nevadensis*, and react aggressively toward it (Haverty and Thorne, 1989). Morphologically, *Z. n. nuttingi* remains indistinguishable from *Z. n. nevadensis* (Thorne and Haverty, 1989). Their distributions are, for the most part, allopatric or parapatric (Thorne et al., 1993; Haverty et al., unpublished data).

The hydrocarbon mixture of *Z. nevadensis nuttingi* has been published previously (Haverty et al., 1988). This subspecies has since been more thoroughly characterized, and several compounds have been identified that were not previously reported. In particular, numerous olefins were identified by their mass

spectra. These compounds can become unstable if not analyzed promptly after extraction, and this may account for their absence in the previous report (Haverty et al., 1988). Handling and extraction of specimens has been standardized to include drying of termites before extraction, thereby improving the recovery of hydrocarbons, especially olefins, from the cuticle (Haverty et al., 1996b).

The general pattern of the chromatograms of *Z. nevadensis nuttingi* (Figure 8) was similar to that of *N. connexus* (Figure 1), where the great majority of the hydrocarbons have 21 to 31 carbons in the parent chain. *n*-Alkanes present were *n*-C21, *n*-C22, *n*-C23, *n*-C24, and *n*-C25 (Table 9). *n*-C23 and *n*-C25 were the most abundant, comprising 15.3% and 9.1% of the total hydrocarbon, respectively (Figure 9). The remaining *n*-alkanes comprised 7.9% of the hydrocarbons.

The unsaturated components constituted 46.2% of the total hydrocarbon. Olefins with 23, 43, and 45 carbons predominated among the olefins. C23:2, C23:1, C43:3, and C45:3 accounted for 29.2%, 3.2%, 4.7%, and 3.0% of the total hydrocarbon, respectively (Figure 9; Table 9). The remaining unsaturated components amounted to only 6.1% of the total hydrocarbons.

2- and 3-Methylalkanes were identified for C21 to C25, with 3-meC23, representing 10.5% of the total hydrocarbon, being the most abundant (Table 9). These terminally branched monomethylalkanes represented 15.9% of the total hydrocarbon. A small amount of the internally branched 11-meC23 was seen. Dimethylalkanes were represented by a homologous series of 3,7-dimeCXX (Table 9).

Hydrocarbons from Termite Fecal Pellets. In general, the hydrocarbons from whole-body extracts of drywood termites were represented qualitatively and quantitatively in extracts of fecal pellets from the same species. Extracts of fecal pellets were more similar to those of their producers than they were to extracts of any other termite species or their fecal pellets. Cluster analysis with all hydrocarbons, using Euclidean distance, consistently paired the hydrocarbon mixture from fecal pellets with the hydrocarbon mixture from termites of the species that produced the pellets (Figure 10).

The hydrocarbon mixtures from whole-body extracts and that of fecal pellets were not identical; minor differences did occur and were likely the result of one or more factors. For example, some termite/pellet pairs were not from the same colony or location (*N. connexus*, *I. immigrans*, and *C. brevis*); thus, the minor qualitative and quantitative differences between termites and fecal pellets could reflect intraspecific variation. When certain hydrocarbons were found in the termites, but not in the fecal pellets, and *vice versa*, they often occurred in small quantities, usually less than 1.0% of the total hydrocarbon component (Tables 1–9). Therefore, the lack of certain hydrocarbons could also be a function of the concentration of the extracts.

Paraffins, a series of *n*-alkanes, were found in both termite and fecal pellet extracts, but long-chain *n*-alkanes were found only in extracts of termite fecal

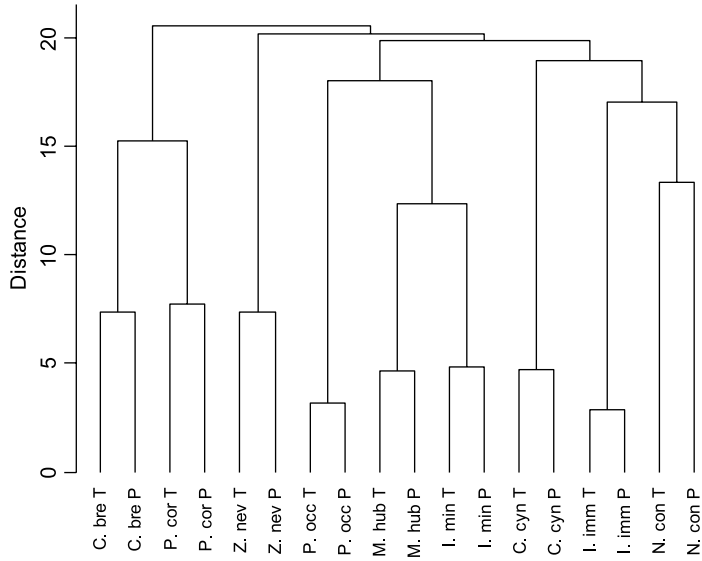

FIG. 10. Dendrogram from cluster analysis based on Euclidean distance of hydrocarbons extracted from eight species of drywood termites and one species of dampwood termite and their fecal pellets. N. con = *Neotermes connexus*, I. imm = *Incisitermes immigrans*, C. bre = *Cryptotermes brevis*, C. cyn = *Cryptotermes cynocephalus*, I. min = *Incisitermes minor*, P. cor = *Procryptotermes corniceps*, P. occ = *Pterotermes occidentis*, M. hub = *Marginitermes hubbardi*, and Z. nev = *Zootermopsis nevadensis nuttingi*.

pellets (Tables 1, 2, 7, 8; Figure 1). *n*-Alkanes with more than 31 carbon atoms are not usually seen in insects; insects apparently cannot synthesize *n*-alkanes containing more than 34 carbon atoms (Hadley, 1985). In plants, *n*-alkanes are the principal hydrocarbon fraction of the cuticlar lipids, ranging in length from 21 to 37 carbon atoms (Hadley, 1981). It is possible that the long-chain *n*-alkanes from drywood termite fecal pellets were a contaminant from plant origin, from the ubiquitous Parafilm® used in most laboratories, or even contamination from hand lotion. Such contamination complicated evaluation of chemotaxonomic characters of two species of Central American *Nasutitermes* (Howard et al., 1988).

Olefins comprised the majority of the hydrocarbon components in six of the nine termites discussed in this paper. Olefins are the least stable and most reactive of the cuticular hydrocarbons. However, for some reason, they remain stable in or on the cuticles of insects. For example, when hydrocarbons were extracted from 70-year-old museum specimens of scolytid cone beetles, *Conophthorus ponderosae* Hopkins, the mixtures were qualitatively identical to those collected and extracted by Page et al. (1990a). On the contrary, once

extracted, cuticular hydrocarbons of *Reticulitermes* spp. containing mono-, di- and trienes, lost much of the olefin component when dried and left in a vial for 24 hr, particularly the di- and trienes (Nelson and Haverty, unpublished observations). Thus, something prevents the oxidation of olefins while still in the cuticle. On or in fecal pellets, the olefins appeared to be as stable as they are within the cuticle of the termites.

The composition of the hydrocarbon mixture of insects is genetically controlled (Toolson and Kuper-Simbrón, 1989; Kaib et al., 1991; Page et al., 1991; Coyne et al., 1994). This composition can be slightly affected by diet and environmental conditions (Hadley, 1977; Espelie et al., 1994; Chapman et al., 1995; Howard, 1998; Woodrow et al., 2000). Because cuticular hydrocarbon mixtures are generally species-specific (see references in the Introduction), we did not expect to observe qualitative differences in cuticular hydrocarbon mixtures between recently collected *Z. nevadensis nuttingi* and those (Phenotype III) described by Haverty et al. (1988). Sevala et al. (2000) found similar qualitative differences between recently collected *Z. nevadensis nevadensis* and those (Phenotype I) reported by Haverty et al. (1988). In the present study, termites were extracted that had been dried, while Haverty et al. (1988) extracted freshly frozen (i.e., moist) termites. This could explain the difference, as drying termites first has been shown to enhance extraction of cuticular hydrocarbons, especially the late-eluting olefins (Haverty et al., 1996b). In addition, the olefin component of all four taxa reported by Haverty et al. (1988) was likely underreported, as samples were extracted, the extract dried under nitrogen, then sent to the University of Nevada for identification by GC-MS. Therefore, many (or all) of the olefins could have oxidized and no longer been present in the sample for identification. Furthermore, the equipment used by Haverty et al. (1988) (Finnigan 4023 mass spectrometer) was less sensitive than that used by Sevala et al. (2000) and in this study (HP5890 GC coupled with an HP5989 or 5970 MS, respectively). Thus, minor components and coeluting isomers could have been missed by Haverty et al. (1988).

From where do the hydrocarbons in drywood termite fecal pellets come? The answer to this question could be contamination from the cuticles of the termites moving within the gallery system strewn with fecal pellets. Three other scenarios are more likely, however. First, controlled cannibalism is a key to the nitrogen economy of a termite colony. Exuviae, injured or dead individuals, and excess members of any caste are commonly eaten (Moore, 1969). The hydrocarbons ingested could pass through the gut undigested, only to be compacted in the fecal pellets, although this has not yet been tested. Second, the termites moving and storing fecal pellets could secret a mixture of hydrocarbons from the various glands (labral, mandibular, labial, or salivary) associated with the mouthparts (Noirot, 1969). Third, and most likely, is that hydrocarbons are deposited onto the fecal pellets within the rectum during or after the fecal dehydration

process. Oenocytes transmit hydrocarbons within the hemolymph to their epicuticular destinations (Schal et al., 1998; Sevala et al., 2000). Because the rectum is lined with cuticle (Child, 1934), hydrocarbons are likely transferred to the epicuticular cells of the rectum and then transferred to the surface, or lining.

What is the significance of fecal hydrocarbons that are similar to those found in termite cuticles? With more sampling across additional taxa, diagnostic hydrocarbons could be identified (Haverty et al., 1997) and a key to species developed (Haverty et al., 2000) for either nondiagnostic castes, such as larvae, nymphs, and pseudergates or workers, or even fecal pellets alone. This knowledge could potentially have applications for quarantine operations to identify species or damage to wood products or solid wood packing material and contain the spread of invasive termite species. In addition, our findings open up the possibility of other chemicals, such as semiochemicals, being deposited on or within fecal pellets with the pellets functioning as a slow-release mechanism (Souto and Kitayama, 2000). The possibility also exists that other wood-destroying insects could also be identified to species according to the hydrocarbons in their feces.

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CIRCADIAN RHYTHMS OF SEXUAL BEHAVIOR AND PHEROMONE TITERS OF TWO CLOSELY RELATED MOTH SPECIES *Autographa gamma* AND *Cornutiplusia circumflexa*[1]

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Abstract—Two closely related plusiinae moths, *Autographa gamma* Linnaeus and *Cornutiplusia circumflexa* Linnaeus, are sympatric in Israel. Both species use identical sex pheromone components but in different ratios, and do not attempt to mate with each other. In addition to the effective reproductive separation by their sex pheromones, the sexual behavior of both species was compared to determine whether the lack of selection pressure might create additional barriers to cross-attraction and cross-mating. We found the gamma moth to be sexually active almost equally throughout the scotophase, whereas the sexual activity of *C. circumflexa* was limited to a short period at the end of the scotophase when most of the gamma moths had already mated. Higher levels of calling were observed with older females. There was a close relationship between pheromone titer and calling activity in both species.

Key Words—*Autographa gamma*, gamma moth, *Cornutiplusia circumflexa*, Noctuidae, Plusiinae, calling behavior, male response, pheromone gland titer, circadian rhythm.

INTRODUCTION

The two closely related lepidopteran species, the gamma moth *Autographa gamma* Linnaeus and *Cornutiplusia circumflexa* Linnaeus (Noctuidae: Plusiinae) are sympatric in Israel. Both species are migratory, and adult migratory

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flights are recorded in Israel in autumn and spring (Yathom and Rivnay, 1968; Kitching, 1987; Pedgley and Yathom, 1993). Overlapping populations of these moths have also been recorded in Egypt (Harakly, 1975; Etman, 1989) and Saudi Arabia (Wiltshire, 1990). The two species belong to the subtribe Plusiina of the tribe Plusiini (Kitching, 1987). The gamma moth is a palearctic species and is a serious polyphagous pest, particularly in the eastern part of Europe (Ghizdavu et al., 1979). In Israel, this moth damages potato and celery crops. *C. circumflexa* is a poorly studied paleotropical species, feeding mainly on Solanaceae, but it is of no economic importance.

The sex pheromones of both species contain (*Z*)-7-dodecenyl acetate (*Z*7-12:Ac) and (*Z*)-7-dodecenol (*Z*7-12:OH), with *Z*7-12:Ac being the major component of the *A. gamma* (Dunkelblum and Gothilf, 1983; Tóth et al., 1983), whereas *Z*7-12:OH is the major component of the *C. circumflexa* pheromone (Mazor et al., 1991). No cross-attraction was found between the two species in both wind tunnel and field test experiments (Mazor et al., 1991; Dunkelblum and Mazor, 1993).

This study was conducted to determine whether temporal factors may also contribute to reproductive isolation of these two species.

METHODS AND MATERIALS

Insect Rearing. Laboratory colonies of *A. gamma* and *C. circumflexa*, started in 1983, were maintained at 26 ± 2°C, 60 ± 5% relative humidity under a 14:10 light–dark (LD) photoperiod, and wild adults, captured by light trap, were introduced annually into the colony. Larvae were reared on a modified artificial diet for Noctuidae (Shorey and Hale, 1965). Pupae were segregated by sex and placed in 30 × 30 × 30 cm screen cages in different rooms under the same rearing conditions. One room was used for observations of calling and copulation behavior and the other for wind tunnel experiments. Both rooms were lit with two to three red incandescent light bulbs, with a light intensity of approximately 0.5 lx during the scotophase. The intensity of the light in the wind tunnel room was measured at several points on the outer surface of the transparent wind tunnel. Adults were provided with a 10% sucrose solution. Adults emerging between 0 and 24 hr were considered as 1-d-old.

Observations of Calling Behavior. Observations were conducted during the 10 hrs of the scotophase with two groups of 30 *A. gamma* females because mating appeared to occur throughout the scotophase. The first group was examined from the beginning of the scotophase for 5 hrs, and the second group was examined during the 5 hrs from the 6th hr of the scotophase to its end. Females, 0- to 24-hr-old, were placed individually in 500-ml glass jars containing a steel mesh support and a 10% sucrose solution. Observations were

made every 10 min during the 1st, 2nd, 3rd, 4th, 5th, 8th, and 10th nights. The mean value of the six recordings per hour was taken to calculate the proportion of calling females. Twenty *C. circumflexa* females were tested every 15 min during the 1st, 2nd, 4th, 5th, 7th, 9th, and 11th night during the last 4 hrs of the scotophase because preliminary observations indicated that sexual activity of this moth occurred at this time. The mean value of the four recordings per hr was used to calculate the proportion of calling females. Dead individuals were removed from the cages. The proportions of females calling in both species were calculated according to the number of surviving females.

Wind Tunnel Experiments. The response of males toward the appropriate synthetic sex pheromone was tested in a 195 × 60 × 60 cm wind tunnel with a wind speed of 0.5 m/sec (Snir et al., 1986). Rubber septa (Arthur Thomas Co., 5 × 9 mm) containing 10 μg *Z*7-12:Ac were used as lures for *A. gamma* males because no difference was found between males' responses to *Z*7-12:Ac alone or in combination with the minor component *Z*7-12:OH (Mazor and Dunkelblum, 1992). Septa containing a blend of 10 μg *Z*7-12:OH and 2 μg *Z*7-12:Ac were used as lures for *C. circumflexa* males. Males were tested individually, recording the following behavioral parameters: takeoff (within 1 min), directed flight, landing, and copulation attempts. The response of 30 *A. gamma* males was tested daily for moths aged 1- to 8-d- and 11-d-old, and the response of 20 *C. circumflexa* males was tested daily for 1- to 7-d- and 10-d-old moths. The responses of 45 *A. gamma* and 20 *C. circumflexa* males were tested at different hours of the scotophase at the age of maximal sexual activity (6-d-old for *A. gamma* and 7-d-old for *C. circumflexa*).

Copulation. Two groups of 75 *A. gamma* pairs were tested during each of the two halves of the scotophase on three different nights. Observations were conducted at 15-min intervals. Each group was divided to three subgroups of 25 pairs. Each subgroup was placed in a 30 × 30 × 30 cm screen cage and was supplied with 10% sucrose solution. Females were 4- to 6-d-old and males were 6- to 8-d-old, the appropriate age of maximal sexual activities. Mating pairs were gently transferred into a 5-cm diam petri dish for the measurement of copulation duration. This procedure did not disturb the mating pair or the remaining moths. The same procedure was conducted with *C. circumflexa*. Two groups of 10 6-d-old *C. circumflexa* pairs and 14 6- to 7-d-old pairs were placed in individual screen cages as described for *A. gamma*. Observations of 15-min intervals were conducted during the second half of the scotophase on two different nights. The percentages of pairs starting to copulate at different times of the scotophase were calculated for all the groups of each of the species, (75 pairs for *A. gamma* and 24 pairs for *C. circumflexa*).

Pheromone Titer Determination. Batches of 25 pheromone glands (considered as one replicate because of low amount of pheromone per gland) were excised and immersed for 15 min in approx. 150 μl hexane. The supernatant

solutions were transferred into small conical vials, sealed with a Teflon-lined screwcap. For quantification, 100 ng of 10-undecenyl acetate (10-11:Ac) and 100 ng of 10-undecenol (10-11:OH) were added as internal standards for *A. gamma* and *C. circumflexa*, respectively. Samples were concentrated to ~1 μl by passive evaporation in a fume hood before analysis. Quantitative analyses were conducted by capillary GC on a Carlo Erba 5300 instrument equipped with an FID detector and a splitless injector, using a 30 m × 0.25 mm DB-225 column (J&W Scientific, Folsom, CA) held at 60°C/2min, then programmed to 150°C at 10°C/min. After 21 min, the temperature was raised to 200°C at 10°C/min. The extracts were analyzed for *Z*7-12:Ac, *Z*7-12:OH, and the two internal standards. Changes in pheromone titer at different moth age were examined for *A. gamma* at the 7th hr of the scotophase and for *C. circumflexa* at the 9th hr of the scotophase. Changes in pheromone titer at different times of the scotophase were examined with glands of 4- to 5-d-old *A. gamma* females and with 5- to 6-d-old *C. circumflexa* females in 7 replicates for *A. gamma* and 4–14 replicates for *C. circumflexa*. The timing of the extractions was selected in accordance with the moths' maximal sexual behavior.

Statistical Analysis. Data for *A. gamma* calling behavior were analyzed using the GLM procedure available on SAS software. Repeated measures analysis of variance was conducted where repeated observations were conducted for each female at the various ages during each of the two halves of the scotophase. Multiple comparisons were conducted to determine differences between any pairs of ages at each of the two halves of the scotophase. The data for *C. circumflexa* calling behavior, which consisted of repeated measurements at various consecutive ages, were analyzed with a paired *t*-test to compare differences between means of all pairs of age levels. The Bonferroni procedure of multiple comparisons was used, and each *t*-test was declared significant at $P = 0.003$. Data from wind tunnel experiments were analyzed with Fisher's exact test. The percentages of the responding males for each parameter at k group (ages or different times during the scotophase) were compared at the $P = 0.05$ level of significance. If differences were significant, a closed testing procedure for multiple comparisons among l groups ($l < k$) was conducted (Marcus et al., 1976). Changes in pheromone titer were analyzed by ANOVA, and the means were separated by Student–Newman–Keuls multiple range test at $\alpha = 0.05$ level of significance.

RESULTS

Observations on Calling Behavior. Calling behavior was measured as the proportion of calling females and the duration of calling by each female during

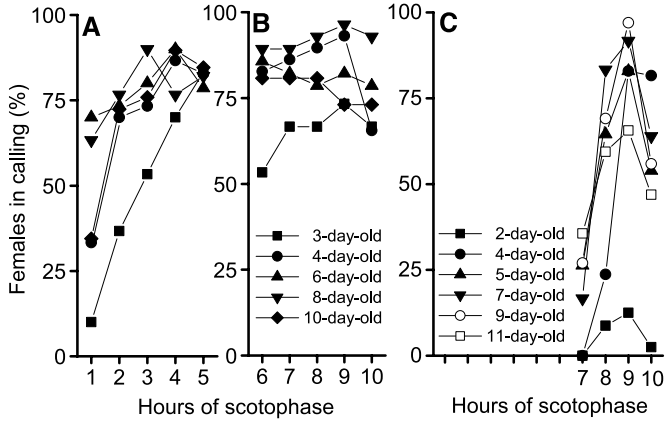

FIG. 1. The effect of age and time in scotophase on calling by *Autographa gamma* and *Cornutiplusia circumflexa* females. Observations on *A. gamma* were made on two groups (A and B) during the two halves of the scotophase at 10-min intervals ($N = 30$). Values represent the mean percentage of six recordings per hour. Observations on *C. circumflexa* were made during the last 4 hr of the scotophase (C) ($N = 20$). Values represent the mean percentage of four recordings per hour.

the scotophase. The pattern of *A. gamma* calling females at different ages during the two halves of the scotophase is shown in Figure 1A and B. The females called throughout the scotophase. However, there was marginally more calling due to more time spent in calling during the second half than during the first half

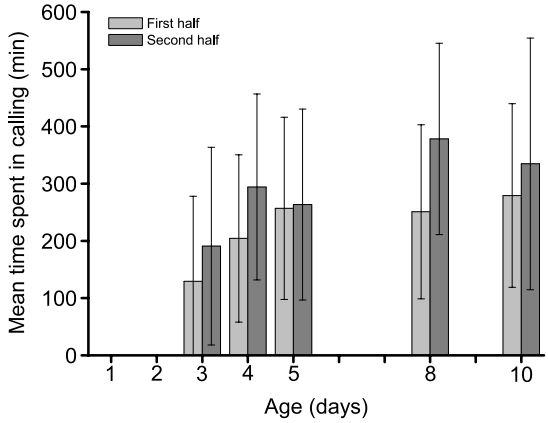

FIG. 2. The mean time (± SD) spent in calling by *A. gamma* females of different ages during the two halves of the scotophase ($N = 30$).

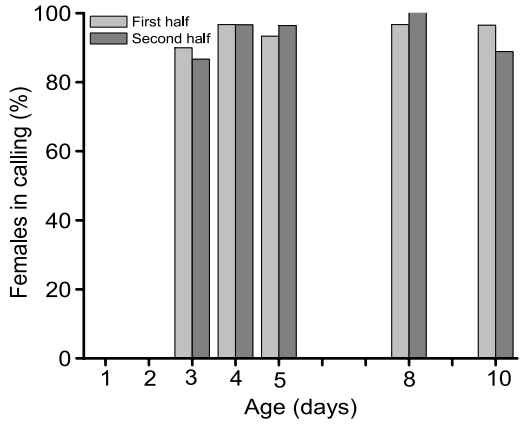

FIG. 3. The percentage of *A. gamma* calling females of different ages during the two halves of the scotophase ($N = 30$).

of the scotophase ($P = 0.053$) (Figure 2). Calling activity started on the 3rd night after emergence, and almost constant calling activity was observed thereafter (Figure 3). The effect of age on the time that was spent in calling was significant ($P < 0.001$) with more calling at advanced ages (Figure 4A).

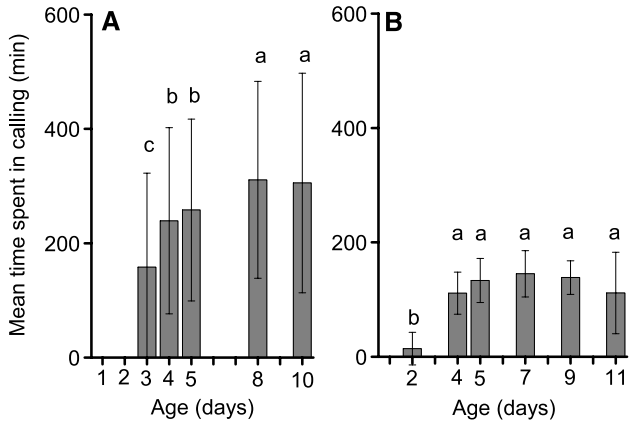

FIG. 4. The mean time spent in calling by (A) *A. gamma* ($N = 30$) and (B) *C. circumflexa* ($N = 20$) females of different ages. For *A. gamma*, GLM procedure followed by multiple comparisons were conducted. Columns with different letters are significantly different ($P < 0.001$). For *C. circumflexa*, a paired *t*-test followed by the Bonferroni procedure of multiple comparisons were conducted. Columns with different letters are significantly different at $\alpha = 0.05$.

TABLE 1. EFFECT OF AGE ON RESPONSE (%) OF *Autographa Gamma* AND *Cornutiplusia Circumflexa* MALES TO THEIR SYNTHETIC SEX PHEROMONE IN A WIND TUNNEL

	A. gamma[a]				*C. circumflexa*[a]			
Age (days)	Takeoff	Directed flight	Landing	Copulation attempts	Takeoff	Directed flight	Landing	Copulation attempts
1	0c				0c			
2	0c				0c			
3	70.0b	46.7	33.3	33.3	55b	0c		
4	73.3b	60.0	53.3	53.3	50b	20bc	10b	0b
5	90.0a	66.7	63.3	56.7	80a	35b	15b	0b
6	90.0a	66.7	63.3	63.3	100a	95a	65a	65a
7	100.0a	66.7	60.0	60.0	100a	90a	80a	80a
8	93.3a	66.7	66.7	63.3				
9								
10					95a	90a	90a	90a
11	100.0a	66.7	63.3	63.3				

[a] For *A. gamma*, observations were conducted during the second half of the scotophase ($N = 30$). For *C. circumflexa*, observations were conducted during the 9th hr of the scotophase ($N = 20$). Percentages within columns followed by different letters are significantly different at $P = 0.05$ according to the Fisher's exact test followed by closed testing procedure for multiple comparisons.

TABLE 2. EFFECT OF TIME IN SCOTOPHASE ON RESPONSE (%) OF *A. Gamma* AND *C. Circumflexa* MALES TO THEIR SYNTHETIC SEX PHEROMONE IN A WIND TUNNEL

	A. gamma[a]				*C. circumflexa*[a]			
Hour of scotophase	Takeoff	Directed flight	Landing	Copulation attempts	Takeoff	Directed flight	Landing	Copulation attempts
2	88.9a	55.5	51.1	51.1				
4	91.1a	57.8	55.5	55.5	95a	0c		
6	84.4a	62.2	60.0	55.5	75b	55b	35b	15b
7	88.9a	55.5	51.1	51.1				
8	91.1a	62.2	60.0	60.0	100a	100a	85a	75a
9					100a	90a	80a	80a
10	51.1b	51.1	44.4	44.4	100a	95a	85a	85a

[a] For *A. gamma*, tested males were 6-d-old ($N = 45$). For *C. circumflexa*, tested males were 7-d-old ($N = 20$). Percentages within columns followed by a different letter differ significantly at $P = 0.05$ according to Fisher's exact test followed by closed testing procedure for multiple comparisons.

Sexual activity of *C. circumflexa* occurred at the end of the scotophase, peaking during the 9th hr (Figure 1C). Only 25% of the females called on the 2nd night after emergence, whereas all females called on subsequent nights. A decrease in percent calling was observed on the 11th night after emergence when only 81% of females called. The total time spent in calling by females was low during the 2nd night (<20 min) and increased to >100 min for the rest of the period (4th night to the 11th night) (Figure 4B). There were no differences among the mean times spent in calling between the 4th night to the 11th night (Figure 4B).

Wind Tunnel Experiments. A. gamma males began to respond to their synthetic sex pheromone on the 3rd night after emergence and continued to respond well until the 11th and last night of the experiment (Table 1). The temporal pattern of response of *A. gamma* males during the scotophase paralleled the calling activity of the females with males responding to pheromone throughout the scotophase (Table 2). In contrast, the first weak response of *C. circumflexa* males was noticed during the 3rd night after emergence, with peak response not occurring until the 6th night (Table 1). Responses then remained unchanged until the end of the observations on the 10th night. *C. circumflexa* males responded strongly to their synthetic sex pheromone during the last 3 hrs of the scotophase (Table 2).

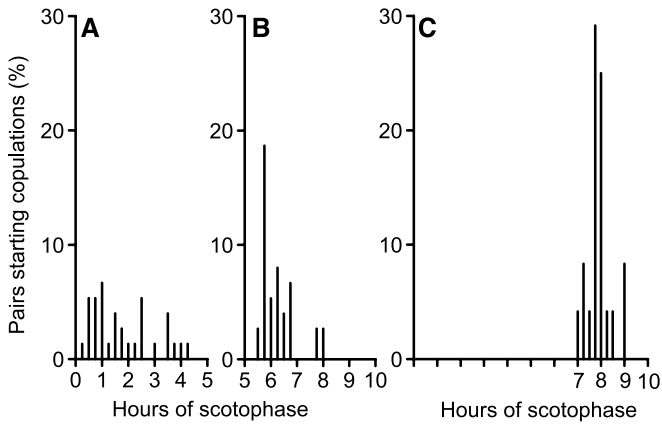

FIG. 5. The effect of time in scotophase on the percentage of *A. gamma* and *C. circumflexa* couples starting to mate. For *A. gamma*, observations were made at 15-min intervals during the two halves of the scotophase (A and B) on three groups of 25 couples each on three different nights. For *C. circumflexa*, observations were made at 15-min intervals during the last 4 hr of the scotophase (C) on two groups of 10 and 14 couples on two different nights.

TABLE 3. EFFECT OF AGE ON PHEROMONE CONTENT IN PHEROMONE GLANDS OF *A. GAMMA* AND *C. CIRCUMFLEXA* FEMALES

	Pheromone titer, *A. gamma*[a] (ng/gland, mean ± SD)			Pheromone titer, *C. circumflexa*[a] (ng/gland, mean ± SD)			
Age (days)	*N*	*Z*7-12:Ac	*Z*7-12OH	*N*	*Z*7-12:Ac	*N*	*Z*7-12OH
2	7	0.66 ± 0.48c	0.09 ± 0.04b				
3	7	1.75 ± 0.46b	0.42 ± 0.37ab				
4	7	2.09 ± 0.91b	0.39 ± 0.16ab	5	0.41 ± 0.28	10	0.59 ± 0.36b
5	7	3.38 ± 0.60a	0.49 ± 0.34a	9	0.63 ± 0.45	9	1.07 ± 0.65ab
6				8	0.60 ± 0.25	10	1.22 ± 0.48a
7	7	2.97 ± 0.44a	0.34 ± 0.21ab	12	0.64 ± 0.27	14	1.26 ± 0.35a
8				9	0.42 ± 0.24	10	1.06 ± 0.44ab
10	7	2.77 ± 0.42a	0.09 ± 0.02b	11	0.72 ± 0.43	10	1.07 ± 0.63ab

[a] Twenty-five glands were considered as one replicate. For *A. gamma*, female glands were extracted during the second half of the scotophase. For *C. circumflexa*, female glands were extracted during the 9th hr of the scotophase. Means followed by different letters differ significantly at $P = 0.05$ (Student–Newman–Keuls test).

Copulation. Copulations of *A. gamma* started almost at the onset of the scotophase. A gradual increase, reflecting the calling activity of the females, was observed during the 1st hour of the scotophase (Figure 5A). The percentage of copulations initiated during the 1st hour of the second half of the scotophase was higher than that of the first half (Figure 5B) and is probably the result of more females ready to mate at the middle of the scotophase than at the beginning. Forty-four percent of the pairs initiated copulation during the first

TABLE 4. EFFECT OF TIME IN SCOTOPHASE ON PHEROMONE TITER IN PHEROMONE GLANDS OF *A. GAMMA* AND *C. CIRCUMFLEXA* FEMALES

Hour of scotophase	Pheromone titer, *A. gamma*[a] (ng/gland, mean ± SD)			Pheromone titer, *C. circumflexa*[a] (ng/gland, mean ± SD)			
	N	*Z*7-12:Ac	*Z*7-12OH	*N*	*Z*7-12:Ac	*N*	*Z*7-12OH
1	7	1.05 ± 0.12c	0.27 ± 0.09				
4	7	1.79 ± 0.42b	0.34 ± 0.19				
6				4	0.35 ± 0.28b	10	0.29 ± 0.21b
7	7	3.38 ± 0.65a	0.49 ± 0.34				
8				8	0.47 ± 0.38ab	13	0.63 ± 0.43b
9				12	0.64 ± 0.27ab	14	1.26 ± 0.35a
10	7	1.36 ± 0.52bc	0.36 ± 0.11	9	0.85 ± 0.56a	10	1.34 ± 0.72a

[a] Twenty-five glands were considered as one replicate. For *A. gamma*, glands of 4- to 5-d-old females were extracted. For *C. circumflexa*, glands of 5- to 6-d-old females were extracted. Means followed by different letters differ significantly at $P = 0.05$ (Student–Newman–Keuls test).

half of the scotophase, and 49% copulated during the second half of the scotophase. The average duration of a copulation in the first half of the scotophase was 48.7 ± 9.9 min (mean ± SD; $N = 33$) and 42.6 ± 7.4 min in the second half of the scotophase ($N = 37$).

In *C. circumflexa*, copulation reflected the sexual activities of both sexes during the scotophase determined in our previous experiments (Figure 1C and Table 2). Copulations started at the beginning of the 8th hr, reached a peak about an hour later, and stopped an hour before light onset (Figure 5C). The average duration of copulation for *C. circumflexa* was 58.24 ± 12.12 min ($N = 18$).

Pheromone Titer Determination. The amount of *Z*7-12:Ac, the main pheromone component of *A. gamma*, reached a peak on the 5th night after emergence and remained unchanged until the 10th night. The amount of the minor component *Z*7-12:OH remained practically unchanged (Table 3). *Z*7-12:Ac titer peaked during the 7th hr of scotophase, whereas the minor component *Z*7-12:OH remained unchanged (Table 4).

The amount of the major pheromone component of *C. circumflexa*, *Z*7-12:OH, increased between the 4th and 6th night but otherwise remained unchanged (Table 3). Its titer was highest during the last 2 hr of the scotophase (Table 4). The amount of the minor component *Z*7-12:Ac remained constant for all tested ages (Table 3) and varied little throughout the calling period (Table 3).

DISCUSSION

The sexual behavior of *A. gamma* was studied in central Europe by Szöcs and Tóth (1979) and Tomescu et al. (1985), and in Great Britain by Hill and Gatehouse (1992). The origin of the moths examined, the environmental conditions during rearing and during the experiments, and the parameters studied differed from ours. Szöcs and Tóth (1979) and Tomescu et al. (1985) obtained similar results to ours concerning the sexual activity of the moths, which occurred throughout most of the scotophase. Discrepancy was observed between the above reports and ours as to the age at which sexual activity commenced. Szöcs and Tóth (1979) reported that sexual activity of the gamma moth began on the 1st night after emergence, whereas in our study, sexual activity started only on the 3rd night. Possible explanations for the discrepancy are the different strains used in the two studies, and the different photoperiods used in the two studies. The photoperiod used by Szöcs and Tóth (1979) was 18:6 LD, the typical photoperiod in central Europe during the summer when *A. gamma* populations are at their peak. In Israel, the moth is most common during spring and autumn; therefore, we used a photoperiod of 14:10 LD. Hill and Gatehouse (1992) examined two photoperiods, short (12:12 LD) and long (16:8

LD), and obtained significantly different mean precalling periods of 3.47 and 1.97 days, respectively. The fact that *A. gamma* is a migratory insect (Fisher, 1938; Yathom and Rivnay, 1968; Kitching, 1987; Pedgley and Yathom, 1993) may explain the impact of photoperiod on its sexual maturation. Migration usually takes place during the prereproductive period of adult life (Johnson, 1960; Dingle, 1972). Hill and Gatehouse (1992) confirmed that photoperiod has a significant effect on the prereproductive period of *A. gamma*, and that females have longer precalling periods under shorter day photoperiod. A similar photoperiodic effect on calling was reported by Delisle and McNeil (1986) for the true armyworm, *Pseudaletia unipuncta*, and by Del Socorro and Gregg (1997) for the Australian common armyworm, *Mythimna convecta*. Both are migratory species. McNeil (1986) suggested using the photoperiodic effect to identify migratory pest species.

The calling behavior of *A. gamma* and *C. circumflexa* females was age dependent. For both species, more calling was observed by older females, mainly due to advanced onset and longer periods of calling, because the percentage of calling females during the calling period was almost constant. An advanced onset of calling with age has been reported for several other moths (Swier et al., 1977; Webster and Cardé, 1982; Turgeon and McNeil, 1982; Howlader and Gerber, 1986; Schal and Cardé, 1986; Kou and Chow, 1987; Dunkelblum et al., 1987; Kamimura and Tatsuki, 1993; Delisle and Royer, 1994; Edmonds et al., 2000). It has been suggested that the advance in the onset of time of calling with age in lepidopteran species is an adaptation that permits older females to increase their chances of attracting mates before younger females start calling (Swier et al., 1977; Delisle, 1995). Delisle (1992) showed that the mean onset time of calling in *Choristoneura rosaceana* (Harris) advances with age, and that older females attract more males than younger ones, although less pheromone is produced by older females (Delisle and Royer, 1994). Older females would be at a disadvantage compared to younger ones if they did not expand their calling period.

The amount of pheromone and its composition in *A. gamma* and *C. circumflexa* were measured from extracts of excised glands at different ages and different times during the scotophase. The amount of pheromone in the gland may not be indicative as to the release rates (Shorey et al., 1968), but attempts to obtain more accurate information on the relationship between sexual behavior and pheromone release by collecting effluvia failed because of the low amounts of pheromone emitted by females of both species.

There was a synchronous relationship between the amount of pheromone in the glands and the calling activity at different ages for the two species. For *A. gamma*, calling started on the 3rd night, increased to the 5th night, and remained constant thereafter. The increase in pheromone titer paralleled the calling activity. For *C. circumflexa*, some calling was observed on the 2nd night from emergence, but a distinct increase in calling was seen only from the 4th night

onwards. The pheromone titer increased until the 5th night and remained almost constant thereafter.

The relationship between calling and pheromone titer during the scotophase also was almost synchronous in both species. The sexual activity of *A. gamma* was almost constant throughout the scotophase. However, there was more calling during the second half of the scotophase, accompanied by an increase in pheromone titer at the 7th hour of the scotophase. This might indicate intensified sexual activity during the second half of the scotophase. For *C. circumflexa*, calling and the pheromone titer were higher at the end of the scotophase, but calling declined during the last hour, whereas the amount of pheromone remained unchanged. Delisle and Royer (1994) summarized the literature available on the relationship between calling as a function of age and pheromone production, as well as between the diel pattern of calling and pheromone production in Lepidoptera. Some of the cases showed a synchronous relationship between calling and the amount of pheromone with age, as was found for *A. gamma* and *C. circumflexa*, whereas in others, such as the oblique-banded leafroller, *Ch. rosaceana*, the amount of pheromone decreased with age, although calling remained stable (Delisle and Royer, 1994). In the black cutworm, *Agrotis ipsilon* pheromone quantity coincided with calling, but unlike *A. gamma*, *C. circumflexa*, and *Ch. rosaceana*, the two parameters reached a peak for 2-d-old females during the scotophase and decreased progressively in older females (Gemeno and Haynes, 2000). Overall, 15 out of 25 spp. showed close synchrony of the two parameters. For the remaining 10 species, two different asynchronous patterns have been reported. In seven species, the peak of pheromone production occurred before the peak of calling, whereas in *P. unipuncta* (Delisle and McNeil, 1987) and the plusiinae species *Chrisodeixis chalcites* (Snir et al., 1986), the peak of pheromone production occurred after the peak of calling. In the last species, *Helicoverpa zea*, the relationship is not clear because of conflicting data (Raina et al., 1986, 1991).

Overlapping populations of *A. gamma* and *C. circumflexa* are frequently found in Israel during spring and autumn. The gamma moth is sexually active throughout the scotophase, whereas *C. circumflexa* is sexually active only during a short period at the end of the scotophase. There is no sharp temporal separation between the sexual activities of the two species, but most of the gamma moths have already been mated by the time *C. circumflexa* moths begin with their sexual behaviors. Furthermore, the inverse ratio between the two sex pheromone components, *Z*7-12:Ac and *Z*7-12:OH, seems to provide effective reproductive isolation (Mazor and Dunkelblum, 1992; Dunkelblum and Mazor, 1993). However, individual variations in the ratios of two components in both pheromone blends, combined with wider latitude of responsiveness of males as has been reported for other moth species (Ono and Orita, 1986; Löfstedt, 1990; Liu and Haynes, 1994; Löfstedt et al., 1994; Evenden et al., 2002), might limit

reproductive isolation based on different pheromone blend ratios between *A. gamma* and *C. circumflexa*. The temporal separation in sexual activity may provide an additional barrier in the reproductive isolation process between the two species. An analogous situation was reported for two plusiinae sibling species, *Diachrysia chrysitis* and *D. tutti* (Löfstedt et al., 1994). These two species are sympatric in large parts of their ranges and use sex pheromone blends in an almost opposite ratio, but despite the differences in blends, cross-attraction and potential hybridizations under natural conditions were confirmed.

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CAPTURE OF FEMALE *Hylotrupes bajulus* AS INFLUENCED BY TRAP TYPE AND PHEROMONE BLEND

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Abstract—Three different types of traps were evaluated for a semiochemical-based trapping method for *Hylotrupes bajulus* (L.) (Cerambycidae). One, designated the ground trap, was the most efficient under both laboratory and natural conditions and had an active space of about 3.5 m. Significantly more beetles were captured in traps baited with a mixture of synthetic pheromones, (3*R*)-3-hydroxy-2-hexanone [(3*R*)-ketol] + 1-butanol, than in those with either single compound or with racemic mixtures. Furthermore, the synthetic lure captured more females than two virgin males in a laboratory bioassay. In addition, doubling the concentration of the synthetic pheromone significantly increased trap catches. The present findings have potential value of the management of this pest.

Key Words—*Hylotrupes bajulus*, Coleoptera, Cerambycidae, trap designs, effective distance, pheromone lure, pheromone mixture.

INTRODUCTION

Long-horned beetles have received attention in the recent past because of their pest potential (Hanks, 1999; Allison et al., 2004; Nerg et al., 2004, and references therein). The old-house borer, *Hylotrupes bajulus* (L.) (Coleoptera: Cerambycidae), is a cosmopolitan insect of high economic importance due to its

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great potential to damage wood in houses. The beetle normally only feeds on sapwood, but in severe infestations may also attack the heartwood (White, 1954). Attacks often begin in the roof where larvae bore through rafters made of pine, spruce, and fir (Cannon and Robinson, 1982). Infestations spread when beetles fly from house to house in hot, sunny weather (Mares and Robinson, 1985), so an effective semiochemical-based trapping method would help to monitor and control *H. bajulus* populations.

Schröder et al., (1994) demonstrated that *H. bajulus* males produce a sex pheromone and identified (3*R*)-3-hydroxy-2-hexanone [(3*R*)-ketol] as a major component. Subsequently, chemical analysis of hexane extracts of dissected prothoracic glands, the source of the sex pheromone (Noldt et al., 1995), and headspace samples of both sexes revealed several male-specific compounds that were attractive to virgin females: (3*R*)-ketol, 2-hydroxy-3-hexanone, the diastereomeric alcohols [(2*R*, 3*R*)-2,3-hexanediol and (2*S*, 3*R*)-2,3-hexanediol, 2,3-hexanedione], and 1-butanol (Fettköther et al., 1995).

The objectives of this study were to determine the optimal pheromone dose, develop dispensers baited with pheromone mixtures, define the effective distance of the pheromone lure, and determine the efficiency of pheromone mixtures for use in trapping strategies.

METHODS AND MATERIALS

H. bajulus larvae are reared in darkness at 25°C and 75% RH at the Institute of Wood Biology and Wood Protection, Hamburg (Germany). The original culture was established in 1965, with adults collected at various roof sites in Schleswig-Holstein and Hamburg, and regularly restocked with specimens from other locations (Noldt et al., 1995). Newly emerged males and females were placed in individual Bellaplast boxes (Bellaplast AG, Altstätten, Switzerland) lined with moistened filter paper and then held in separate rooms at 20°C, 12 L:12 D photoperiod until needed. Food was not provided, since adult beetles do not feed under natural conditions (Becker, 1944). Beetles used in any given experiment were of the same age; however, age ranged from 5 to 15 days across all experiments.

Experiments were carried out in a greenhouse from April 15 to June 20, 1999, between 1200 and 1730 hours when insects are likely to be producing and responding to pheromones. Traps were evaluated in a gauze cage (450 cm long $\times$ 230 cm wide $\times$ 100 cm high) with six openings to insert or remove both traps and beetles. A ventilator was installed to facilitate a constant airflow of 2.5 m/sec and the contaminated air was removed during the experiment with an exhaust fan located on top of the cage.

Three trap types were evaluated, named ground, hanging, or wall traps, according to where they are placed. The ground trap (Figure 1A) measured 120 by 65 cm and had a slitted 50 by 8-cm baffle. The hanging trap (Figure 1B), suspended 75 cm from the floor, measured 50 by 12 cm and had a baffled slit on the upper surface. Wall traps (Figure 1C) were box shaped (40 cm high and 11 × 11 cm in cross section) with two 22-cm-long opening slits on the sides adjacent to the wall. All were constructed from 5-mm-thick brown cardboard, with a baffle near the openings to ensure that beetles caught did not escape.

Pheromone dispensers were made from 1-ml brown glass screw-top vials (N 8-1, Macherey-Nagel, Duren, Germany) capped with a 15 × 8-mm rubber septum (Thomas Scientific, Swedesboro, NJ, USA). Each vial was filled with 1,000 ml of pheromone mixture (purity ≥98.5%) obtained from the Department of Chemistry, Cornell University (USA) and the Institute of Organic Chemistry, University of Hamburg (Germany). Test chemicals were (3*R*)-ketol, 1-butanol (3 mg/vial/d), or racemic 3-ketol [(±)-3-ketol] (2.8 mg/vial/d), either alone at 1:100 or combined at 1:1:100 (by volume in hexane), respectively. Two

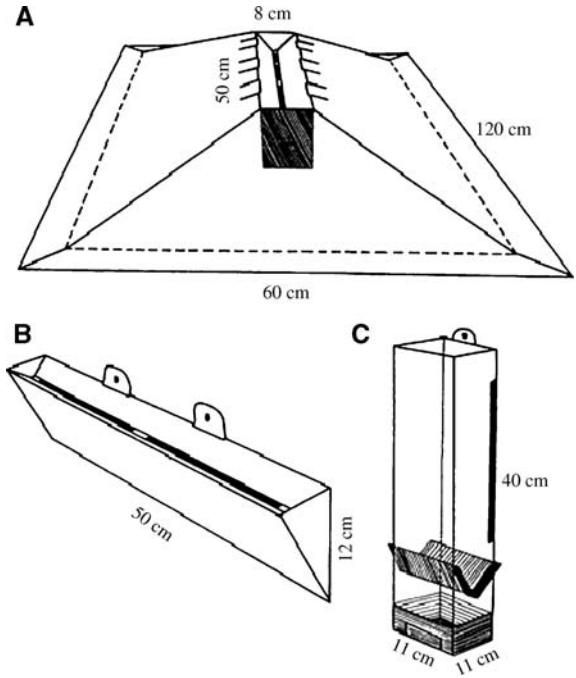

FIG. 1. Schematic diagram of three different pheromone traps tested for monitoring *H. bajulus*: (A) Ground trap, (B) hanging trap, and (C) wall trap.

dispenser vials were used per trap, unless otherwise stated. Dispensers were prepared the day before the experiment to allow volatile compounds to penetrate the rubber septa.

General Trapping Method. A trap baited with the test mixture was placed inside the greenhouse cage 2 hr before the release of the beetles so that pheromone could spread throughout the cage. Ten virgin female *H. bajulus* were released downwind of the trap at 1230 hr, and the number captured was counted after 3 hr. Temperature, relative humidity, and wind speed were measured with Meteo Digit III (Lambrecht, Klimatologische Messtechnik, Gottingen, Germany), whereas light intensity was recorded using a Lux-Meter (Testo GmbH & Co., Lenzkirch, Germany). All laboratory experiments were replicated eight times.

Radius of Maximum Attraction of Synthetic Pheromone Mixture. To determine the radius of maximum attraction of the synthetic pheromone, the ground trap was used, since it proved the most efficient in our previous experiment. Ten unmated females were released 1, 2, 3, 3.5, or 4 m downwind of a trap baited with (3*R*)-ketol + 1-butanol.

Effect of Pheromone Lure Composition on Capture of Unmated Females. Each trap was baited with (3*R*)-ketol (1.4 mg/vial/d), 1-butanol (1.6 mg/vial/d), (±)-3-ketol (1.2 mg/vial/d), or either two or four vials of (3*R*)-ketol + 1-butanol (standard; 3.0 mg/vial/d) or (±)-3-ketol + 1-butanol (2.6 mg/vial/d)). In each replicate, ten unmated females were released 3 m downwind of the trap.

Trapping Efficiency of Pheromone and Racemic Mixtures in the Presence of Males. Ten unmated females (5–15 d-old) were released 3 m downwind from two competing pheromone sources that were 50 cm apart. One was a ground trap baited the pheromone mixture (3*R*)-ketol + 1-butanol or (±)-3-ketol + 1-butanol and the other, two virgin males (5–15 d-old). The males, placed in a cavity within two pieces (13 × 13 × 13 cm) of pine wood held together with a rubber band were enclosed in a 30 × 20 × 20 cm nylon screen cage to prevent them from escaping. The beetles were replaced every 2 d. Data on the number of females approaching and entering the trap or coming into contact with the nylon cage containing the males were recorded. Females remaining on the nylon cage for 5 min were considered as trapped. Trapped beetles were removed from the experimental arena.

Efficiency of Traps under Natural Infested Conditions in the Field. The three trap designs were evaluated from July to October, 1999, in five naturally infested sites around the town of Bayreuth, Germany. Four ground, 30 wall, and 21 hanging traps were deployed in each of the five sites. The intertrap distance was 10 m, and their positions were re-randomized between replicates once every week. Traps were coated on the inner side with Hostaflon TF 5035 (polytetrafluoro ethylene) (Werk Gendorf, Burgkirchen, Germany) to prevent

trapped beetles from escaping. Four unbaited control traps of each type were used in the experiments. The number of beetles captured was recorded every morning.

Effect of Different Pheromone Blends in Recapturing H. bajulus in the Field. Ground traps baited with either the standard pheromone mixture (3*R*)-ketol + 1-butanol (3.0 mg/vial/d), an 86:14 blend of (3*R*)-ketol, and (3*S*)-ketol + 1-butanol (2.8 mg/vial/d), a 1:1 mixture of (±)-3-ketol + 1-butanol (2.6 mg/vial/d), as well as (3*R*)-ketol (1.4 mg/vial/d) or 1-butanol (1.6 mg/vial/d) as single components. Experiments were set up in barns at four different locations near Bayreuth, Germany, during July to October, 1999, with one trap in each location. The intertrap distance was 10 m in each location, whereas interlocation distance was approximately 2 km. Ten unmated females marked with color were released 2 m from the trap. Releases were made in the afternoon and the number of trapped beetles was recorded the following morning. The untrapped beetles were collected at the end of the experiment.

Statistical Analysis. Data were analyzed by Kruskal–Wallis nonparametric tests to compare the efficiency of traps, effective distance of pheromone mixture, or effect of pheromone compositions in recapturing *H. bajulus*. Mann–Whitney *U* tests (Sokal and Rohlf, 1995) were used to test for significant differences between the treatments.

RESULTS

Evaluation of Traps. In the greenhouse arena, the ground trap captured significantly more females than other traps baited with identical pheromone mixture ($P \leq 0.05$, Table 1). A similar trend was seen when tests were conducted in the infested roofs, trusses, and barns ($P = 0.001$, Table 1). Traps without pheromone mixtures, used as control, captured no beetles so they were not included in the analyses.

Radius of Maximum Attraction of Synthetic Pheromone Mixture. The baited ground trap was equally effective at distances up to 3.5 m, distances greater than previously reported for this species (Fettköther et al., 1995, 2000). However, there is a significant decline in captures when females are released 4m from the trap ($P \leq 0.05$; Table 2). The average temperature, relative humidity and light intensity prevailing during the study were 29.5°C, 30.5% and 1297.0 lx.

Effect of Different Pheromone Lures on Capture of Unmated Females. Ground traps baited with pheromone mixtures, except (±)-3-ketol + 1-butanol, were generally more attractive than those baited with any single compound ($P \leq 0.05$; Table 3). Increasing the dose increased trap catches with both blends,

TABLE 1. THE EFFICACY OF DIFFERENT TRAP DESIGNS, BAITED WITH (3*R*)-KETOL AND 1-BUTANOL, IN CAPTURING FEMALE *H. bajulus*

	Median captures of ten released females in a screen cage[a]	Capture of females in infested roof trusses and barns	
Trap design	Median ± MAD/trial (N = 8)	No. of traps used	Mean number of females caught ± SE
Ground trap	7.5 ± 0.5a	4	2.8 ± 0.2a
Wall trap	6.0 ± 0.5b	30	1.2 ± 1.0b
Hanging trap	3.5 ± 0.5c	21	0.1 ± 0.5c

[a] Captures in both greenhouse assays (Kruskal–Wallis ANOVA, P = 0.001) and naturally infested sites (Kruskal–Wallis ANOVA, P = 0.001) were significantly different. Median/Means followed by the same letter are not significantly different (Mann–Whitney U test, $P \leq 0.05$).
MAD = median absolute deviation.

but for any given concentration the (3*R*)-ketol + 1-butanol blend caught significantly more beetles than the corresponding racemic mixture (Table 3). The average temperature, relative humidity, and light intensity prevailing during this experimental period were 31°C, 25%, and 971 lx, respectively.

Trapping Efficiency of Two Pheromone Blends in the Presence of Males. Ground traps baited with the pheromone mixture captured significantly more females (median of six beetles captured) than two live males (median of four beetles captured) when tested concurrently in the greenhouse (F = 36.22, $P \leq$ 0.05; data not shown). In contrast, traps baited with (±)-3-ketol + 1-butanol captured significantly (F = 28.14, $P \leq 0.05$) fewer females than the males (medians of 3.0 and 5.0, respectively). The temperature, relative humidity, and

TABLE 2. EFFECT OF DISTANCE ON THE NUMBER OF *H. bajulus* FEMALES CAPTURED IN GROUND TRAPS BAITED WITH THE STANDARD PHEROMONE MIXTURE IN A GREENHOUSE TRIAL

	Median captures of ten released females in screen cage trial[a]
Distance (m)	Median ± MAD/trial (N = 8)
1	9.0 ± 0.5a
2	8.0 ± 1.0a
3	7.0 ± 1.0a
3.5	6.5 ± 1.5a
4	3.5 ± 0.5b

[a] Captures were significantly different (Kruskal–Wallis ANOVA; P = 0.011). Medians followed by the same letters are not significantly different (Mann–Whitney U test; $P \leq 0.05$).
MAD = median absolute deviation.

TABLE 3. EFFECT OF DIFFERENT PHEROMONE LURES ON THE CAPTURE OF UNMATED *H. bajulus* FEMALES IN GROUND TRAPS

Attractants	Quantity (no. of vials)	No. of females captured[a] Median ± MAD/trial
Single pheromone		
1-butanol	2	2.0 ± 1.5c
(3*R*)-ketol	2	2.0 ± 0.5c
(±)-3-ketol	2	1.0 ± 0.5d
Blend of pheromone		
(3*R*)-ketol + 1-butanol (standard)	2	6.0 ± 1.0b
(3*R*)-ketol + 1-butanol	4	8.0 ± 0.5a
(±)-3-ketol + 1-butanol	2	3.0 ± 0.5c
(±)-3-ketol + 1-butanol	4	5.0 ± 1.0b

[a] Captures were significantly different (Kruskal–Wallis ANOVA, $P = 0.012$). Medians followed by the same letters are not significantly different (Mann–Whitney U test; $P \leq 0.05$).
MAD = median absolute deviation.

light intensity prevailing during this experimental period were 31.5°C, 21.5%, and 983.0 lx, respectively.

Efficiency of Different Pheromone Mixtures in the Field. As with the greenhouse experiments, traps baited with pheromone mixtures were more efficient than those baited with either single pheromone component ($P \leq 0.05$; Table 4). Furthermore, the standard blend was more effective that any of the racemic mixtures (Table 4).

TABLE 4. PERCENTAGE OF FEMALE *H. bajulus*, RELEASED 2 M FROM GROUND TRAPS BAITED WITH DIFFERENT PHEROMONE MIXTURES, WHICH WERE RECAPTURED IN BARNS AT FOUR DIFFERENT LOCATIONS

Attractants	Percent females captured				Average ± SE[a]
	Barn 1a	Barn 1b	Barn 1c	Barn 2	
(3*R*)-ketol + 1-butanol (standard)	70	60	52	73	63.7 ± 8.3a
Mixture of 86% (3*R*)-ketol +14% (3*S*)-ketol + 1-butanol	50	45	45	56	49.0 ± 4.5b
(±)-3-ketol + 1-butanol	45	35	28	53	40.2 ± 9.5c
Mixture of (±)-3-ketol + (±)-2-ketol + 1-butanol	27	24	40	50	35.2 ± 10.4c
(3*R*)-ketol	20	30	10	24	21.0 ± 7.2d
1-butanol	15	40	25	33	28.2 ± 9.3d

[a] Catches were significantly different (Kruskal–Wallis ANOVA, $P = 0.001$). Means followed by the same letters are not significantly different (Mann–Whitney U test; $P \leq 0.05$).

DISCUSSION

The ground trap proved to be the most effective design tested partly because although females initially fly upwind in the pheromone plume they generally walk the final distance (about 50 cm) to the source, whereas the relative response of different beetles to racemic and pure pheromone blends varies with species (Iwabuchi et al., 1986; Hallett et al., 1995; Giblin-Davis et al., 1996; Miller et al., 1997). *H. bajulus* females are clearly less attracted to racemic blends. Furthermore, the racemic blend proved less effective than virgin males. Thus, we believe that ground traps baited with (3*R*)-ketol + 1-butanol offer a viable approach for the monitoring and control of *H. bajulus*.

However, additional work is required to further refine the system. For example, research examining the possible effects of physical characteristics such as color on the efficacy of the ground traps merits attention (Suckling et al., 2005). With respect to the lure, we saw an increase in trap catch when the concentration of the pheromone was doubled (Table 3). Thus, we need to determine if doses higher than those tested in this study increase the numbers of beetles caught. This merits attention because different dose-dependent responses were reported on other beetle species (Fadamiro, 1996; Fukaya and Honda, 1996). In addition, if trap catches do increase with increasing concentrations of pheromone, one needs to examine if this is related to having a larger active space. Furthermore, our previous studies (Fettköther et al., 2000) showed that the addition of α-pinene to the pheromone increased attraction and orientation of both sexes toward the source. Therefore, trials need to be carried out to determine how this and other compounds such as (−)-verbenone, *trans*-pinocarveol, and terpinen-4-ol (Fettköther et al., 2000) might be used with the pheromone to increase trap efficacy.

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ALLELOPATHIC POTENTIAL OF *Robinia pseudo-acacia* L.

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Abstract—*Robinia pseudo-acacia* L. (black locust) is a nonindigenous species currently invading the central part of Japanese grasslands. Several allelochemicals were identified and characterized from the leaf tissue. The growth of both radicle and hypocotyl in the tested species (barnyard grass, white clover, lettuce, and Chinese cabbage) was reduced when grown in soil mixed with the leaves of *R. pseudo-acacia* at various concentrations. Aqueous leaf extracts, when bioassayed, exhibited a significant suppression of radicle growth. Chromatographic separation of an ethanolic extract of *R. pseudo-acacia* leaves resulted in isolation of three compounds, identified as robinetin (**1**), myricetin (**2**), and quercetin (**3**) by nuclear magnetic resonance and mass spectroscopy. All inhibited root and shoot growth of lettuce. Robinetin, found in a large amount, caused 50% suppression of the root and shoot growth of lettuce at 100 ppm. The presence of these bioactive substances in leaf tissue suggests a potential role for flavonoids in *R. pseudo-acacia* invasion in introduced habitats.

Key Words—Allelopathy, allelochemicals, quercetin, robinetin, myricetin invader, *Robinia pseudo-acacia*, growth inhibitors, black locust.

INTRODUCTION

Plants produce a variety of secondary metabolites that are released into the soil, either as exudates from living plant tissues or by decomposition and leaching from plant residues (Rice, 1984; Putnam and Tang, 1986). Some may be associated with allelopathy and play a role in chemical interactions in natural communities (Einhellig, 1996; Seigler, 1996; Dayan et al., 2000). Various interactions among plant species are responsible for natural selection in plant

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communities. Some exotic invasive plants show competitive behavior that is different or not present in the natural community they invade. Allelopathy has been considered as a probable mechanism for the remarkable success of invader plants (Callaway and Aschehoug, 2000) because native species may be devoid of those chemicals produced by the invader plants. It is suspected that *Centaurea* species, one of the most destructive exotic invaders in North America, is using allelochemicals to displace native species (Goslee et al., 2001; Ridenour and Callaway, 2001; Bais et al., 2002).

Robinia pseudo-acacia L. belongs to the Fabaceae and is commonly known as black locust. It is widely distributed in temperate and subtemperate zones. It is a nitrogen fixer, has rapid juvenile growth (Hanover et al., 1992; Moon et al., 2001), and can propagate by seeds or sprouts. It is widely planted as an ornamental for shelterbelts and for land reclamation. This early successional plant spreads rapidly, creates dense stands that shade, and outcompete the nearby vegetation. It has interesting biological properties and medicinal activities (Fielder, 1975; Foster and Duke, 1990). It was brought to Japan about 100 years ago as an ornamental tree. It has been observed that in places where these trees dominate, other plants are lacking, and the vegetation beneath the tree is poor. It is known that 500 trees in one hectare will suppress the growth of other plants. In the central part of Japan, *R. pseudo-acacia* is reported to establish itself in monocultures on the river side and it cause damage to the apple trees that are the main fruit trees in this area (Maekawa and Nakagoshi, 1997; Maekawa, 1998). Based on this field observation and a previous bioassay study of fallen leaves of this plant by sandwich methods and of root exudates by plant box methods (Uraguchi et al., 2003), it was suggested that *R. pseudo-acacia* releases allelopathic compounds that inhibit the growth of other plants.

METHODS AND MATERIALS

Plant Material. R. pseudo-acacia is grown in the National Institute for Agro-Environmental Sciences, Tsukuba, Japan. Fresh and fallen leaves (1.5 kg) were collected from two sites in early autumn. The soil type was andosol, analyzed as 70% sand, 6% silt, 24% clay (pH 4.6), and 0.58% organic matter. The seeds of alfalfa (*Medicago sativa* L.), Italian rye grass (*Lolium multiflorum* Lam), barnyard grass (*Echinochloa crus-galli* P. Beauv. var. *crus-galli*), timothy (*Phleum pretense* L.), lettuce (*Lactuca sativa* L.), Chinese cabbage (*Brassica campestris* L.), and white clover (*Trifolium repens* L.) were used as bioassay species. All seeds were purchased from Takii Seed Co. Ltd., Kyoto, Japan.

Concentration-Dependent Phytotoxic Effects. Studies were conducted to determine the effect of leaf debris on the growth of test plants under fieldlike

conditions. Andosol soil (30 g each) was mixed with 0.17, 0.34, and 0.68 g of *R. pseudo-acacia* dried leaves. The soil was placed in black vinyl pots (7.5 cm diameter and 6.5 cm deep) (TO-R2, Kuromaru Nippori, Mitsubishi Chem. Co. Ltd., Japan). Four plant species, including two edible crop species (lettuce and Chinese cabbage) and two weed species (barnyard grass and white clover) were selected for the concentration-dependent pot experiments. The treatment is expressed as 0, 0.6, 1.14, and 2.3% (w/w) based on soil dry weight. These are consistent with those used for phytotoxic studies of other species (Bhowmik and Doll, 1982; Touchette et al., 1988; White et al., 1989). There were five replications of each concentration for each bioassay species. Pots placed in a greenhouse were watered daily. Temperature was maintained at 26°C day and 18°C night. Emergence was recorded after 4 d of planting, and after 7 d, each pot was thinned to the five healthiest plants. Plants were harvested after 3 wk, and the soil was carefully washed from the roots. Root/shoot length, and fresh/dry weight of roots were measured.

Phytotoxic Effects of Aqueous Extracts of Leaves. Aqueous extracts of leaves were tested for allelopathic potential on lettuce and other weed species. Ten grams of oven-dried leaves were soaked in 100 ml water in a flask and agitated for 24 hr on an orbital shaker (150 rpm) at room temperature (25 ± 3°C). The extract was strained through two layers of cheesecloth and then two layers of Whatman No. 2 filter paper. In an 8.5 × 8.5 × 1.5-cm culture dish lined with Whatman No.1 filter paper, ten seeds each of barnyard grass, white clover, Chinese cabbage, lettuce, timothy, and alfalfa were placed separately. Treatments were applied in 2.5 ml volumes per dish, and distilled water was used as control. Petri dishes were sealed with parafilm (Parafilm "M" laboratory film, American National Can) and incubated in the dark in the growth chamber for 72 hr at 25°C. Treatments were replicated three times. Root and shoot lengths were recorded after 72 hr.

Extraction and Isolation of Compounds. Fresh leaves (1.5 kg) were soaked in 80% aqueous ethanol (5 l) for 7 d. The ethanolic extract was concentrated to a crude, dark brown gum (100 g). The crude extract was partitioned against hexane and water. The original crude, aqueous, and hexane extracts were bioassayed using lettuce as a test plant. Comparison of bioassay results revealed that the major activity of the crude extract was in the aqueous extract. Bioassay directed silica gel (Wakogel C-200, 74–149 μm, Wako Pure Chemical Industries, Osaka, Japan) column chromatographic resolution of a portion of the aqueous fraction gave 12 main fractions (Figure 1). Each of these was bioassayed with lettuce, and among these Fr-1-6, Fr-1-9, and Fr-1-10 were active. A portion of fraction Fr-1-9 (500 mg) was therefore subjected to Sephadex gel LH-20 using $MeOH/H_2O$ (9:1) followed by reverse-phase chromatography on YMC gel (ODS-AQ 120-S50) and eluting with 90% aqueous methanol. Four main fractions were obtained, which were bioassayed,

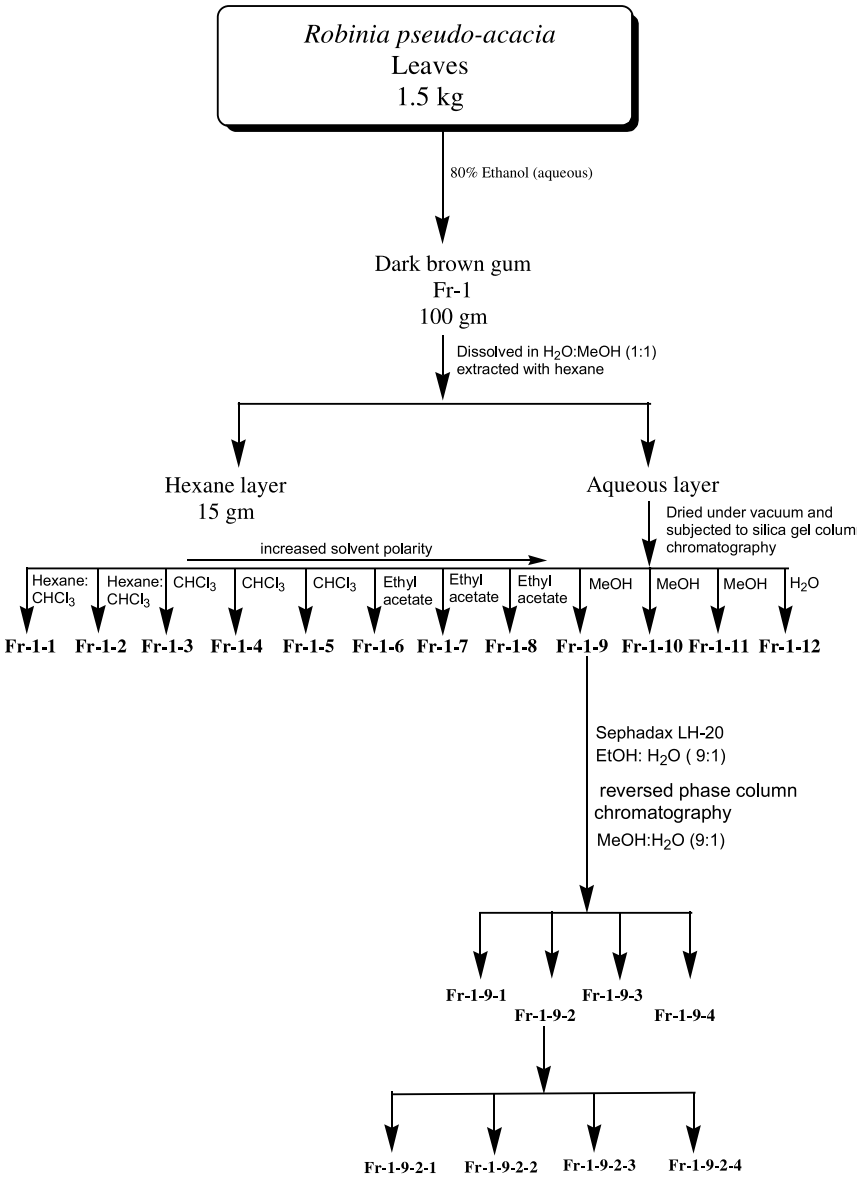

FIG. 1. Isolation scheme of active constituents of *R. pseudo-acacia* from the crude ethanolic extract of leaves.

and the most active one, Fr-1-9-2, upon further purification yielded compounds **1**, **2**, and **3** (Figure 2).

Identification. Structures of compounds **1**–**3** were determined by nuclear magnetic resonance (NMR) and mass spectroscopic techniques. NMR spectra were recorded using a JEOL JNMα-600 spectrometer operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C using standard JEOL software (Alpha Data System). Spectra were recorded using acetone-d_6 as a solvent and tetramethyl silane (TMS) as internal reference. FAB-MS spectra were recorded on a JEOL

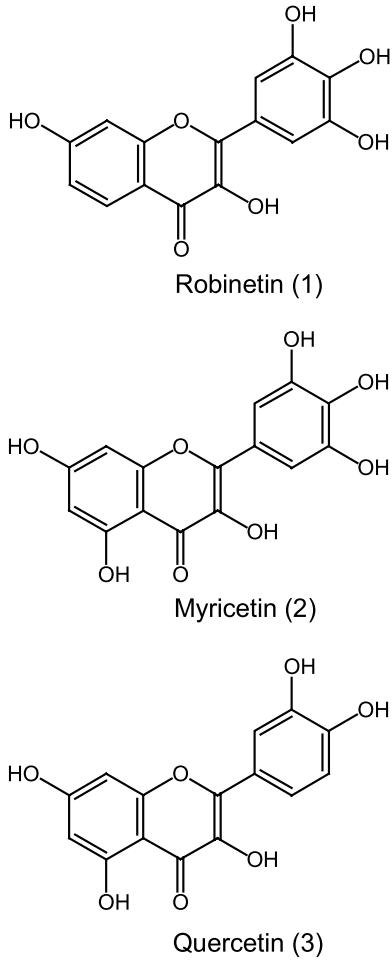

FIG. 2. Structures of robinetin (**1**), myricetin (**2**) and quercetin (**3**).

SX102A spectrometer. Samples were mixed with glycerol as a matrix and analyzed using a direct inlet system.

Bioassay. The crude ethanolic extract and its subfractions obtained after chromatographic resolution were subjected to bioassay with lettuce as the test species. Seeds were germinated on wet filter paper (No. 1, Toyo Ltd., Tokyo, Japan) for 18 hr at 25°C in the dark. The resulting five germinated seeds (2 mm radicle length) were placed on filter paper (4 cm diameter, No. 1, Toyo Ltd.) containing appropriate concentrations of test solution in a Petri dish (4 cm ID). The seedlings were grown at 25°C in the dark for another 2 days, followed by root and shoot length measurement. Elongation ratio was calculated by using the formula $(A/B) \times 100$, where A is the mean elongation of the hypocotyls or radicles in the presence of test compound, and B is the mean elongation length of the hypocotyls or radicles of control. Each treatment was replicated three times ($N = 15$).

Purified compounds **1**, **2**, and **3** were also tested for growth inhibitory activity at 100, 50, and 10 ppm, respectively, against lettuce. Experiment design and incubation conditions were the same as described above in the bioassay of crude extracts.

Statistics. The analysis of variance for all data was measured using the general linear model procedure by SAS/STAT (1990). Effective concentration required for 50% inhibition (EC_{50}) of test species was calculated based on fitted regression equations.

RESULTS

Concentration-Dependent Phytotoxicity. Radicle and hypocotyl growth of all tested weeds (barnyard grass and white clover) and edible crops (lettuce and Chinese cabbage) species was strongly inhibited when grown in soil mixed with *R. pseudo-acacia* leaves at three different concentrations. Seedling growth decreased as the concentrations of leaves increased in soil, and the highest inhibition was observed at 2.3% (w/w). The inhibitory effect was greatest on weed species, particularly on barnyard grass (Figure 3a). Incorporated leaves at a concentration of 0.17 g/30 g soil inhibited the root and shoot length of barnyard grass by 87 and 71% of control, respectively. Root and shoot growth of Chinese cabbage, however, was less sensitive at this concentration.

Fresh and dry weights of radicles in all tested plants significantly decreased when grown in soil mixed with *R. pseudo-acacia* leaves. Weed species, especially barnyard grass, experienced the highest weight decrease (Figure 4b). Total dry weight per plant was reduced to 86 and 78% for barnyard grass and white clover among weeds, and 79 and 75% for lettuce and Chinese cabbage

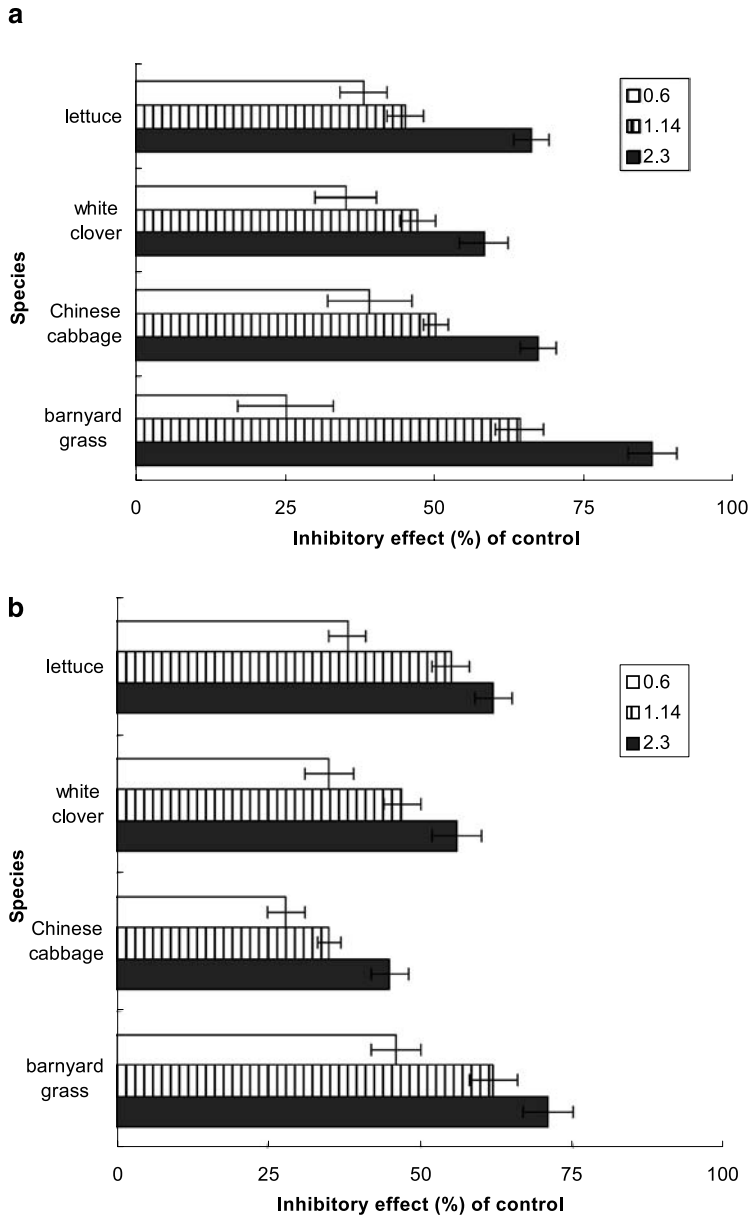

FIG. 3. Inhibitory effect of soil incorporated with leaves of *R. pseudo-acacia* on the root (a) and shoots (b) elongation of weed and crop species at three different concentrations. Error bars represent ± standard deviation ($N = 50$).

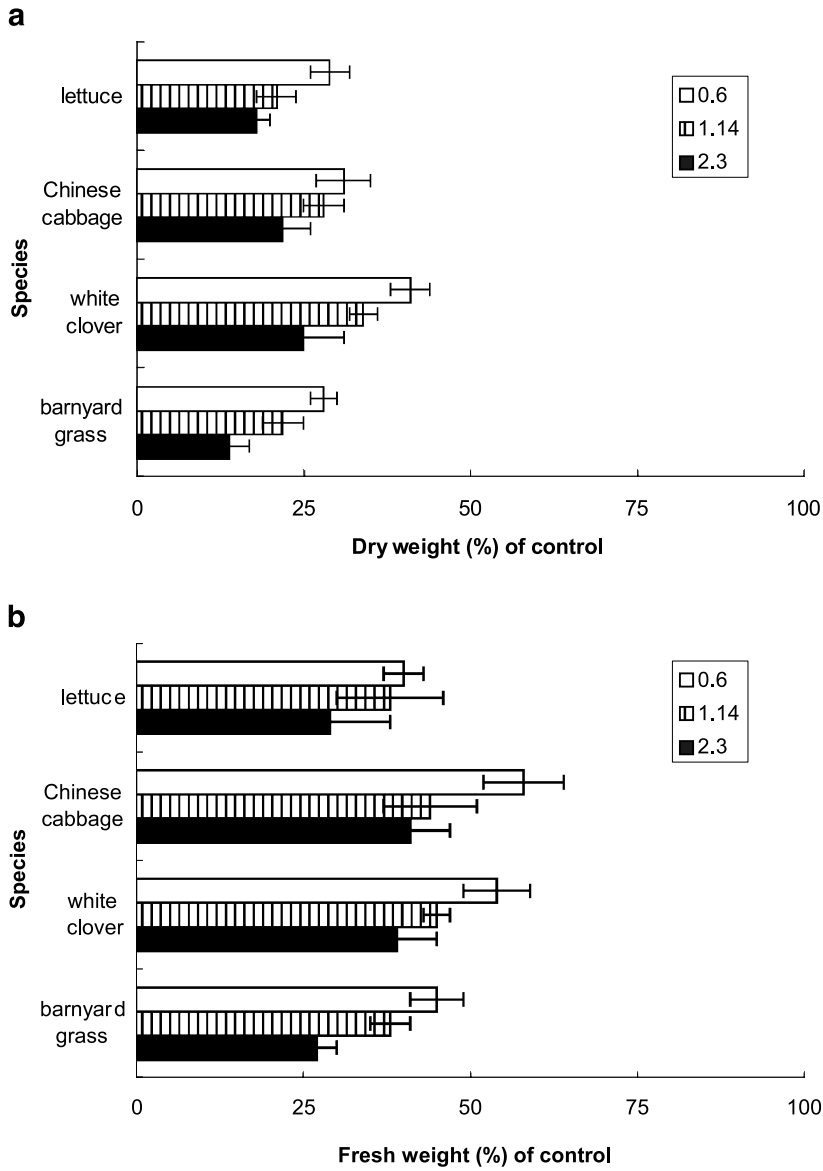

FIG. 4. Inhibitory effect of soil incorporated with leaves of *R. pseudo-acacia* on the root dry weight (a) and root fresh weight (b) of weed and crop species at three different concentrations. Error bars represent ± standard deviation ($N = 50$).

TABLE 1. EFFECT OF AQUEOUS EXTRACT OF *R. pseudo-acacia* LEAVES ON THE ROOT GROWTH OF TEST SPECIES (%) OF CONTROL

Extract (mg g^{-1} fw)	Alfalfa	Chinese cabbage	Barnyard grass	Lettuce	White clover	Timothy
0 (Control)	100 ± 4.3	100 ± 8	100 ± 6	100 ± 8	100 ± 8	100 ± 6
17	0	0	0	0	0	0
8.5	0	0	0	0	0	0
1.7	22 ± 5	23 ± 4	16 ± 5	23 ± 5	24 ± 5	15 ± 8
0.8	28 ± 6	29 ± 8	18 ± 7	34 ± 9	32 ± 6	20 ± 8
0.17	35 ± 8	40 ± 8	21 ± 8	46 ± 10	50 ± 8	31 ± 8

among the edible crops, respectively. The estimated leaf concentration required for a 50% reduction in root dry weight was less than 0.17 g/30 g of soil.

Phytotoxicity of Aqueous Extracts. Aqueous extracts of *R. pseudo-acacia* leaves [17, 8.5, 1.7, 0.8, 0.17 mg g^{-1} fresh weight (fw)] were applied in Petri dishes to six plant species. Growth of all test plants was completely inhibited at the two highest applied concentrations. However, at the other three concentrations, selective inhibitory effects on both hypocotyls and radicles were observed (Tables 1 and 2). In comparison, the inhibition of seedling growth was stronger in weeds than in the edible crop species and ranged between 65–79% and 40–63% in radicles and hypocotyls, respectively.

Isolation of Allelochemicals. The crude aqueous ethanolic extract inhibited the radicle elongation of lettuce seedlings more than 70% relative to the control (Figure 5A). After chromatographic resolution, inhibitory activity was found mostly in the methanol-eluted fraction Fr-1-9 (Figure 5B), which caused significant inhibition of radicle length in lettuce seedlings after 72 hr. Successive bioassay-directed chromatography of Fr-1-9 on Sephadex LH-20 and reverse-phase silica gel columns gave four main fractions. Among these, Fr-1-9-2 was the most active (Figure 5C). The fraction Fr-1-9-2 was therefore subjected to reverse phase column chromatography using MeOH–H_2O (9:1) as

TABLE 2. EFFECT OF AQUEOUS EXTRACT OF *R. pseudo-acacia* LEAVES ON THE SHOOT GROWTH OF TEST SPECIES (%) OF CONTROL

Extract (mg g^{-1} fw)	Alfalfa	Chinese cabbage	Barnyard grass	Lettuce	White clover	Timothy
0 (Control)	100 ± 4.3	100 ± 8	100 ± 6	100 ± 8	100 ± 8	100 ± 6
17	0	0	0	0	0	0
8.5	0	0	0	0	0	0
1.7	33 ± 4	29 ± 8	22 ± 6	32 ± 6	34 ± 5	25 ± 6
0.8	44 ± 11	45 ± 6	29 ± 6	43 ± 9	46 ± 6	33 ± 7
0.17	53 ± 8	57 ± 8	44 ± 6	55 ± 10	58 ± 8	38 ± 3

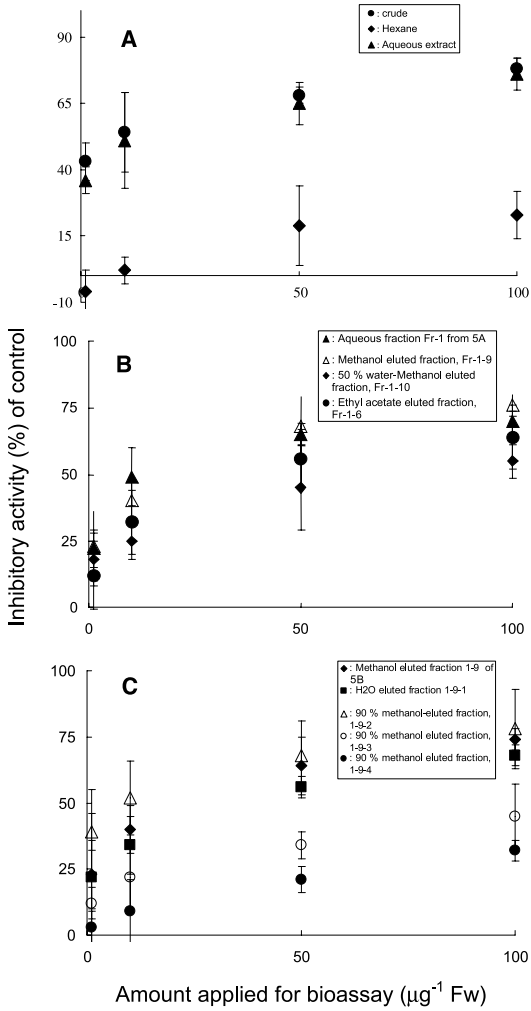

FIG. 5. Comparison of inhibitory effect of the crude aqueous ethanolic extract and its sub fractions on root elongation of lettuce seedlings. (A) Crude extract and its fractions obtained after liquid–liquid partitions. (B) Aqueous fraction Fr-1, of (A) and its subfractions obtained after silica gel column chromatography. (C) Pure methanol eluted fraction Fr-1-9 and its subfractions after chromatographic resolution. Error bars represent ± standard deviation (N = 15).

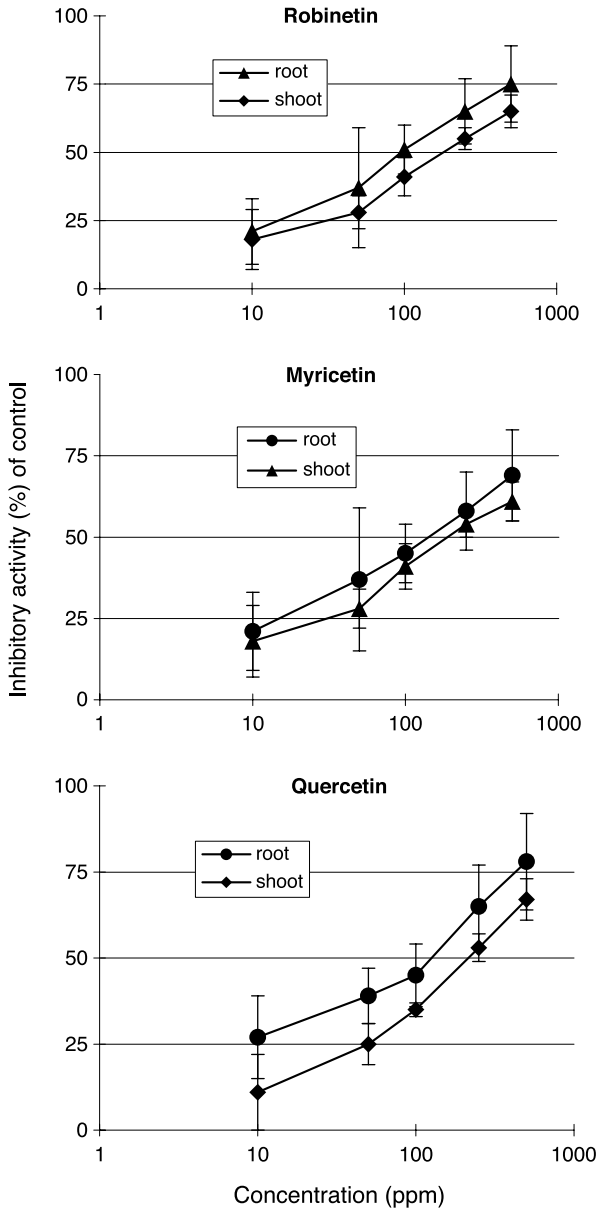

FIG. 6. Inhibitory effect of robinetin (a), quercetin (b) and myricetin (c) on the root and shoot growth of lettuce seedlings at five different concentrations. Error bars represent ± standard deviation ($N = 6$).

the eluting solvent to obtain four fractions, which were bioassayed, and the most active fraction, Fr-1-9-2-1, was further purified on a lobar column using methanol/water (9:1) to obtain the flavonoid **1**. The second most active fraction, Fr-1-9-2-2, yielded two pure compounds, **2** and **3**. These were identified as robinetin, myricetin, and quercetin (Figure 2). The spectroscopic data of **1** were in accordance with those reported in the literature (Mabry et al., 1970; Coetzee et al., 1995). The ^{1}H and ^{13}C NMR data of **2** and **3** were identical to those of authentic samples.

Compounds identified from *R. pseudo-acacia* exhibited inhibitory effects on radicle and hypocotyl growth of lettuce seedlings (Figure 6). The order of potency of growth inhibition was robinetin, myricetin, and quercetin with EC_{50} values of 100, 108, and 110 ppm, respectively.

DISCUSSION

A number of plants have inhibitory effects on the growth of neighboring or successional plants by releasing phytotoxic chemicals into the soil, either as exudates from living tissues or by decomposition of plant residues (Putnam and Tang, 1986; Chou and Leu, 1992; Chou et al., 1998; Chaves et al., 2001). The presence of phytotoxins in leaves of *R. pseudo-acacia* was confirmed by four plant species grown in soil mixed with leaves of this plant at three different concentrations (0.17, 0.34, and 0.68 w/w). In all tested species, growth of both radicles and hypocotyls was inhibited. The magnitude of inhibition increased with an increase in concentration (w/w) of *R. pseudo-acacia* leaf debris (Figure 3a). Weed species, particularly barnyard grass, was the most sensitive to allelochemicals released from the decomposing leaves. Furthermore, a gradual decrease in fresh and dry weight was observed in all tested species. The reduction in fresh and dry weight ranged from 35–76% and 40–65% in roots and shoots, respectively. In these pot experiments, leaves of the tested plants changed to a yellowish-green color. This phenomenon is speculated as being due to a decline in total chlorophyll contents. *R. pseudo-acacia* leaf aqueous extract showed strong phytotoxicity in terms of radicle and hypocotyl growth inhibition when the aqueous extract was applied in five different concentrations (w/w) (Tables 1 and 2). In all bioassay species, both radicle and hypocotyl growth decreased proportionally with increasing concentration of leaf water-soluble extract. Compounds identified from *R. pseudo-acacia*, robinetin (**1**, 0.990 μmol g^{-1} fw), myricetin (**2**, 0.062 μmol g^{-1} fw), and quercetin (**3**, 0.099 μmol g^{-1} fw) exhibited inhibitory effects on radicle growth of lettuce. Robinetin was isolated in larger amounts and was identified as one of the major inhibitors in the leaves. Therefore, the major portions of the crude extract's inhibitory activity may

partially be attributed to robinetin and partially to other compounds. The EC_{50} of robinetin was 100 ppm, which is not especially low. However, the purified compound with its low specific inhibitory activity may play a greater role in the expressed inhibitory potential than those of high specific inhibitory activity by virtue of its being present in larger quantity (Lehle and Putnam, 1983). The other compounds also inhibited the root and shoot elongation of lettuce seedlings. Allelochemicals are present in all parts of plants including leaves, stems, and roots, and under appropriate conditions these are released into the environment and likely act additively or synergistically to affect the growth of neighboring plants (Weston and Duke, 2003).

In summary, this study demonstrates that allelochemicals released into the environment through accumulated fallen leaves inhibit the growth of various weeds and crop species. A species-selective inhibitory effect was observed. Allelopathy may play a role in the successful invasion of *R. pseudo-acacia*, but field trials are required to examine the significance of the allelochemicals on target species that co-occur with *R. pseudo-acacia* in nature.

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VOLATILE ALLELOCHEMICALS IN THE *Ageratum conyzoides* INTERCROPPED CITRUS ORCHARD AND THEIR EFFECTS ON MITES *Amblyseius newsami* AND *Panonychus citri*

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Abstract—*Ageratum conyzoides* L. weed often invades cultivated fields and reduces crop productivity in Southeast Asia and South China. However, intercropping this weed in citrus orchards may increase the population of predatory mite *Amblyseius newsami*, an effective natural enemy of citrus red mite *Panonychus citri*, and keep the population of *P. citri* at low and non-injurious levels. This study showed that *A. conyzoides* produced and released volatile allelochemicals into the air in the intercropped citrus orchard, and these volatiles influenced the olfactory responses of *A. newsami* and *P. citri*. At test temperature (25°C), *A. conyzoides* fresh leaves, its essential oil, and major constituents, demethoxy-ageratochromene, β-caryophyllene, α-bisabolene, and E-β-farnesene, attracted *A. newsami* and slightly repelled *P. citri*. Field experiments demonstrated that spraying *A. conyzoides* essential oil emulsion in an *A. conyzoides* nonintercropped citrus orchard increased the population density of *A. newsami* from below 0.1 to over 0.3 individuals per leaf, reaching the same level as in an *A. conyzoides* intercropped citrus orchard. However, this effect could not be maintained beyond 48 hr because of the volatility of the essential oil. In contrast, in the *A. conyzoides* intercropped citrus orchard, *A. conyzoides* plants continuously produced and released volatile allelochemicals and maintained the *A. newsami* population for a long time. The results suggest that intercropping of *A. conyzoides* not only made the citrus orchard ecosystem more favorable for the predatory mite

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A. newsami, but also that the volatile allelochemicals released from *A. conyzoides* regulated the population of *A. newsami* and *P. citri*.

Key Words—*Ageratum conyzoides* L., volatile allelochemical, citrus orchard, intercropping, predatory mite *Amblyseius newsami*, citrus red mite, olfactory response.

INTRODUCTION

The Compositae weed *Ageratum conyzoides* L. originated from Central America and is now widely spread in Southeast Asia and South China. It invades cultivated fields and reduces crop productivity in tropical and subtropical agroecosystems (Roder et al., 1997; Singh et al., 2003). However, when grown in citrus orchards, it effectively suppresses the growth of other weeds and spore germination of several fungal pathogens through release of allelochemicals into the soil (Kong et al., 2004c). Additionally, intercropping *A. conyzoides* with citrus makes the orchards more favorable for predatory mites (*Amblyseius* spp.), which effectively control the major arthropod pest citrus red mite (*Panonychus citri* McG) in citrus orchards in South China (Tao and Luo, 1992). Therefore, *A. conyzoides* has been advocated for intercropping in citrus orchards and is utilized on more than 150,000 ha of citrus orchards in South China (Liang and Huang, 1994). The natural biochemical interactions between *A. conyzoides* and other organisms in the intercropped citrus orchard remain obscure.

A. conyzoides produces and releases volatile allelochemicals into the environment at various growth stages (Kong et al., 1999, 2002, 2004a,b). The concentration of volatiles in *A. conyzoides* intercropped citrus orchards is so high that an unpleasant odor can be detected. Studies have shown that volatile allelochemicals released by many plant species play an important role in tritrophic systems that include the host plant, herbivore, and parasitoid or predator (Dicke et al., 1990; Bruin et al., 1992; Dicke, 1999; Sabelis et al., 1999; Agrawal, 2000; Bruin and Sabelis, 2001; Kessler and Baldwin, 2001). It is possible that volatiles released by *A. conyzoides* may affect the behavior and population of mites in intercropped citrus orchards. Accordingly, our objectives in this study were to determine and identify the volatile allelochemicals in the *A. conyzoides* intercropped citrus orchard and to evaluate their effects on the predatory mite (*Amblyseius newsami* Evans) and the citrus red mite (*P. citri*).

METHODS AND MATERIALS

Sampling Citrus Orchards. Two orchards planted with *Citrus sinensis* (L.) Osb. (plants, 2.5 × 4 m) in Guangzhou Suburb, China (23°06′N, 113°18′E),

were selected. One orchard had been intercropped with *A. conyzoides* since 2001. The *A. conyzoides* plants grew luxuriantly and covered the orchard floor that was free from other weeds. Another citrus orchard without *A. conyzoides* served as the control. The major weeds in this orchard were *Bidens pilosa*, *Digitaria sanguinalis*, and *Cyperus difformis*. If any *A. conyzoides* appeared in the control site, they were hand-weeded since October 2002. The experimental orchards were in a subtropical climate zone, with a mean annual temperature of 21.8°C and a mean annual rainfall of 1682 mm. There were no significant differences in pH, organic matter content, and fertility status between the intercropped and control site soils (pH 5.6; organic matter 19.7 g kg^{-1}; total N 0.97 g kg^{-1}; available N 40.8 mg kg^{-1}; total P 0.48 g kg^{-1}; available P 13.8 mg kg^{-1}; total K 102.9 g kg^{-1}; available K 98.7 mg kg^{-1}).

Population Densities of Mites. A. newsami and *P. citri* were used because *A. newsami* is the most common *Amblyseius* spp. predatory mite and *P. citri* is a major citrus arthropod pest in citrus orchards in South China. Fifteen citrus plants, whose canopies did not touch each other, were randomly selected from two orchards, respectively. For the population census, 50 representative spatial leaves of each citrus plant were selected, and adult mites were counted twice a day (10: 00 a.m. and 4:00 p.m.) for three consecutive days (Tao and Luo, 1992). Data were subjected to ANOVA with Duncan's multiple range test. The mean number of individual mites (*A. newsami* or *P. citri*) per leaf indicated their population densities in the orchards.

Effect of the essential oil on the mites was studied in a nonintercropped citrus orchard (i.e., control without *A. conyzoides*). Twenty-seven citrus plants (3 × 9), whose tree canopies were separated from one another by at least 15 m, were randomly selected. Then, a 5% essential oil emulsion (10 kg) of *A. conyzoides* was sprayed on 18 citrus plants (2 × 9). Ten kg water served as a control (1 × 9 citrus plants). After 24 and 48 hr, the population densities (mean individuals per leaf) of adult mite *A. newsami* and *P. citri* on each of 50 representative spatial leaves of each plant were counted as described above.

Experiments were carried out in June 2003, when *A. conyzoides* was at its peak flowering stage, and mites *A. newsami* and *P. citri* were active and abundant. Sampling dates were cloudy with a 27 ± 5°C air temperature and 70 ± 10% relative humidity. Pesticides have never been applied in either citrus orchard since October 2002.

Collection and Analysis of Volatiles. Collection and analysis of volatiles in the orchards was as follows (Kong et al., 2004a). Nine sampling locations were randomly selected from both citrus orchards. Air samples 1 m above the ground at 2 × 9 locations each were collected by Timing Minipump air samplers with a flow meter TMP-1500 (China Electrical Instruments Co.) and pumped through the sampling tubes (Tekmar Co., USA) packed with Tenax and Carbonsieve at a flow rate of 0.5 l/min for 30 min. The volatile chemicals in air samplers were

adsorbed onto the solid adsorbents, and sampling tubes were installed into a Tekmar 6032 Aero-trap Autosampler for thermal desorbing. The tubes were rapidly heated to 225°C, and their volatiles were desorbed and carried by helium to a Tekmar 3000 Purge & Trap Concentrator where they were trapped again. The concentrator was heated at a rate of more than 400°C/min, and the trapped volatiles were transported to a Hewlett-Packard P 5972 GC/MSD with an HP-5 bonded stage fused-silica capillary column (30 m × 2.5 mm) for analysis. The initial oven temperature (70°C) was maintained for 2 min and then increased to 180°C at a rate of 10°C/min. The column was maintained at 180°C for 5 min, and then the oven temperature was raised to 250°C at 15°C/min. Mass spectra were repetitively scanned from 35 to 450 amu every 2 sec. Ionization was selected in the electron impact mode (EI) at 70 eV. The chemical constituents were identified by peak matching against standards in the NIST 95 Computer Library or by spectral similarity to an authentic reference compound (Aldrich Chemical Co.). The relative amounts of the chemical constituents were calculated by integrating all peaks with an area greater than 0.1%.

Collection and analysis of volatiles from fresh leaves and the essential oil of *A. conyzoides* were performed using the technique of multiple headspace solid-phase microextraction (Augusto et al., 2003; Ezquerro et al., 2003). GC/MSD and manipulation was the same as described above.

The essential oil was obtained by placing fresh leaves of *A. conyzoides* into a desiccator with a liquid nitrogen cold trap and pumped into a vacuum (Kong et al., 2002). It was then subjected to silica gel column chromatography with *n*-hexane/ether (9:1 and then 4:6; v/v) mixture. It produced five major components, ageratochromene, demethoxy-ageratochromene, β-caryophyllene, E-β-farnesene, and α-bisabolene.

Olfactory Responses of Mites to Volatiles. Adult mites of both *A. newsami* and *P. citri* were collected from citrus orchards and reared in a petri dish (9-cm diam.; 1.8-cm depth) at 25°C and 50% relative humidity. The dish was placed in a transparent container over a pad of black colored velvet and a sponge (8-cm diam.; 1 cm thick). Because the mites are translucent, the black background enhanced visual detection. The sponge was allowed to imbibe and maintain water by partially adding water daily.

The olfactory response of the mites to volatiles was studied with a slightly altered airflow four-armed olfactometer designed by Pettersson (1970). The size of the exposure chamber was halved (ray of crescents: 67.5 mm, inner height: 5 mm, inner diameter of stainless tubes: 3 mm). A flow rate of 300 ml/min through each arm was maintained to create odor fields with sharp boundaries. The exposure chamber and TV monitor screen (21″) were interfaced with a multifunctional MC-1 camera (China Optical Co.) with the following features: sensitive element, 1/3″, 500(H) × 582(V) lines; resolution, 420 lines; optical attachment, eyepiece adapter (39-mm diam for stereomicroscope and 23.3-mm

diam for the biological microscope); minimum illumination, 3 lx; shutter, 1/50–1/1000 sec autocontrol; objective, $F = 8$ mm, focus from 10 mm to infinity. A vacuum pump/compressor was connected to a hole in the center of the floor of the exposure chamber by silicone rubber tubing and was used to suck air through the arms of the device into the exposure chamber. Test mites were introduced into the exposure chamber through this hole with the extractor tube removed. Activated charcoal enclosed in dry cotton wool at each flow meter purified incoming air.

Trials were conducted in accordance with the protocols suggested by Vet et al. (1983). Each arm of the exposure chamber was connected to a set of three 50-ml glass vials. The vial closest to the chamber served as the trap to catch mites, the second one provided an odor source, and the outside vial contained distilled water over which the incoming air was passed to create uniform humidity. The odor source, *A. conyzoides* fresh leaves (500 mg), the essential oil (100 mg), or five components (100 mg) were each placed in the odor vial. An olfactometer with a single arm of humidified air served as control. With proper air pressure, four air streams of 300 ml/min were introduced into the central region through the flow meters into each of the different arms of the olfactometer. Fifty adult mites were individually introduced into the chamber, and they could make two choices. The first choice was made when a mite reached the arbitrary transverse diagonal of one of the odor compartments. Before the first choice was made, each mite was given 2 min in the exposure chamber, at the end of which it was regarded as unmotivated and not recorded. The remaining 8 min was allocated for the second choice that was recorded as the mite stayed in any of the odor compartments beyond the first choice line. Thus, a mite could express both choices or the first choice only. The total number of mites that expressed the first choice or both choices was recorded. To neutralize any asymmetry in the experimental setup, vials containing odor sources were reordered after testing 10–15 mites, followed by a thorough cleaning of the chamber with absolute alcohol and allowing the solvent to dry for 10–15 min. The experimental environment was at 25°C and 60–70% relative humidity.

Olfactory responses were analyzed using ANOVA with Duncan's multiple range test. The impacted degree (ID) was calculated based on the following formula (Mumcuoglu et al., 1996): degree of attraction = $[1 - (C/T)] \times 100\%$ and degree of repellency = $[1 - (T/C)] \times 100\%$, where T is number of mites in the treatment and C is number of mites in the control.

RESULTS

Population of Mites in the Citrus Orchards. Figure 1 shows that *A. newsami* and *P. citri* occurred in both the *A. conyzoides* intercropped and

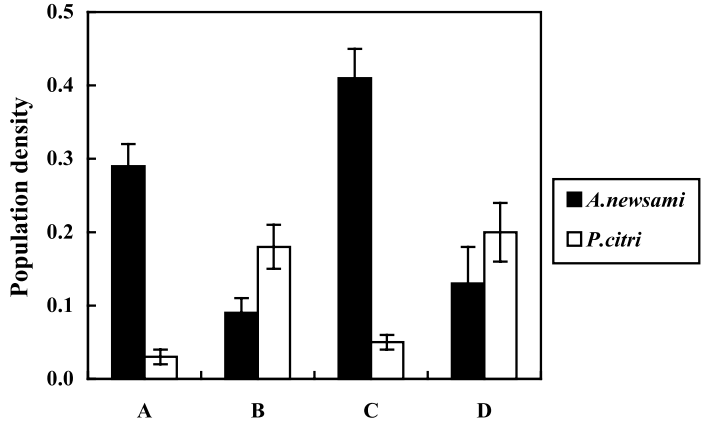

FIG. 1. Population density (mean individuals per leaf) of mites on citrus trees (**A**, in the *A. conyzoides* intercropped citrus orchard; **B**, in the *A. conyzoides* nonintercropped citrus orchard; **C**, in the *A. conyzoides* nonintercropped citrus orchard, after 24 hr of spraying 5% *A. conyzoides* essential oil emulsion; **D**, in the *A. conyzoides* nonintercropped citrus orchard, after 48 hr of spraying 5% *A. conyzoides* essential oil emulsion).

nonintercropped citrus orchards, but that their population densities had obvious differences. In the *A. conyzoides* intercropped orchard, the population density of *A. newsami* reached 0.3 individual per leaf, and the *P. citri* population was markedly decreased. In the *A. conyzoides* nonintercropped orchard, the *A. newsami* population was below 0.1 individual per leaf, and the *P. citri* population was increased over 0.2 individual per leaf. This indicated that intercropping with *A. conyzoides* in citrus orchards increased the *A. newsami* population and reduced the population density of citrus red mite to low and noninjurious levels. This was possibly correlated with volatiles in the air.

Volatile Allelochemicals and Their Effects on Mites. Several terpenes, such as carene, cubebene, copaene, limonene, myrcene, and pinene, were detected in the air in both the *A. conyzoides* intercropped and nonintercropped orchards. Abundant ageratochromene, demethoxy-ageratochromene, α-bisabolene, β-caryophyllene, and E-β-farnesene were found in samples from the *A. conyzoides* intercropped orchard, fresh leaves, and the essential oil of *A. conyzoides*, but these were not detected in the air samples above the *A. conyzoides* nonintercropped orchard (Table 1). These volatile components in the orchards might come from understory plants, soil, or other organisms, but ageratochromene, demethoxy-ageratochromene, β-caryophyllene, α-bisabolene, and E-β-farnesene were all major components of *A. conyzoides* fresh leaves and essential oil. Particularly, ageratochromene and demethoxy-ageratochromene are specific in the *Ageratum* genus (Kong et al., 2002, 2004a; Okunade, 2002). These

TABLE 1. CHEMICAL CONSTITUENT OF VOLATILES IN AIR SAMPLES COLLECTED FROM DIFFERENT SOURCES

Chemical constituent	Nonintercropped citrus orchard*	Intercropped citrus orchard*	Fresh leaf of *A. conyzoides* (headspace)	Essential oil of *A. conyzoides* (headspace)
Ageratochromene	0a	6.6 ± 1.3b	10.3 ± 1.0c	31.2 ± 5.1d
Demethoxy-ageratochromene	0a	8.9 ± 1.7b	9.8 ± 1.1b	21.5 ± 4.4c
β-Caryophyllene	0a	19.6 ± 4.5b	28.1 ± 2.4c	16.9 ± 3.3b
α-Bisabolene	0a	9.8 ± 1.9b	12.5 ± 2.9a	10.1 ± 1.7b
E-β-Farnesene	0a	10.2 ± 2.1b	13.4 ± 2.3c	9.3 ± 1.1b
Carene	6.7 ± 0.9a	2.2 ± 0.9b	0c	0c
Cubebene	8.6 ± 1.1a	7.8 ± 1.7a	9.3 ± 1.5b	3.9 ± 0.3c
Copaene	9.7 ± 2.3a	11.1 ± 2.6a	5.4 ± 1.7b	1.5 ± 0.7c
Myrcene	11.2 ± 1.7a	3.1 ± 1.0b	0c	0c
Pinene	15.0 ± 4.1a	2.3 ± 0.7b	2.3 ± 0.5c	0.9 ± 0.2d
Limonene	33.2 ± 6.8a	5.5 ± 1.2b	0c	0c
Other unknown components	15.6 ± 3.8a	12.9 ± 2.1b	8.9 ± 2.2c	4.7 ± 1.9d

Data were the relative amounts (%) of constituents from each sample.

* Means ± SE from three independent experiments with nine sampling locations in the *A. conyzoides* intercropped or nonintercropped citrus orchards for each determination are shown. Data not followed by the same letter in a line are significantly different at $P < 0.05$, with Duncan's multiple range test.

chemicals did not occur in the air samples of the citrus orchard without *A. conyzoides*, indicating that they were primarily released from *A. conyzoides*. The presence of these volatile allelochemicals thus may make a contribution toward regulating the population of the mites in the *A. conyzoides* intercropped orchard.

Olfactometer trials showed that *A. conyzoides* fresh leaves, the essential oil, and its four components had different effects on the olfactory responses of *A. newsami* and *P. citri* (Table 2). The fresh leaves and essential oil as well as demethoxy-ageratochromene, β-caryophyllene, α-bisabolene, and E-β-farnesene strongly attracted *A. newsami* and slightly repelled *P. citri* at the test temperature (25°C). It is noted that an olfactory response of mites to ageratochromene was not achieved because ageratochromene is a crystal (mp 46–47°C) and difficult to volatilize at this test temperature. The olfactory response of *A. newsami* to each individual component was clearly lower than to those of *A. conyzoides* fresh leaves and the essential oil (Table 2), suggesting that this effect could be intensified with the combinations of several components from *A. conyzoides*.

Field experiments (Figure 1) showed that spraying the essential oil of *A. conyzoides* on the citrus trees in the *A. conyzoides* nonintercropped citrus

TABLE 2. OLFACTORY RESPONSE OF MITES ON VOLATILES AND ITS MAJOR CONSTITUENTS FROM *A. conyzoides*

Volatiles	Degree of attraction or repellency (%)	
	A. newsami	*P. citri*
Fresh leaf	61.11A (attraction)	7.69A (repellency)
Essential oil	95.45B (attraction)	14.81B (repellency)
β-Caryophyllene	59.09C (attraction)	7.69A (repellency)
α-Bisabolene	40.91D (attraction)	7.69A (repellency)
E-β-Farnesene	38.89D (attraction)	7.69A (repellency)
Demethoxy-ageratochromene	22.22E (attraction)	0.00C (repellency)

Data were mean value of three independent experiments with 50 mites for each determination and not followed by the same letter in a column are significantly different at $P < 0.05$, with Duncan's multiple range test.

orchard increased the *A. newsami* population greatly. It increased from below 0.1 to over 0.3 individual per leaf at 24 hr after the essential oil emulsion spray, which reached the same level as in the *A. conyzoides* intercropped citrus orchard. It then decreased to 0.13 individuals per leaf after 48 hr. The results indicate that the volatiles from *A. conyzoides* attract the predatory mite *A. newsami* and increase its population on citrus tree leaves in the field. This attraction was time-dependent, however, and could only be maintained for 48 hr because of the oil's volatility. The results suggest that the stable population density of *A. newsami* in an *A. conyzoides* intercropped citrus orchard is correlated with the continued release of volatiles from understory *A. conyzoides* plants. Thus, intercropping *A. conyzoides* may increase the predatory mite *A. newsami* population in citrus orchards.

DISCUSSION

The role of orchard ground cover plants in improving habitat and enriching natural enemies of pests has been known for a long time (Bugg et al., 1990; Fye, 1983; House and Alzugaray, 1989; Lawton, 1982). It is necessary to select beneficial plant species and pay attention to their chemical interactions with understory plants and other organisms in the orchards. A citrus orchard is a relative stable and complicated ecosystem. The population dynamics of mites in citrus orchards often depends on the associated organisms, atmospheric temperature, precipitation, and other factors (Liang and Huang, 1994). In this study, *A. conyzoides* produced and released volatile allelochemicals into the air. These were at least, in part, responsible for the population variations of mites,

particularly the predatory mite *A. newsami*. However, it remains obscure why and how *A. conyzoides* manipulates the relationship between predatory mite and citrus red mite through releasing volatiles.

Many plants defend themselves from herbivores by releasing volatiles that manipulate the relationships among plants, herbivores, and their natural enemies as soon as the plants are under attack (Dicke et al., 1990; Bruin et al., 1992; Dicke, 1999; Sabelis et al., 1999; Agrawal, 2000; Bruin and Sabelis, 2001; Kessler and Baldwin, 2001; Rausher, 2001). Many predatory mites are known to respond to odors released by plants (Janssen, 1999; Shimoda and Dicke, 1999; Maeda et al., 2001). However, most studies on the attraction of natural enemies have been carried out with host plants infested with herbivores. Our research suggests that the chemical interactions among predator, pest, and citrus can also be achieved through intercropping.

It is noted that allelopathic inhibition was not apparent in the intercropped citrus orchard. Generally, allelopathy occurs at the time of seed germination and in early growth stages of plant species. Perennially adult citrus trees may resist the allelochemicals released from understory *A. conyzoides* plants, or bulk rhizosphere and soil microflora of the citrus may be able to rapidly biodegrade any allelochemicals released into the soil from *A. conyzoides* (Liang and Huang, 1994; Kong et al., 2004c).

A. conyzoides has been a historical folk medicine in China and in several countries of the world. It contains a wide range of secondary metabolites including flavonoids, chromenes, benzofurans, and terpenoids that serve as antimicrobial, insecticidal, and nematocidal agents (Ming, 1999; Okunade, 2002). This species also appears to be a valuable agricultural resource. Its intercropping in citrus orchards has become popular in South China, and substantial ecological and economic benefits have been achieved. Our results suggest that there are natural chemical interactions among the organisms in *A. conyzoides* intercropped citrus orchards, and that further clarification and utilization of these interactions should be beneficial to integrated pest management in citrus orchards.

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RAPID COMMUNICATION

BIOLOGICALLY RELEVANT CONCENTRATIONS OF PETROMYZONOL SULFATE, A COMPONENT OF THE SEA LAMPREY MIGRATORY PHEROMONE, MEASURED IN STREAM WATER

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Abstract—Adult sea lampreys locate spawning streams in the Great Lakes by using a migratory pheromone that is released by stream-resident larval conspecifics. Behavioral, electrophysiological, and biochemical analyses of larval release water have suggested that this pheromone is composed of several components, one of which is petromyzonol sulfate (PS), a known lamprey-specific bile acid. Its precursor, allocholic acid (ACA), has also been implicated. In this study, we employed high-performance liquid chromatography and mass spectrometry to look for both bile acids in various stream waters, thereby testing whether they might have a role in natural pheromone function. Although PS was measured at picomolar concentrations in streams known to contain larval lampreys and attract migratory adults, ACA was not. Neither compound was measured in streams lacking larvae. This finding indicates that PS is a component of the natural pheromone, and it suggests that ACA has little relevance.

Key Words—*Petromyzon marinus*, pheromone, petromyzonol sulfate, allocholic acid, migration, bile acid, sea lamprey.

INTRODUCTION

The sea lamprey (*Petromyzon marinus*) is an ancient fish whose larvae live in freshwater streams but, following a metamorphosis, spends 1–2 years in oceans or large lakes parasitizing other fish before eventually entering streams as

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mature adults to spawn. Field and laboratory tests have provided evidence that migrating adult lampreys locate spawning streams by using a migratory pheromone released by stream-resident larval lampreys (Sorensen and Vrieze, 2003). Coordinated biochemical, physiological, and behavioral studies have demonstrated that adult lampreys detect and respond to a mixture of compounds released by larvae including the lamprey-specific bile acid, petromyzonol sulfate (PS) (Sorensen et al., 2003, unpublished results). The other components have not yet been identified, but two appear to be novel and more important than PS, whereas a potential fourth, allocholic acid (ACA), has uncertain function. These olfactory cues seem to function as a synergistic mixture (Sorensen et al., 2003). Although laboratory estimates of larval bile acid release rate indicate that lamprey streams in the Great Lakes Basin likely contain PS at concentrations of about 10^{-12} M, and ACA at even lower concentrations (Polkinghorne et al., 2001), their presence in streams has yet to be confirmed. In this study, we developed a method that employed high-performance liquid chromatography (HPLC) in conjunction with mass spectrometry to identify and measure PS and ACA (the components for which we have synthesized standards) in streams containing populations of larvae.

METHODS AND MATERIALS

Sample Collection and Purification. We selected nine Great Lakes streams that are routinely censused by sea lamprey biologists (Table 1). One-liter water samples were collected in carboys containing preservative and extracted using reversed-phase C18 (RPC18) within 2 d following established protocols (Polkinghorne et al., 2001). Extracts were dried under nitrogen and stored at −20°C until analyzed. When possible, samples were collected in triplicate.

Identification and Quantification. Because direct analysis of bile acids using liquid chromatography–mass spectrometry was precluded by the buffering system needed to separate these compounds, we developed a two-step process that used HPLC fractionation followed by RPC18 to purify them so that they could be identified and quantified by mass spectrometry (MS). Briefly, dried stream water extracts were reconstituted in 110 μl of methanol/water (60:40), spiked with an internal standard [^{14}C-chenodeoxycholic acid (CDCA), New England Nuclear, Massachusetts, USA], and then injected onto an HPLC equipped with an inflow scintillation monitor, and fractionated by following established protocols (Fine et al., 2004). We collected three 5-min fractions centered on the retention times of PS, ACA, and CDCA as determined by previous experience (Polkinghorne et al., 2001) and pilot HPLC runs that used ^{14}C-CDCA alone. Fractions were extracted by using RPC18, dried under nitrogen, and reconstituted in 35 μl methanol. Extraction/recovery efficiencies

TABLE 1. STREAMS EXAMINED IN THIS STUDY

Stream	Sampling date(s)	Latitude	Longitude	Discharge (m^3/sec)[a]	Number of larval sea lampreys[b]	Native lamprey present?[c]
Valley Creek	June 2001	44.9126°N	92.8237°W	0.15	0	No
Nagel	June 1999	45.4592°N	83.871°W	0.013	0	No
Lone Pine	June 1999	45.6229° N	84.201° W	0.006	0	No
Ocqueoc	June 1999	45.4894°N	84.0748°W	2.75	Unknown	Yes
Cheboygan	June 1999	45.655°N	84.4649°W	23.97	72,000	Yes
Big Garlic	October 1999	47.8904°N	87.4579°W	1.73	31,622	No
Misery	October 1999	46.9984°N	88.9803°W	4.18	170,644	Yes
Middle	October 1999	46.6907°N	91.8523°W	6.94	259,591	Yes
Rock	October 1999	46.4652°N	86.9153°W	3.83	134,650	Yes
St. Mary's	March 2001	45.5064°N	84.3524°W	2122.5	1,600,000	Yes

[a] Nagel and Lone Pine represent a single springtime estimate by Vrieze and Sorensen (2001). Valley Creek is base flow (Newman, Department of Fisheries, Wildlife and Conservation Biology, University of Minnesota, personal communication). Values for the other rivers are averages and do not reflect seasonal variation (Schleen and Klar, 2000, 2002).

[b] Estimates for Nagel, Lone Pine, Ocqueoc, and Cheboygan Rivers are based on surveys by the U.S. Fish and Wildlife Service which have been extrapolated using their "Empiric River Treatment Ranking System" (Slade, Ludington Biological Station, MI, personal communication). Estimates for other streams are based on historical survey records (Schleen and Klar, 2000, 2002).

[c] Native lampreys include either *Lethenteron appendix*, *Ichthyomyzon fossor*, or both.

were estimated to be 64 ± 11.3% (mean ± SD). Identification and quantification of PS and ACA in the appropriate fractions were accomplished with MS. Briefly, a 5-μl aliquot of each fraction was injected into an ion trap mass spectrometer (LCQ Classic, Thermo Electron Corporation, Waltham, MA, USA) equipped with an electrospray ionization source. Because the molecular ion of PS did not fragment into daughter ions that could be measured by our instrument, profiles of 10 *m*/*z* units centered on the molecular ion of PS at 473.4 *m*/*z* were examined before employing selected ion monitoring centered on the molecular ion (see Fine et al., 2004). Selective reaction monitoring was used on those fractions thought to contain ACA because its molecular ion (407.4 *m*/*z*) consistently fragments at a normalized collision energy of 30% to produce a quantifiable ion at 380.9 *m*/*z* that we could measure. Peak sizes were measured for quantification and values measured compared to a standard curve that was generated by adding known amounts of PS and ACA (Toronto Research Chemicals, Canada) to 1-l water samples collected from a small inland stream (Valley Creek, Minnesota, USA) known to lack lampreys and to have a similar organic load. The 9-point standard curves we generated demonstrated a linear increase in peak size ($r^2 = 0.99$) between 250 and 6400 pg for each compound ($N = 4$). The detection limit of our assay was slightly less than 10^{-12} M for PS and ACA.

RESULTS AND DISCUSSION

Although a compound with an *m*/*z* ratio of 473.4, the same as PS, was discernable in six of the seven lamprey rivers, there was no indication of ACA in any sample. In those samples with good signal-to-noise ratios (about half), the isotopic ratio associated with the peak at 473.4 *m*/*z* matched that of authentic PS (Figure 1a), seemingly confirming its identity. Concentrations of PS in lamprey streams ranged from about 10^{-12} to 5×10^{-12} M (Figure 1b). These values are in close agreement with those estimated to be present by Polkinghorne et al. (2001) and above the estimated detection limit of the sea lamprey olfactory system which is approximately 10^{-12} M (Li et al., 1995). Experiments also find PS to have behavioral activity at these concentrations (Bjerselius et al., 2000; Vrieze and Sorensen, 2001; Fine and Sorensen, unpublished results). However, PS's activity is not strong. Its function may be to synergize that of other components as is sometimes seen with insect pheromones (Sorensen et al., 2003). Alternatively/additionally, this cue's function may be associated with short-range orientation within streams (i.e., at "higher" concentrations) vs. searching for cues from lakes/oceans. Finally, although our estimates did not show a clear correlation between larval density and PS concentration, this may be a consequence of the native lamprey species (which

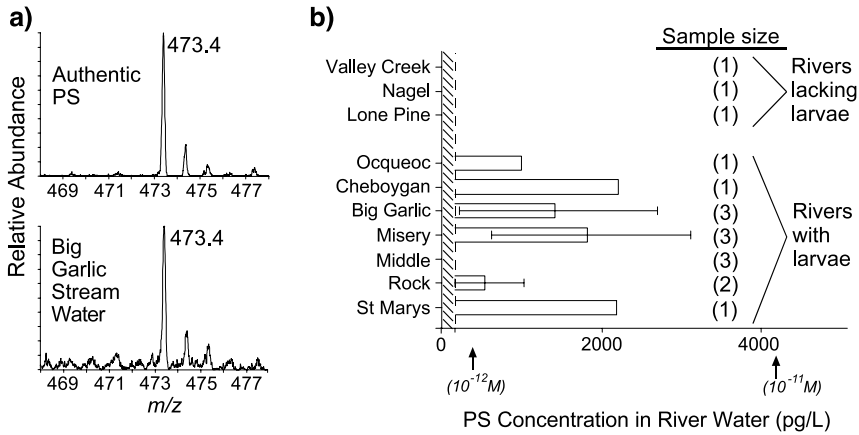

FIG. 1. (a) Electrospray ionization–mass spectrometry (ESI-MS) of 1 ng authentic petromyzonol sulfate (PS) added to methanol and an extract isolated from Big Garlic stream water. (b) Mean concentrations of PS (± standard deviation) measured in lamprey and nonlamprey streams as measured by ESI-MS. Concentrations are also noted as their log molar equivalents in parentheses. The putative detection limit of PS by our assay is represented by the dashed vertical line.

also release PS) also found in these rivers (Table 1; Fine et al., 2004), possible seasonal variation in larval metabolism (Polkinghorne et al., 2001), and/or degradation/binding of PS to organics in stream waters. We are presently attempting to adopt other more sensitive forms of MS so that we can evaluate how environmental variables influence natural concentrations of PS with greater precision and ease.

In summary, this study has demonstrated that PS is present in streams inhabited by larval sea lamprey at biologically relevant concentrations, thereby providing evidence that PS functions as a component of the natural pheromone. This appears to be the first time a fish pheromone, or component thereof, has been measured *in situ*. The lack of measurable ACA suggests that it is not a component of the cue. Recent pilot studies have added larval extracts containing $\sim 10^{-12}$ M PS and the other (unidentified) components to natural streams and have proven attractive to migratory adults (Jones, Michigan State University, personal communication). This work may provide guidance to fishery biologists attempting to employ extracts of the entire larval pheromone as an attractant to develop a means to remove sea lamprey from the Great Lakes where it is invasive (Twohey et al., 2003). Future studies will seek to identify and measure all components so they too might be employed in a pheromone-based sea lamprey control program.

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RAPID COMMUNICATION

METHYL JASMONATE APPLICATION INDUCES INCREASED DENSITIES OF GLANDULAR TRICHOMES ON TOMATO, *Lycopersicon esculentum*

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Abstract—This study was designed to address whether applications of methyl jasmonate (MJ) or Benzothiadiazole (BTH) to cultivated tomato, *Lycopersicon esculentum*, induced elevated densities of defense-related glandular trichomes on new leaves. Four-leaf tomato plants were sprayed with MJ, BTH, or control solutions, and the density of type VI glandular trichomes on new leaves was subsequently determined at 3, 7, 14, 21, and 28 d. At 7, 14, and 21 d, the density of type VI glandular trichomes on new leaves was significantly higher on MJ-treated plants than on BTH- or control-treated plants. At 7 and 14 d after treatment, the mean density of glandular trichomes on new leaves of MJ-treated plants was ninefold higher than on leaves of control-treated plants. We observed entrapment of immature western flower thrips in trichomes on MJ-treated plants at higher rates than on BTH or control plants. Studies to evaluate potential trade-offs between reductions in pest populations by increased trichome density and possible negative impacts of trichome induction on biological control agents are needed.

Key Words—*Lycopersicon esculentum*, tomato, methyl jasmonate, induced resistance, glandular trichomes.

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INTRODUCTION

Plants have evolved numerous defensive strategies to reduce herbivory or its effects on plant fitness (Karban and Baldwin, 1997). These defenses may be constitutively expressed or may be induced following attack by herbivorous arthropods. Induced responses to herbivory have been widely documented and may involve changes in plant secondary chemistry, reductions in plant nutritional quality, emission of volatiles that attract predators and parasitoids of herbivores (Kessler and Baldwin, 2002), or increases in trichome density (Traw and Dawson, 2002). Responses to herbivores may be mediated by the jasmonic acid (JA) or salicylic acid (SA) signaling pathways (Kessler and Baldwin, 2002).

Trichomes occur on the surfaces of many plants and can make a contribution to plant resistance against herbivores (Simmons and Gurr, 2004). Trichomes play important roles in arthropod resistance within the plant family Solanaceae and particularly within the genus *Lycopersicon* (Kennedy, 2003). Seven types of trichomes occur on *Lycopersicon* spp., including glandular trichomes (types I, IV, VI, and VII) and nonglandular trichomes (types II, III, and V) (Luckwill, 1943). Glandular trichomes have heads containing various sticky and/or toxic exudates that may be secreted onto the plant surface or may rupture on contact with herbivores, causing irritation, entrapment, or death (Simmons and Gurr, 2004). Nonglandular trichomes do not have heads and affect herbivores by mechanically obstructing their movement across the plant surface (Simmons and Gurr, 2004). Four-lobed type VI glandular trichomes are associated with high levels of arthropod resistance in *L. esculentum* (Kennedy, 2003).

Recent studies of the tomato mutant *jasmonic acid insensitive* 1 [*jai*1], which is defective in JA-based signaling, revealed several defense-related phenotypes, including abnormal glandular trichome production, suggesting a role for JA in glandular trichome-based defenses (Li et al., 2004). JA was recently shown to induce trichomes in Arabidopsis (Traw and Bergelson, 2003).

To our knowledge, jasmonates have not previously been demonstrated to induce elevated trichome densities in tomato. In preliminary studies, we found applications of methyl jasmonate (MJ) and BTH (Actigard™) to tomato reduced populations of *Myzus persicae* (Sulzer) (Homoptera: Aphididae) in the greenhouse (Boughton et al., unpublished data). Here, we present evidence showing that exogenous application of methyl jasmonate, but not BTH, induced dramatic increases in densities of type VI glandular trichomes on new leaves of *L. esculentum*. We suggest that resistance to herbivores induced by these elicitors probably operate by different mechanisms.

METHODS AND MATERIALS

Plants. Tomato plants (*L. esculentum* c.v. Trust) (DeRuiter Seeds) were grown in 4-in. plastic pots in sterile soil mix (peat–perlite–vermiculite, 55–20–25; Penn State Seed, Dallas, PA, USA). Plants were grown in the greenhouse under natural lighting with day and night temperatures varying between 21 and 33°C. Plants were irrigated daily with fertilizer solution (N–P–K, 4–18–38, Chem-Gro Tomato Formula; Hydro-Gardens Inc., Colorado Springs, CO, USA) containing supplemental magnesium sulphate and calcium nitrate.

Effect of Elicitors on Induction of Glandular Trichomes. Fourth-leaf tomato plants (45 per treatment) selected at random were treated with (1) 7.5 mM MJ (Bedoukian Research, Danbury, CT, USA), or (2) 0.1 mM BTH (Actigard®-Syngenta, Greensboro, NC, USA) (both in 0.8% ethanol and water), or (3) control solution (0.8% ethanol and water). Elicitor concentrations were within the range used in other studies and were selected in dose response trials as the lowest concentrations that induced resistance. Plants receiving different treatments were moved to opposite sides of the greenhouse and sprayed until leaves were saturated. After 24 hr, plants were arranged on three benches according to a randomized block design. At 3, 7, 14, 21, and 28 days post-treatment (DPT), three plants from each treatment were selected at random on each of the three benches, and the youngest terminal leaflet at least 5 cm in length on each plant was removed for sampling. This leaf selection process yielded terminal leaflets of similar sizes, which ranged from 5.8 to 8.0 cm in length, and 11.7 to 21.8 cm^2 in area. Two leaf disks (0.6 cm diam.) were punched midway between the leaf tip and leaf base, one on either side of the midrib, taking care to preserve trichomes on the upper leaf surface. Numbers of

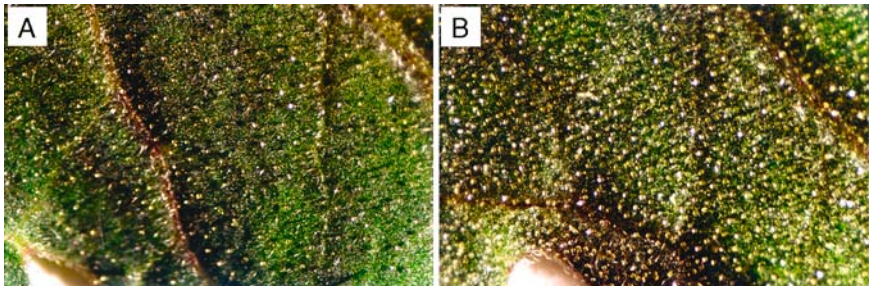

FIG. 1. Type VI glandular trichomes on upper surface of leaves from (A) control or (B) MJ-treated tomato plant at 12 DPT. Photographs show same field of view at 15× magnification.

TABLE 1. EFFECT OF ELICITOR TREATMENT ON GLANDULAR TRICHOME DENSITY ON NEW TOMATO LEAVES

	Mean trichome density[a] (no./mm^2 ± SE)			
Time point[b] (DPT)	Control-treated	BTH-treated	MJ-treated	*P* value[c]
3	0.7 ± 0.1 a	0.8 ± 0.1 a	0.9 ± 0.1 a	0.105
7	1.1 ± 0.2 a	1.5 ± 0.3 a	9.4 ± 0.9 b	<0.001
14	2.1 ± 0.2 a	2.2 ± 0.4 a	17.9 ± 1.8 b	<0.001
21	3.1 ± 0.4 a	3.7 ± 0.4 a	12.5 ± 1.0 b	<0.001
28	5.3 ± 0.7 ab	4.7 ± 0.6 a	7.2 ± 0.5 b	0.027

MJ = Methyl jasmonate, BTH = Benzothiadiazole.

[a] Mean density of type VI glandular trichomes on upper surface of new leaves. $N = 9$ plants per treatment. One leaf examined per plant. Plants sampled only once. Densities calculated from counts of trichomes on two leaf disks per leaf. Means within the same row followed by different letters are significantly different by Tukey's-test ($P < 0.05$).

[b] Days post-treatment (DPT).

[c] One-way ANOVA evaluating effect of treatment on trichome density.

type VI trichomes were determined by using a dissecting scope. Preliminary observations had suggested MJ treatments increase type VI trichome density. Other trichome types were not examined in this study. Plants were sampled only once. Trichome density data were evaluated with one-way ANOVA.

RESULTS

Results showed that application of MJ to tomato plants induced increased densities of type VI glandular trichomes on new leaves expanding after treatment (Figure 1). BTH treatment had no effect on trichome densities (Table 1). Highest densities of trichomes were present on leaves produced 14 d after MJ treatment. At 7 and 14 DPT, trichome densities were ninefold higher on MJ- than control-treated plants. Trichome densities were significantly higher (Tukey's test, $P < 0.05$) on MJ plants than on BTH or control plants at 7, 14 and 21 DPT. MJ-treated plants were observed to entrap higher numbers of immature western flower thrips than BTH- or control-treated plants (Boughton et al., unpublished data).

DISCUSSION

Increasing interest has focused on the use of elicitors of natural plant defensive responses, such as JA and MJ, to induce resistance to herbivores and

plant pathogens (Thaler, 1999). In tomato, jasmonate-induced resistance to herbivores has been documented in several field studies and has frequently been attributed to the action of defensive proteins such as polyphenol oxidase and proteinase inhibitors (Thaler, 1999). These proteins are induced within 24 hr of elicitor treatment, before any changes in type VI trichome density are detectable. However, over subsequent weeks it seems likely that a component of jasmonate-induced resistance may be a result of increased densities of type VI glandular trichomes, contributing to elevated polyphenol oxidase levels and increased repellency, entrapment, or mortality of arthropod herbivores. We suggest that elevated trichome densities were likely responsible for increased thrips entrapment observed in our studies.

Although it is likely that jasmonate-induced resistance in crop plants will have direct negative impacts on arthropod pests, which may be beneficial from a pest management perspective, it is also possible that induced plant defenses may have indirect effects on natural enemies, such as insect parasitoids and predators, which contribute to the regulation of pest populations (Thaler, 1999; Simmons and Gurr, 2004). Trichomes, for example, may have differing impacts on natural enemies ranging from positive effects, such as increased searching efficiency, to negative effects, such as hindered movement, irritation by trichome exudates, and entrapment (Obrycki, 1986).

The use of jasmonates to induce plant resistance to arthropod pests as a management tactic will require careful evaluation to ensure that benefits arising from negative impacts on pest fitness are not outweighed by adverse effects that might reduce the efficacy of biological control.

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RAPID COMMUNICATION

PLANT–PLANT SIGNALING: ETHYLENE SYNERGIZES VOLATILE EMISSION IN *Zea mays* INDUCED BY EXPOSURE TO (*Z*)-3-HEXEN-1-OL

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Abstract—Leaf alcohol (*Z*)-3-hexen-1-ol (Z-3-ol) is emitted by green plants upon mechanical damage. Exposure of intact maize plants to Z-3-ol induces the emission of a volatile blend that is typically released after caterpillar feeding and attracts natural enemies of the herbivores [herbivore-induced volatile organic compounds (HI-VOC)]. Thus, it has been suggested that Z-3-ol might have a function in indirect plant defense mediating plant–plant signaling and intraplant information transfer. Here, we demonstrate that HI-VOC induction by Z-3-ol is synergized by the phytohormone ethylene. Exposure to Z-3-ol at doses of 100 and 250 nmol induced HI-VOC emission in intact maize plants. HI-VOC emissions increased by 2.5-fold when ethylene was added. The effect of ethylene was more pronounced (5.1- to 6.6-fold) when only total sesquiterpene release was considered. In contrast, ethylene alone had no inductive effect but rather decreased the emission of the constitutive maize volatile linalool. We suggest that ethylene plays a synergistic role in plant–plant signaling mediated by green leaf volatiles.

Key Words—Plant–plant signaling, ethylene, (*Z*)-3-hexen-1-ol, herbivore-induced volatile organic compounds, sesquiterpenes, *Zea mays*.

INTRODUCTION

Evidence is increasing that volatiles emitted from damaged plants can induce defense responses in intact neighbor plants. This phenomenon has been interpreted as a prophylactic reaction toward impending herbivore attack (Dicke and Bruin, 2001). Among the compounds discussed as mediators for this information transfer are volatile phytohormones such as methyl jasmonate, methyl salicylate, and ethylene (Farmer, 2001) and also green leaf volatiles (GLV)

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(Arimura et al., 2001), a series of six-carbon aldehydes, alcohols, and esters that are released from green plants after mechanical damage. (*Z*)-3-Hexen-1-ol (Z-3-ol), a major component of the GLV bouquet, is derived from linolenic acid by enzymatic degradation during the octadecanoid pathway (Hatanaka et al., 1995). Exposure of maize plants (*Zea mays* L.) to several different GLV [among them (*Z*)-3- and (*E*)-2-configured as well as saturated derivatives] induces the emission of a volatile blend consisting mainly of terpenoids, which is typically associated with caterpillar feeding [herbivore-induced volatile organic compounds (HI-VOC); Engelberth et al., 2004; Ruther and Fürstenau, unpublished data]. HI-VOC attract natural enemies of the caterpillars and have been shown to decrease oviposition rates of insect herbivores, thus representing both an indirect and a direct defense mechanism (Engelberth et al., 2004). Volicitin, *N*-(17-hydroxylinolenoyl)-L-glutamine, occurring in the caterpillars' oral secretion, and the phytohormone jasmonic acid (JA) are potent elicitors of HI-VOC when applied to artificially damaged maize leaves. Both HI-VOC induction by volicitin and JA can be synergized by ethylene (Schmelz et al., 2003). The present study demonstrates that ethylene also synergizes HI-VOC emission in maize plants exposed to Z-3-ol but has no volatile inducing effect on its own.

METHODS AND MATERIALS

Plant Material. Maize seeds (cv. Delprim) were planted individually in plastic pots (650 ml, 9 × 9 × 8 cm) filled with potting soil. Plants were grown in a green house at 20–25°C and 60% relative humidity. Supplemental artificial lighting was provided between 6 and 22 hr by SON-T Agro 400 high-pressure sodium vapor lamps (Royal-Phillips Electronics, Holland). After 3–4 wk, plants used in the experiments were approximately 40 cm high and had five to six fully developed leaves. The aboveground biomass of the tested plants was 14.4 ± 0.3 g (mean ± 1SE).

Plant Treatment and Volatile Collection. Plant treatment and volatile collection was performed in a glass cylinder (volume 8.8 l, 15-cm diam, 50 cm high) that was closed at the top and the bottom by Teflon plates (20-cm diam, 10 mm thick). The lower Teflon plate had a hole in the center (8-mm diam) and was split into two parts. By closing the two parts loosely around the stem of the maize plant, the intact aboveground part was placed into the glass cylinder whereas the pot was kept outside. For volatile collection, charcoal-purified air (1.2 l hr^{-1}) was pumped into the container via Teflon tube. The volatile-laden headspace was sucked at a flow rate of 1 l hr^{-1} through an adsorption tube (charcoal filter 5 mg, Gränicher and Quartero, Daumazan, France) connected to a mini vacuum pump (Neolab, Heidelberg, Germany) by another Teflon tube. Four identical cylinders were used simultaneously.

Plants were introduced into the container and exposed to the different chemicals: (1) 10 μl dichloromethane (solvent control), (2) 100 or 250 nmol of Z-3-ol dissolved in 10 μl dichloromethane, (3) 200 μl ethylene, (4) 100 or 250 nmol of Z-3-ol plus 200 μl ethylene. Solutions were applied onto the inner surface of the glass cylinder, and direct contact with the plants was avoided. GLV doses were in the range as released from maize seedlings after artificial or herbivore damage (Engelberth et al., 2004). Experiments were started between 2 and 3 P.M.; volatiles were collected for 8 hr after an incubation time of 14 hr. Trapped compounds were eluted from the filter with 20 μl of dichloromethane containing 50 ng μl^{-1} methyl nonanoate as an internal standard.

Chemical Analysis. Extracts (1 μl) were analyzed by GC-MS. Analyses were performed on a Fisons 8060 GC, and mass spectra were obtained on a Fisons MD800 quadrupole MS (EI mode at 70 eV). Volatiles were separated on a 30 m × 0.32 mm i.d. DB-5ms column, film thickness 0.25 μm (J&W Scientific, Folsom, CA, USA) with helium as carrier gas (2 ml/min). The temperature program was 40°C for 4 min, followed by a rate of 3°C min^{-1} to 280°C. Identification was performed by comparison of MS and retention data with authentic reference compounds or with those compiled in the essential oil library of Massfinder 3.12 scientific software (Detlef Hochmuth Consulting, Hamburg, Germany). Quantification (ng per g fresh weight) was performed by relating peak areas of individual compounds to the internal standard (50 ng μl^{-1}).

Statistical Analysis. Individual amounts, total sesquiterpenes, and total volatile amounts were analyzed by a nonparametric Kruskal–Wallis *H*-test followed by Mann–Whitney *U*-test for individual comparisons using Statistica 5.5 scientific software (StatSoft Inc., Tulsa, OK, USA). (*Z*)-3-Hexenyl acetate was not considered for estimation of total amounts because maize plants are able to transform externally applied Z-3-ol to (*Z*)-3-hexenyl acetate (Farag et al., 2005), and thus, transformed and induced amounts were not distinguishable.

RESULTS AND DISCUSSION

Intact control plants released only small amounts of volatiles (Figure 1; Table 1). Exposure to Z-3-ol at doses of 100 and 250 nmol resulted in a significant increase of HI-VOC emission. By exposure to combinations of Z-3-ol and ethylene, total HI-VOC emissions were increased by 2.5-fold when compared to Z-3-ol only. The effect of ethylene was more pronounced (5.1- to 6.6-fold) when only the total sesquiterpene release was considered. Exposure to ethylene alone did not induce any HI-VOC emission. Interestingly, the amounts of constitutively released volatiles (mainly linalool) dropped after just ethylene exposure.

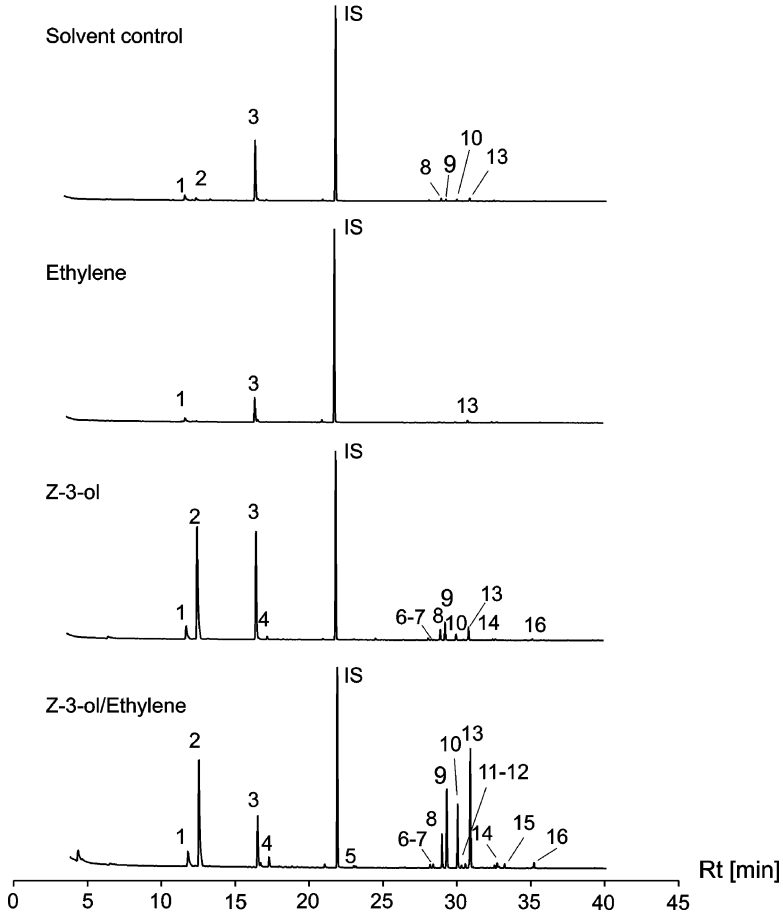

FIG. 1. Total ion current chromatograms of volatile extracts from maize plants (var. Delprim) exposed to 10 µl dichloromethane, 200 µl ethylene, 250 nmol Z-3-ol, and to 200 µl ethylene + 250 nmol Z-3-ol. Peak numbers correspond to Table 1; IS = internal standard.

Ethylene is a multifunctional signal molecule in plants also involved in various plant defense responses (Bleecker and Kende, 2000). Like Z-3-ol, it is released by plants upon mechanical damage (Tscharntke et al., 2001; Arimura et al., 2002). It has been suggested as mediator of plant–plant signaling because exposure of intact elder leaves to ethylene induced an increase of direct defense molecules such as phenols and proteinase inhibitors (Tscharntke et al., 2001). In maize, ethylene is selectively emitted upon insect feeding rather than from

TABLE 1. INDIVIDUAL AND TOTAL AMOUNTS OF VOLATILES ESTIMATED IN THE HEADSPACE OF DIFFERENTLY TREATED MAIZE PLANTS[a]

Compound	Control ($N = 9$)	Ethylene ($N = 6$)	Z-3-ol 100 nmol (−) Ethylene ($N = 11$)	Z-3-ol 100 nmol (+) Ethylene ($N = 11$)	Z-3-ol 250 nmol (−) Ethylene ($N = 12$)	Z-3-ol 250 nmol (+) Ethylene ($N = 8$)	Kruskal–Wallis ($df = 5$, $N = 57$)
1. β-Myrcene	3.2 ± 0.4a	2.3 ± 1.0a	5.9 ± 0.9b	5.4 ± 0.6b	5.0 ± 0.5b	5.4 ± 0.6b	$H = 22.09$, $P < 0.001$
2. (*Z*)-3-Hexenyl acetate	0.3 ± 0.1a	0.1 ± 0.0a	4.3 ± 1.6b	3.9 ± 1.0b	28.5 ± 11.3c	40.2 ± 8.5c	$H = 40.27$, $P < 0.001$
3. Linalool	20.7 ± 4.8bc	8.9 ± 3.6a	21.2 ± 2.1c	13.8 ± 2.3ab	36.6 ± 3.2d	12.9 ± 1.8ab	$H = 31.69$, $P < 0.001$
4. (3*E*)-4,8-Dimethyl-1,3,7-nonatriene	0.7 ± 0.2a	0.2 ± 0.1a	4.2 ± 1.0b	8.7 ± 2.1c	2.9 ± 0.5b	9.7 ± 2.7c	$H = 34.99$, $P < 0.001$
5. 2-Phenylethyl acetate	0a	0a	0a	0.1 ± 0.0a	0.3 ± 0.1b	0.8 ± 0.2b	$H = 41.26$, $P < 0.001$
6. Geranyl acetate	0.2 ± 0.1a	0.1 ± 0.1a	0.2 ± 0.0a	0.4 ± 0.1ab	0.6 ± 0.1bc	0.7 ± 0.1c	$H = 27.51$, $P < 0.001$
7. 7-Epi-sesquithujene	0.1 ± 0.0a	0.1 ± 0.0a	0.3 ± 0.1b	1.1 ± 0.2cd	0.7 ± 0.1c	1.8 ± 0.4d	$H = 40.63$, $P < 0.001$
8. Sesquithujene	1.6 ± 0.8a	1.0 ± 0.4a	1.2 ± 0.3a	12.5 ± 3.5bc	7.2 ± 2.0b	15.8 ± 4.8c	$H = 33.21$, $P < 0.001$
9. β-Caryophyllene	0.9 ± 0.2a	0.6 ± 0.3a	5.3 ± 0.8b	26.0 ± 6.2c	13.9 ± 3.6b	46.0 ± 9.4d	$H = 44.83$, $P < 0.001$
10. (*E*)-α-Bergamotene	0.7 ± 0.3a	0.3 ± 0.1a	1.5 ± 0.4b	9.7 ± 2.6d	4.4 ± 1.6c	42.9 ± 12.6e	$H = 42.63$, $P < 0.001$
11. Sesquisabinene	0a	0a	0a	0.4 ± 0.1b	0.2 ± 0.1b	1.8 ± 0.6c	$H = 41.55$, $P < 0.001$
12. α-Humulene	0a	0a	0.4 ± 0.1b	1.3 ± 0.3cd	0.7 ± 0.2bc	2.4 ± 0.5d	$H = 42.73$, $P < 0.001$
13. (*E*)-β-Farnesene	2.0 ± 1.4ab	0.5 ± 0.2a	2.0 ± 0.5b	19.3 ± 5.6d	8.5 ± 3.3c	71.3 ± 18.7e	$H = 40.24$, $P < 0.001$
14. β-Bisabolene	0.1 ± 0.1a	0.1 ± 0.0a	0.2 ± 0.0b	2.0 ± 0.7cd	1.1 ± 0.3c	3.1 ± 1.1d	$H = 33.62$, $P < 0.001$
15. β-Sesquiphellandrene	0a	0a	0.1 ± 0.0b	0.8 ± 0.2c	0.4 ± 0.1b	3.4 ± 0.9d	$H = 42.18$, $P < 0.001$
16. (3*E*,7*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene	0.9 ± 0.8a	0.4 ± 0.1a	0.9 ± 0.3ab	3.5 ± 1.1c	1.6 ± 0.5bc	3.6 ± 1.1c	$H = 21.14$, $P < 0.001$
Total sesquiterpenes	5.6 ± 2.9a	2.7 ± 1.1a	11.0 ± 1.9b	73.0 ± 17.8c	37.0 ± 11.3c	188.5 ± 59.d	$H = 43.99$, $P < 0.001$
Total volatiles[b]	31.2 ± 8.5b	14.7 ± 6.0a	43.6 ± 3.9c	105.2 ± 22.2d	84.1 ± 9.6d	222.6 ± 50.0e	$H = 39.03$, $P < 0.001$

[a]Values (ng per g fresh weight) represent means ± 1SE. Different lowercase letters indicate significant differences at $P < 0.05$ within each line (Mann–Whitney *U*-test).
[b]Without (*Z*)-3-hexenyl acetate.

artificial damage, and it has been shown to be involved together with volicitin and jasmonic acid in HI-VOC emission (Schmelz et al., 2003). The present study demonstrates that ethylene synergistically increases the responsiveness of maize to Z-3-ol. We suggest that ethylene plays an important role in plant–plant signaling mediated by GLV by synergizing HI-VOC emission in neighboring plants. The active ethylene might also be produced by the receiver plant itself because exposure to GLV and other HI-VOC has been shown to induce genes related to ethylene biosynthesis in intact lima bean plants (Arimura et al., 2002). However, GLV and ethylene might not only mediate plant–plant signaling but also be involved in the information transfer between damaged and intact parts of the same plant, thus enabling a fast systemic defense response.

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RAPID COMMUNICATION

ATTRACTIVE PROPERTIES OF AN ISOFLAVONOID FOUND IN WHITE CLOVER ROOT NODULES ON THE CLOVER ROOT WEEVIL

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Abstract—The clover root weevil, *Sitona lepidus*, frequently feeds on N_2 fixing rhizobial root nodules of white clover (*Trifolium repens*), which may contain isoflavonoids with defensive and plant regulatory properties. This study investigated the isoflavonoids present in N_2 fixing (active) root nodules, root nodules that were not fixing N_2 (inactive), and roots without nodules, and tested the behavioral responses of neonatal *S. lepidus* larvae to aglycones of the identified compounds. Formononetin concentrations were higher in the active nodules compared with inactive nodules and roots alone. Moreover, there was a statistically significant attraction to formononetin by *S. lepidus* in arena experiments, whereas the other isoflavonoids were unattractive. It is suggested that *S. lepidus* may have become tolerant to the toxic effects of formononetin with repeated exposure, and that it may play a role in root nodule location. Such coevolutionary relationships are widely reported for aboveground insects and plants, but the present study suggests they may also occur belowground.

Key Words—7,4′ Dihydroxyflavone (DHF), chemotaxis, flavonoids, formononetin, genistein, semiochemical, *Sitona lepidus*, *Trifolium repens*.

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INTRODUCTION

The clover root weevil *Sitona lepidus* Gyllenhal (Coleoptera: Curculionidae) is a destructive pest of white clover (*Trifolium repens* L.), attacking the plant aboveground as adults and belowground as soil-dwelling larvae. The adults lay eggs aboveground that ultimately fall to the soil surface, giving rise to soil-dwelling larvae that attack root nodules as neonates, before feeding on progressively larger roots. We recently reported that the neonatal larvae could distinguish white clover roots from nonhost plant roots in the soil from up to 6 cm (Johnson et al., 2004). Hackell and Gerard (2004) demonstrated that neonatal larvae showed a strong preference for root nodules that were fixing N_2, compared with those that were not, suggesting that compounds associated with N_2 fixation may be phagostimulatory or even enable larvae to locate nodules. While not obligate feeders on such root nodules, survival and development is drastically reduced when neonatal *S. lepidus* larvae do not feed on root nodules (Gerard, 2001). The larvae possibly benefit from the high concentrations of amino acids in root nodules.

Isoflavonoids are a particularly important group of chemicals in white clover, underpinning many microbial symbiotic relationships. Several play important roles during root nodule organogenesis and subsequent N_2 fixation. For instance, 7,4′-dihydroxflavone (DHF) accumulates in nodule progenitor cells where it inhibits auxin breakdown during cell division. After several rounds of cell division, DHF levels decrease as formononetin levels increase (Mathesius, 2001). This switch is associated with auxin breakdown, caused by the stimulation of auxin catabolites [e.g., indole-acetic acid (IAA) oxidase/peroxidase] by formononetin (Mathesius, 2001). Formononetin levels are, therefore, likely to accumulate in nodules that are fixing N_2 because these nodules are not actively undergoing cell division.

It has been suggested that formononetin also has a defensive role in roots (e.g., Cook et al., 1995), so it is perhaps paradoxical that *S. lepidus* larvae should target active root nodules of white clover, because these are likely to have high levels of formononetin. The effects of isoflavonoids can be variable, however, depending on the species of plant and insect, with some insects becoming tolerant or even capable of sequestering isoflavonoids with repeated exposure (Simmonds, 2003). The efficacy of formononetin as a defensive compound against root-feeding insects, in particular, is equivocal because neither the grass grub (*Costelytra zealandica* White) nor the African black beetle (*Heteronychus arator* F.) was negatively affected by formononetin (Sutherland et al., 1980). *Heteronychus arator* was highly susceptible to the other isoflavonoids tested, so formononetin may be relatively benign (Sutherland et al., 1980).

The objectives of this study were to identify and quantify the isoflavonoids present in white clover roots and root nodules and to test the behavioral responses of neonatal *S. lepidus* larvae to them.

METHODS AND MATERIALS

Plant Growth, Inoculation, and Chemical Analysis. Ten white clover plants (cv. Gwenda) were grown from seed in acid washed sand and supplied with 25 ml of Arnon's nutrient solution per day (see Johnson et al., 2004), modified to encourage nodulation. The plants were grown at 16L:8D at 15°C, and each treated with 2.5 ml of *Rhizobium* inoculate (ca. 0.1 ml of *Rhizobium* culture) on d 14. To make the inoculate, *Rhizobium* bacteria were isolated from nodules taken from field grown *T. repens*. Nodules were surface sterilized and crushed in a Petri dish under aseptic conditions, streaked onto manitol yeast extract agar plates, and incubated at 26°C for 2 d. Purity of cultures was verified by Gram strain and restreaking. After 36 d, two plants were randomly selected and sacrificed to verify infection and N_2 fixation by examining nodules for pink coloration. The remaining plants were harvested after 42 d. The roots were cleaned of sand and all root nodules removed and characterized as active (N_2 fixing) or inactive (not fixing N_2). Inactive nodules had either not yet started to fix N_2, or had become dormant. Roots and root nodules were weighed and immediately frozen (−18°C) pending chemical analysis.

Defrosted material (ca. 100 mg) was extracted with hot methanol (MeOH) (HPLC grade) at 20°C for 24 hr. Plant material was washed with MeOH and extracts concentrated to dryness by rotary evaporation at 40°C after being centrifuged at 13,000 rpm for 3 min. The residue was dissolved in 0.5 ml 100% HPLC MeOH and diluted as necessary for HPLC analysis by linear gradient elution on a Bondapak phenyl C_{18} column (4 × 30 mm; packing particle size, 5 μm) fitted with a Hichrom Hypersil phenyl guard cartridge. Elution was with a 65:35 mixture of 2% aqueous acetic acid (HOAc) and MeOH/HOAc/distilled H_2O (18:1:1) changing to 100% of the second solvent at 20 min. The flow rate was 1 ml min^{-1}, temperature 25°C, and UV detection at λ 270 nm for formononetin and λ 331 nm for DHF and the respective glycosides. Quantification was done by peak height against the following concentrations of authentic markers: DHF, 0.0153 mg ml^{-1}; formononetin, 0.0175 mg ml^{-1}; and formononetin 7-glucoside, 0.039 mg ml^{-1}. The DHF marker was used for the quantification of the respective glycoside, as this was deemed adequate for comparative purposes.

Bioassays. A captive population of adult *S. lepidus* was maintained at 20°C, 85% humidity and 16L:8D. Eggs were collected, placed on dampened filter paper inside sealed Petri dishes, and incubated at 25°C until larvae emerged for bioassays.

DHF, formononetin and genistein (supplied by PlantechUK, Reading, UK) were tested to determine whether they elicited a behavioral response from neonatal *S. lepidus* larvae at 0.1 and 0.01 M. The aglycones of the isoflavonoids were tested as the conjugates identified in the chemical analysis were found in

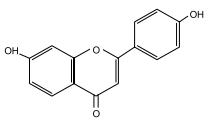
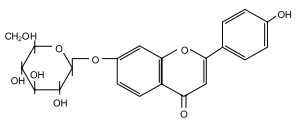
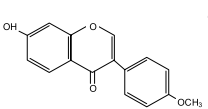
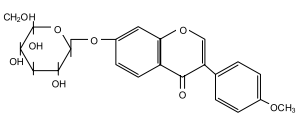
much lower concentrations. While not detected in the Gwenda cultivar, genistein is among the more common isoflavonoids found in white clover, and it has been suggested that it has functional similarities with formononetin in terms of auxin breakdown (Mathesius, 2001). Bioassays were conducted in circular arenas (90 mm diam) lined with moistened (1.5 ml distilled H_2O) filter papers inside a darkened chamber (20°C ± 1°C). Stock solutions of isoflavonoids were made by dissolving in 1 ml ethanol and diluting with 9 ml of distilled H_2O. For each bioassay, 250 μl of the compound were injected onto the filter paper at one side of the arena and a single neonatal larva was placed in the center. The control consisted of 250 μl of a solution of ethanol and distilled H_2O in a 10:90 ratio, respectively. Larvae were tracked using a specialized digital camera (Pro-150ES, Pixera Corp., Los Gatos, CA, USA) above the arena that relayed images at 10-sec intervals for 15 min to an image analysis unit incorporating a tracking algorithm (Image-Pro Plus™, Media Cybernetics Inc., Silver Spring, MD, USA.). Thirty replicates were conducted for each treatment using fresh filter paper, larvae, and test compounds (and control) on each occasion.

RESULTS AND DISCUSSION

Four isoflavonoids were detected in significant quantities in the roots and root nodules of white clover; DHF, DHF glycoside, formononetin, and formononetin 7-glucoside (Table 1). DHF and DHF glycoside concentrations were higher in the roots than the root nodules, whereas formononetin concentrations were higher in active nodules than the roots alone and inactive nodules. Formononetin 7-glucoside was not detected in active nodules and occurred in lower concentrations in the inactive nodules compared with the roots alone (Table 1).

When the test areas in the arenas included 0.1 and 0.01 M formononetin, there was a significant preference by *S. lepidus* larvae for this region. Larval attraction did not differ significantly from the control when the test area included DHF and genistein (Figure 1). These findings differ from studies that have correlated higher formononetin levels in white clover roots with reduced susceptibility to pests (e.g., Cook et al., 1995). Indeed, when *S. lepidus* larvae fed on red clover roots containing formononetin, they gained less weight than those reared on a cultivar of white clover roots containing negligible levels of formononetin. There was, however, no difference in the amount of root tissue consumed for larvae reared on the two types of plant, and there was also no relationship between larval weights and the levels of formononetin in red clover (Gerard, personal communication). It is conceivable that with repeated exposure

TABLE 1. ISOFLAVONOID CONCENTRATIONS IN DIFFERENT COMPONENTS OF WHITE CLOVER ROOTS

	Compound concentration (μg g^{-1} fresh mass)			
	7,4′-Dihydroxyflavone (DHF)	7,4′-Dihydroxyflavone glycoside	Formononetin[1]	Formononetin 7-glucoside
Component of root system				
Active nodules	65.27 ± 5.40[a]	1.41 ± 0.46[a]	983.92 ± 100.37[a]	0
Inactive nodules[2]	88.29 ± 9.52[a]	3.74 ± 1.41[a]	38.28 ± 5.71[b]	3.59 ± 1.53[a]
Roots without nodules	168.66 ± 15.20[b]	8.99 ± 0.88[b]	111.38 ± 7.83[c]	18.70 ± 3.99[b]
	$F_{2,13} = 20.34$, $P < 0.001$	$F_{2,13} = 28.40$, $P < 0.001$	$F_{2,13} = 31.16$, $P < 0.001$	$F_{1,6} = 10.32$, $P = 0.02$

Differences between root system components analyzed using ANOVA tests (plant number treated as a random factor) with Tukey's comparisons between component pairs. Lowercase letters in superscript indicate significant differences between root components.

[1] Bounded transformation of data using Johnson manipulation as they did not conform to normality.

[2] Seven replicates due to loss during analysis.

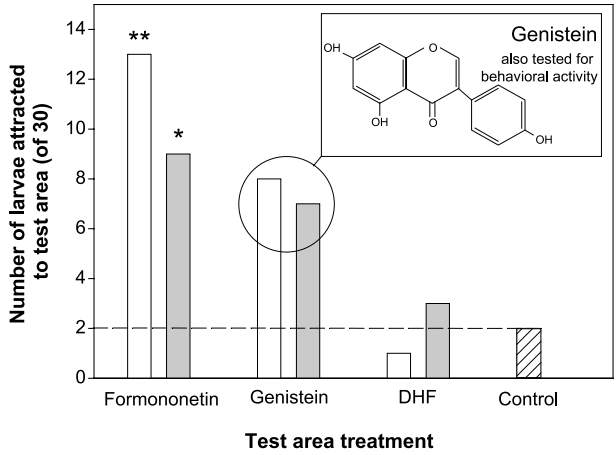


FIG. 1. Number of larvae attracted to the test areas of the arena in which isoflavonoids (0.1 M □, 0.01 M ■) or the control were introduced. Larval response compared to control using Fisher's exact *P* test: *formononetin* 0.1 M $P < 0.01$, 0.01 M $P = 0.04$; *genistein* 0.1 M $P = 0.08$, 0.01 M $P = 0.15$; *DHF* 0.1 M $P = 0.99$, 0.01 M $P = 0.99$. Statistical significance: $^{**}P < 0.01$, $^{*}P < 0.05$. Line indicates larvae moving to test area by chance alone (control).

to formononetin in N_2-fixing root nodules, neonatal *S. lepidus* larvae have become tolerant of its effects, as has been reported for other insects feeding on legume roots (Sutherland et al., 1980). Such a coevolutionary relationship between root defensive compounds and root-feeding insects has been reported elsewhere. For example, carrot root fly larvae (*Psila rosae* F.) use a neurotoxin (falcarindiol) exuded by the roots as a host plant location cue (Maki et al., 1989).

While not statistically significant, it is interesting that a number of larvae were attracted to 0.1 M genistein ($P = 0.08$), which has a similar chemical structure to formononetin (Figure 1). Chemical analogs frequently elicit similar behavioral responses in insect–plant interactions because of the compatibility of molecular binding sites on the insect's sensory organs. Whether formononetin acts as a distinct location cue for neonatal *S. lepidus* remains unclear, but its attractive properties suggest that it may play a role in nodule location when larvae are in close proximity to roots because formononetin is exuded into the immediate rhizosphere (e.g., Mathesius, 2001).

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HERBIVORES, VASCULAR PATHWAYS, AND SYSTEMIC INDUCTION: FACTS AND ARTIFACTS

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Abstract—Over the past 10 years there has been tremendous growth in our understanding of molecular, chemical, and morphological induction of traits involved in the resistance of plants to herbivores. Although it is well established that the patterns of induction can be constrained by a plant's vascular architecture, studies often fail to account for these constraints. Failure to do so has the potential to severely underestimate both the patterns and extent of induction. Here I review (1) the evidence for vascular control of induced responses, (2) how interspecific variation in phyllotaxy influences spatial patterning of induction, (3) the factors, phloem transport and volatile production, that may break down vascular constraints and lead to more widespread induction, and (4) the experimental approaches that could be compromised when vascular architecture is not considered. I show that vascular constraints in systemic induction are commonplace, but vary among species. I suggest that when induction is more widespread than expected from patterns of phyllotaxy, differences in vascular connectivity and volatile production may be responsible. I argue that advances in the mechanisms of systemic induction, cross-talk between different signal transduction pathways, specificity of induction, costs and benefits of systemic induction, and the effects of induced changes on herbivores and their natural enemies require that experiments be designed to examine and/or control for vascular constraints in systemic induction.

Key Words—Systemic induction, vascular architecture, sectoriality, long-distance transport, source–sink dynamics, volatiles, experimental design.

INTRODUCTION

It is widely recognized that constitutive defenses limit the scale of the initial herbivore attack, whereas induced defenses determine patterns of subsequent

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attack (Karban and Myers, 1989; Haukioja, 1990; Jones et al., 1993; Karban and Baldwin, 1997). Induction has been documented in over 100 species of plants (Agrawal and Karban, 1999) and can be morphological, chemical, local, systemic, and highly specific (see Karban and Baldwin, 1997). For the purposes of this review, I define systemic induction as molecular, chemical, or morphological changes that occur in distant undamaged leaves. Recent advances have improved our understanding of plant responses to attack and the effects of these responses to herbivores. We now know that (1) damage results in long-distance transport of signal molecules that elicit changes in distant leaves (Zhang and Baldwin, 1997; Stratmann, 2003); (2) mechanical damage and damage by specific herbivores (and pathogens) generally elicit unique molecular, biochemical, and morphological responses (e.g., Baldwin, 1988; Thomma et al., 1998; Agrawal, 2000; Schittko et al., 2000; Walling, 2000; Voelckel and Baldwin, 2004); (3) induced responses can selectively affect the performance of herbivores and the behavior of natural enemies (Thaler, 1999a,b; Thaler et al., 1999, 2002; Agrawal, 2000; De Moraes et al., 2001; van Poecke et al., 2001); (4) there can be reproductive costs of induction (Baldwin et al., 1990, 1998; Agrawal and Karban, 1999; Heil and Baldwin, 2002; Zavala and Baldwin, 2004; Zavala et al., 2004); and (5) ontogeny sink and strength modify both the strength and pattern of induction (e.g., van Dam et al., 2001; Arnold and Schultz, 2002; Arnold et al., 2004).

We have made excellent progress in our understanding of the mechanisms, costs, and consequences of local and systemic induction. Unfortunately, the importance of spatial variation in systemic induction is often ignored but is critical to future progress. In this review, I focus on herbivore-induced induction within plants and argue that (1) vascular architecture, the pathway of signal movement to and away from the damaged leaf, is a key controller of systemic induction patterns; (2) when induction in some species is more widespread than expected, differences in phloem transport, release, and retrieval along the phloem pathway, and volatile production may allow for greater integration of the induced response; and (3) experimental designs that fail to account for spatial variation in induction are likely to underestimate or even fail to detect systemic induction. The points made apply to signal transduction following pathogen attack provided that the pathogen does not move systemically itself.

VASCULAR ARCHITECTURE

In this section, I first describe how vascular architecture determines patterns of systemic induction and then review inter- and intraspecific factors that control patterns of vascular architecture.

Vascular Architecture and Long-Distance Transport. Botanists have known for years that the transport of photosynthate, signal molecules, and hormones among leaves is controlled by vascular architecture (reviewed by Murray et al., 1982; Watson and Casper, 1984; Watson, 1986; Dickson and Isebrands, 1991; Sachs et al., 1993; Marshall, 1996). There is evidence for both phloem and xylem transport of signal molecules associated with chemical induction (Malone and Alarcon, 1995; Stratmann, 2003).

From a phloem perspective, the anatomy of sieve cells has evolved so that the path of least resistance is longitudinal, through the sieve plates (van Bel et al., 2002), and there is little symplastic transport between mature cells, and this limits movement among sieve cells (reviewed by Thorpe et al., 2005). The bulk of carbohydrate and signal transport is both longitudinal and toward sink leaves, but modified by vascular connectivity (Watson and Casper, 1984; Dickson and Isebrands, 1991; Preston, 1998; Arnold and Schultz, 2002). Connectivity is based on orthostichy—the phyllotactic arrangement of leaves that describes the distribution of vascular traces. Export from one leaf is greatest to orthostichous leaves because these leaves share primary vascular traces. Export to leaves in adjacent orthostichies is intermediate because these leaves have partial connectivity, and export is absent to leaves in opposite orthostichies because they lack vascular connectivity (Murray et al., 1982; Watson and Casper, 1984). There may be xylem transport of signal molecules as well (Malone and Alarcon, 1995). These authors suggest that upon leaf tissue damage, signal molecules can be drawn into the xylem and transported systemically. Since leaf-to-leaf connectivity is similar for xylem and phloem (see Orians et al., 2000; Zwieniecki et al., 2003; and reviewed by Orians et al., 2005), vascular constraints on signal transport are expected by either mechanism.

Whichever pathway of transport, xylem or phloem, within-plant variation in systemic induction is commonplace (Davis et al., 1991; Shulaev et al., 1995; Rhodes et al., 1999; Orians et al., 2000; Schittko and Baldwin, 2003). In cottonwood (*Populus trichocarpa* × *Populus deltoides*, Davis et al., 1991), tobacco (*Nicotiana* sp., Shulaev et al., 1995; Schittko and Baldwin, 2003), and tomato (*Lycopersicon esculentum*, Rhodes et al., 1999), leaves with direct vascular connections to the damaged leaf show greater defensive chemical induction than leaves without vascular connections. Systemic induction in tomato not only exhibits leaf-to-leaf variation, but within-leaf variation as well (Orians et al., 2000). Orians et al. (2000) found that proteinase inhibitor induction in tomato was greatest in leaflets that have direct vascular connections and lower in leaflets without direct connections. That tomato leaflets without direct connections exhibited induction suggest that signal transduction is not completely isolated to specific sectors (see Beyond Vascular Architecture for discussion of possible mechanisms).

Such chemical changes are ecologically relevant. Jones et al. (1993) demonstrated that differential induction alters leaf beetle performance on cottonwood (*Populus deltoides*). More recently, Viswanathan and Thaler (2004) found induced resistance in orthostichous leaves, but induced susceptibility in non-orthostichous leaves of *Solanum dulcamara* when damage was intense. This suggests that induction changes source–sink relationships (*sensu* Arnold and Schultz, 2002) and may result in greater allocation of resources toward resistance traits in the damaged sector and lowered allocation of resources toward resistance traits in other sectors.

Inter- and Intraspecific Variation in Phyllotaxy. Differences in vascular architecture among plants can alter patterns of systemic induction. Most angiosperms have a 2/5 phyllotaxy (Moore et al., 1998). A 2/5 phyllotaxy reflects the fact that every fifth leaf is vertically aligned after two spirals around the plant. In this arrangement, carbon exported from leaf 1 accumulates most in leaves 4 and 6, to a lesser extent in leaf 3, and minimally in leaves 2 and 5 (Dickson, 1991). However, some species have a 1/2 or 3/8 phyllotaxy (Moore et al., 1998). Moreover, since phyllotaxy may change through development—the phyllotactic arrangement of leaves in *Populus* changes from 2/5 to 3/8 to 5/13 with plant development (Larson, 1977)—it is essential to map the phyllotaxy with dyes (Orians et al., 2000) or isotopes (Larson, 1977) to determine the operative vascular connectivity.

BEYOND VASCULAR ARCHITECTURE

Vascular constraints on systemic induction are common; however, there is increasing evidence that induction in some species is less constrained (i.e., more integrated) (Shea and Watson, 1989; Mutikainen et al., 1996). Shea and Watson (1989) found that localized damage to fireweed, *Chamaenerion angustifolium*, despite having a 3/8 phyllotaxy, had little effect on fruit production within that orthostichy. They also report that ^{14}C flow was less restricted. More recently, Orians et al. (2000) and Kiefer and Slusarenko (2003) showed that leaf tissues lacking direct vascular connections are induced in tomato and *Arabidopsis*, respectively. What might lead to induction in other sectors? I suggest that two factors may facilitate greater integration: (1) symplastic and apoplastic pathways in long-distance phloem transport and (2) volatile release at the site of damage and subsequent induction within distant plant tissues.

Throughout development, there is extensive apoplastic exchange—release and retrieval—of phloem constituents along the phloem pathway (Minchin and Thorpe, 1984,1987; van Bel et al., 2002; van Bel, 2003; Thorpe et al., 2005). Such release and retrieval requires an extensive array of specialized transfer

cells and membrane-bound transporters (Pate and Dieter Jeschke, 1995; Fisher, 2000). Most importantly, from the perspective of integration, species differ both in type and in density of transporters (van Bel, 2003). Clearly, transport of damage-induced signal molecules could lead to more widespread induction. Further work is necessary to examine how interspecific variation in apoplastic exchange alters the degree of symplastic isolation and subsequent spatial patterning of systemic induction.

It appears that signals are most likely to reach young developing tissues in other sectors. There is little symplastic exchange among mature sieve elements, but early in tissue development there can be extensive symplastic transport due to high sieve element and plasmodesmal density (van Bel, 2003; Thorpe et al., 2005). This allows for greater movement between adjacent sieve elements in young developing tissues, and modification of microchannel size of the plasmodesmata can facilitate even greater exchange of carbohydrates, viruses, and perhaps signal molecules (Lucas, 1997; Ishiwatari et al., 1998; Ruiz-Medrano et al., 1999). Although many details remain unresolved, recent evidence suggests that modification of microchannel size is a highly regulated and active process (Ruiz-Medrano et al., 2004). Thus, symplastic transport, especially in combination with apoplastic exchange, could result in widespread induction in young developing tissues. Whether plants have evolved mechanisms to facilitate movement of signal molecules from one sector to another requires further study.

I suggest that volatile release from damaged tissues may also result in more widespread induction. In fact, volatiles may be the only mechanism to induce leaves on distant branches where vascular connections are lacking. It is well known that volatiles released from damaged tissues and can cause molecular and chemical changes in adjacent plants, and alter subsequent resistance (Farmer, 2001; Kessler and Baldwin, 2001). The implications of this work are great because they suggest that plants may eavesdrop on their neighbors and adjust their allocation to defenses accordingly. However, it appears that eavesdropping requires that plants be in close proximity (within 15 cm) (Karban et al., 1997). Air turbulence and movement serves to dramatically reduce the concentration of volatiles arriving at distant plants, and as a consequence, there is considerable debate concerning the importance of interplant communication under field conditions (Lerdau, 2002; Dicke et al., 2003). Given that volatile signals become increasingly dilute with distance, volatiles may be more important for intraplant communication. Such tissues are in close proximity and likely to have the appropriate receptors to respond but may be on different branches with no vascular connections (Watson, 1986). Although intraplant volatile communication has been hypothesized to occur (Farmer, 2001), no studies have examined the importance of intraplant volatile communication as a mechanism to bypass vascular constraints in systemic induction.

EXPERIMENTAL APPROACHES

Despite the potential for more widespread induction than would be predicted by vascular architecture alone, the presence of vascular constraints is common. Therefore, failure to account for vascular constraints in systemic induction can: (1) underestimate the strength of induction; (2) lead to erroneous conclusions about specificity of induction; and (3) hide important within-plant heterogeneity in leaf quality. In this section, I review the approaches researchers have taken in their experiments and highlight which of the approaches are likely to underestimate the strength of induction and lead to inappropriate conclusions. It is not my goal to provide an exhaustive literature review, but to use a few case studies to highlight how knowledge of vascular architecture might improve our understanding of systemic induction and ultimately lead to more carefully designed experiments.

To date there have been three approaches to studying induction and each differs in the importance of vascular architecture to the results (Table 1). (1) Some studies have released herbivores on young plants and then, later in development, measured induction of specific traits or induced resistance (hereafter termed "immunization"; Karban, 1986; Agrawal et al., 1999). (2) Other studies have isolated herbivores on specific leaves (or manually damaged the leaf) and then measured induction in undamaged leaves shortly thereafter (hereafter termed "localized damage"; Davis et al., 1991; Jones et al., 1993; Stout et al., 1996a,b). (3) More recently, researchers have sprayed plants with powerful elicitors such as jasmonic acid and salicylic acid (Cipollini and Redman, 1999; Thaler, 1999a,b; Thaler et al., 1999; Moran and Thompson, 2001; Redman et al., 2001; Cipollini, 2002). Most of the elicitor studies applied inducing agents to the entire plant and then measured whole-plant induction (or if applied to a specific leaf, induction was only measured in that same leaf; Cipollini and Redman, 1999). Because most experiments involving elicitors have effectively measured local induction, vascular architecture should have no effect on the outcome of experiments and will not be discussed further.

Immunization studies that give herbivores free access to plants early in development and then measure systemic induction later in development appear robust. For example, Karban (1986, 1987) showed that when young cotton, *Gossypium hirsutum*, seedlings are attacked by mites, the plants often show increased resistance to subsequent mite attack. Agrawal et al. (1999) obtained similar results with cucumber, *Cucumus sativus*. In these studies, cotyledons/leaves were damaged and the whole plant was assayed for subsequent resistance. Since the initial damage was systemic, we would not expect induction to vary among leaves (Table 1).

Problems may arise when initial damaged is localized to specific leaves and induced responses are measured in other parts of the plant. Induction could

TABLE 1. TYPICAL TREATMENTS IMPOSED IN INDUCTION STUDIES, THE TYPES OF RESPONSES MEASURED, AND THE IMPORTANCE OF VASCULAR ARCHITECTURE TO PATTERNS OF SYSTEMIC INDUCTION

Treatment	Response	Importance of vascular architecture	Possible consequences[a]
(1) Immunization e.g., cotyledon inoculation followed by removal of damaging agent	Subsequent leaves, e.g., first true leaves	Minimal	None (if initial damage is dispersed)
(2) Localized damage to specific leaf or leaves	Whole plant	→ Very	Underestimate
	Specific tissue	→ Very	Underestimate or failure to detect
(3) Application of chemical elicitors to whole plant	Whole plant	Not	None
	Specific tissue		(functionally equivalent to localized induction)

[a] Consequences of failure to control for vascular architecture range from none, to underestimation, to failure to detect induction.

be underestimated if damage is concentrated on specific leaves, but induced responses are examined at the whole-plant level or might be severely underestimated in studies that only assay specific leaves for induced responses when the previous damage was concentrated on other leaves (Table 1). As illustration, Agrawal (2000) placed a caterpillar of one of four herbivore species (*Plutella xylostella*, *Spodoptera exigua*, *Pieris rapae*, or *Trichoplusia ni*) on one leaf and then measured how these herbivores performed on the remainder of the damaged plant. He found that previous damage by *Plutella* (a specialist) and *Spodoptera* (a generalist) resulted in increased resistance to all species. In contrast, previous damage by *Pieris* only induced resistance to *Spodoptera* and *Pieris*, and damage by *Trichoplusia* (a generalist) failed to induce resistance to the other herbivores. Although these results clearly indicated specificity of induction, some of the differences in herbivore responses may reflect differences in feeding behavior. Perhaps, *Plutella*—the species that appeared to be least affected by previous feeding—tended to avoid feeding on leaves with direct vascular connections to the damaged leaf and, thus, avoided the most heavily induced leaves. The later explanation is intriguing and deserves further examination.

More recently, Traw and Dawson (2002) released herbivores on four-leafed *Brassica nigra* and then quantified differences in foliar trichome density and sinigrin concentration on specific leaves between damaged and undamaged plants. They found evidence for trichome induction, but the effect was often insignificant and varied among plants. Perhaps differences in initial damage,

which they report, but did not control for in their analysis, limited their ability to detect differences. Because they did not quantify the pattern of initial damage, they could have detected (1) no induction—if damaged was concentrated on a single leaf that did not have direct connections to the sampled leaf; (2) moderate induction—if damage was dispersed; or (3) high induction—if damage was concentrated on the leaf with direct vascular connections to the sampled leaf.

An extreme example of uneven damage exists in studies in which damage is applied to one leaf and induction is measured in other leaves. Since some leaves lack direct vascular connections and others have only partial connections, induction might be underestimated. For example, Stout et al. (1996a) used different leaflets from a leaf adjacent to the damaged leaf to examine how different enzymes are induced following damage. Since some of the leaflets had direct connections whereas others did not, it is possible that differences in induction reflect the experimental design, and not differences in the enzymes. If true, failure to detect induction could be an artifact of the experimental design. It would be better to measure induction in leaflets or leaves known to share direct vascular connections. Alternatively, researchers could distribute damage across several consecutive leaves to make sure all sectors were damaged (as done by Constabel et al., 2000).

CONCLUSION

Whether studying molecular mechanisms of systemic induction, cross-talk between different signal transduction pathways, specificity of induction, costs and benefits of systemic induction, or the effects of induced changes on herbivores and their natural enemies, *a priori* knowledge of vascular constraints on systemic induction will help researchers make robust conclusions. Care should be taken to control for (or to examine) the effects of vascular architecture on induction. Controlling for vascular architecture necessitates that initial damage is dispersed across all sectors of the plant. Examining its effects requires mapping the vascular connections and quantifying the amount of damage to specific leaves. Since within-plant heterogeneity in leaf quality may affect herbivore behavior or performance (Denno and McClure, 1983) and may even influence foraging patterns of natural enemies, we can even use vascular architecture to create and study the effects of heterogeneity on herbivores (reviewed by Orians and Jones, 2001). As we learn more about the mechanisms of within-plant communication, both by long-distance signal transport of dissolved substances within the vascular system and by volatiles, we will gain an even greater understanding of the importance of vascular architecture in systemic induction across species.

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ONTOGENY AND ENVIRONMENT AS DETERMINANTS OF THE SECONDARY CHEMISTRY OF THREE SPECIES OF WHITE BIRCH

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Abstract—This study investigates variation in the secondary chemistry of the bark of three closely related, winter-dormant species of white birch (*Betula resinifera*, *B. pendula*, and *B. platyphylla*) at different ontogenetic stages by using different plant parts (top and base). The experimental birches were grown for 4 years in two growing conditions (pot and field) at different nutrient levels. There was considerable species-specific quantitative and qualitative variation in the secondary chemicals in bark, but this was also affected by fertilization and the age of the plant. In general, there was greater chemical diversity in saplings than in seedlings. The study revealed three new components, secoisolariciresinol 9-*O*-β-glucopyranoside and two of its derivatives, that have not been reported previously for the bark of white birches. Principal component analysis showed that the species studied had a similar chemical composition at the juvenile stage, but as the plants grew, they became more clearly differentiated, which indicates that the species of older plants can be identified by chemotaxonomy. Evidently, the secondary chemistry of birches is under genetic control, but it is affected by properties of growing conditions and ontogeny.

Key Words—*Betula pendula*, *B. resinifera*, *B. platyphylla*, birch, ontogeny, fertilization, phenolic compounds, terpenoids, mountain hare, field vole.

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INTRODUCTION

The concentrations of secondary chemicals in the bark of forest trees are genetically controlled (Rousi et al., 1989, 1993, 1996, 1997; Berenbaum and Zangerl, 1992; Laitinen et al., 2002b, 2004c, 2005), but there are indications that carbon-based secondary chemistry is also modified by environmental variation (Bryant et al., 1983; Waterman and Mole, 1989; Herms and Mattson, 1992; Rousi et al., 1996; Jones and Hartley, 1999; Herms 2002; Laitinen et al., 2002a, 2004c, 2005). Studies have revealed highly variable chemical responses to different growing conditions (e.g., Bryant et al., 1987; Muzika, 1993; Rousi et al., 1996; Haukioja et al., 1998; Mutikainen et al., 2000; Laitinen et al., 2002a). For example, levels of individual phenolics increase after fertilization (e.g., Muzika, 1993), although the total amounts of phenolics decrease (Bryant, 1987; Price et al., 1989; Muzika and Pregitzer, 1992; Muzika, 1993). Concentrations of terpenoids also respond variably to fertilization. Bryant et al. (1987) found reduced terpenoid production in fertilized plants, but Laitinen et al. (2005) found that different birch terpenoids showed different reactions to differences in site fertility. Concentrations of secondary metabolites may also depend on plant part (Palo et al., 1985; Tahvanainen et al., 1991) and ontogeny (e.g., Reichardt et al., 1984; Bryant and Julkunen-Tiitto, 1995; Julkunen-Tiitto et al., 1996; Hartley and Jones, 1997).

Previous studies of secondary chemistry variation in birch bark have generally used plants of the same age grown in the same environment (e.g., Tahvanainen et al., 1991; Vainiotalo et al., 1991), plants of the same age given different treatments (Lavola and Julkunen-Tiitto, 1994; Lavola et al., 1994), or plants of different ages grown under the same conditions (e.g., Julkunen-Tiitto et al., 1996). Our aim was to gain a more comprehensive understanding of the effect of environmental heterogeneity and ontogenetic changes on secondary chemistry of birch, by studying the chemical profiles of 1-, 3-, and 4-yr-old seedlings and saplings of three closely related species of white birch (*Betula resinifera*, *B. pendula*, and *B. platyphylla*) (de Jong, 1993) grown in different environments and given different fertilization treatments.

METHODS AND MATERIALS

Plant Material. We used exotic *B. resinifera* (North American origin), *B. platyphylla* (Japanese origin), and native *B. pendula* species grown at Punkaharju Forest Research Station (61°48′N, 29°20′E), Finland, for four growing seasons. After 1 year of growth in a greenhouse, seedlings were transferred to two outdoor growing sites: some were planted in 3 l pots (pot experiment), and the others were planted in a field (field experiment). Four

fertilization treatments were applied annually on each site for 4 yr. Pot seedlings were fertilized with liquid fertilizer (Superex, Kekkilä Yhtiöt, Eurajoki, Finland) for the first 3 yr, but in the fourth year, granular fertilizer was used, as in the field experiment (Kemira Y1) (Table 1). Pot seedlings were watered when necessary; field seedlings were not. A more detailed growth protocol is presented in Laitinen et al. (2004a).

Pot seedlings were divided into three replicate blocks, each containing 12 plots with 60 seedlings each (species × fertilization treatment). Field seedlings were in three replicate blocks, each divided into 11 plots, because one treatment (highest fertilization) was not available for *B. platyphylla* due to a lack of seedlings. Only healthy seedlings were transplanted to the field, so the number of seedlings/plot varied: most replicate plots contained 30–60 plants, but one replicate plot contained only 13 seedlings (replicate 3 for *B. pendula*, highest fertilization).

After the first and third years, three plants from each treatment (species/fertilization combination) in blocks 1 and 2 of the pot experiment and two plants from each treatment in block 3 were collected for measurement and chemical analysis. In the fourth year, eight saplings were taken randomly from each treatment in the pots and the field. Growth of the 1-yr-old seedlings and 3-yr-old saplings was measured. For the 4-yr-old saplings, previously published growth data were used (Laitinen et al., 2004a). Samples for chemical analyses were taken from the top of the plant (1st, 3rd, and 4th years), 1 cm below the terminal bud, and from the base (1st and 3rd years). The length of the top samples varied in different years. In the first year, it was only 10 cm because the total length of the seedlings was small. In the third year, samples were 15 cm long because there was sufficient material for longer samples. In the fourth year, the diameter of the saplings varied greatly, and to obtain samples with approximately the same biomass, the length of the samples had to vary between 5 and 10 cm. Base samples were 10 cm long. Resin droplets were classified visually into four categories: 0 = no resin droplets, 1 = some resin droplets, 2 = moderate number of resin droplets, and 3 = large number of resin droplets. Samples were stored in plastic bags at −20°C.

Extraction of Phenolics and Triterpenoids. Bark was separated from wood, and buds were removed. Each bark sample was cut into small pieces and methanol was added. The sample was left to stand on ice for 20 min and then homogenized in an Ultra-Turrax homogenizer for 3 min and filtered. The residue was extracted with methanol ×2 for pot samples and once for field samples. The sample was left on ice for 10 min the first time and 5 min the second time. Finally, to ensure extraction of triterpenoids, the residue was extracted once with diethyl ether, allowed to stand for 5 min on ice, and then homogenized for 3 min.

The residue in each of the extractions was washed with methanol. All extracts were filtered and combined, and the methanol and diethyl ether were

TABLE 1. FERTILIZATION SCHEDULE FOR *Betula resinifera, B. pendula* AND *B. platyphylla* SEEDLINGS AND SAPLINGS IN THE POT EXPERIMENT (A) AND IN THE FIELD EXPERIMENT (B) WITH DIFFERENT FERTILIZATION TREATMENTS

(a)

	Growing season							
	First		Second		Third		Fourth	
Fertilization	N g/seedling							
1	0.041		0.000		0.000		0.000	
2	0.056		0.392		0.388		0.355	
3	0.087		0.503		0.733		0.710	
4	0.118		0.733		1.484		1.421	
	Fertil. (Superex)	Time of fertilization	Fertil. (Superex)	Time of fertilization	Fertil. (Superex)	Time of fertilization	Fertil. (Superex)	Time of fertilization
	9	June 17–July 15	9	June 23	9	June 7 and 26	Kemira	June 12
	5	July 22–Aug 12	9	July 7 and 21	9	July 17	Y1	July 12
	7	Aug 19–Sept 9	9	Aug 4	9	Aug 7		

(b)

	Growing season		
		Third	
Fertilization treatment	Second	N kg/ha	Fourth
1	0	0	0
2	10	80	96
3	20	160	192
4	40	320	384

(1 = the lowest, 4 = the highest). N g/seedling (a) and N kg/ha (b) are also shown. Superex9 contained 19.4% N (7.2% NO_3–N), 5.3% P, 20.0% K, and 0.20% Mg; Superex5 contained 10.9% N (9.1% NO_3–N), 4.0% P, 25.3% K, and 1.5% Mg; Superex7 contained 0% N, 6.9% P, 31.9% K, and 1.0% Mg. Micronutrients (Fe, Mn, B, Zn, Cu, Mo, and Co) were included in all Superex fertilizers. Kemira Y1 contained 10.0% N (3.0% NO_3–N), 7.0% P, 14.0% K, and 2.0% Mg. In addition, Kemira Y1 included S, B, Cu, Fe, Mn, Mo, Zn, and Se. For more details, see Laitinen et al. (2004a).

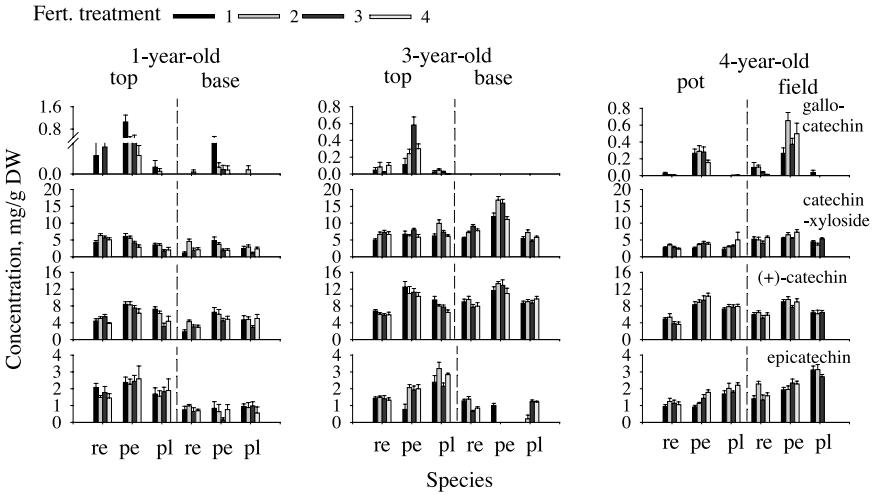

FIG. 1. Concentrations of catechin derivatives (mean ± SE, mg/g of DW = dry weight) in seedlings and saplings (*Betula resinifera* = re, *B. pendula* = pe, and *B. platyphylla* = pl) in top samples (at 1-, 3-, and 4-yr-old) and base samples (ages 1 and 3) in pot and field experiments with different fertilization treatments. The fertilization treatments are: 1 = lowest, 4 = highest. For N g/seedling and N kg/ha in each year, see Table 1.

vacuum-evaporated. Samples were redissolved in 10 ml of methanol, and aliquots of 2 × 1 and 1 × 2 ml were evaporated to dryness under nitrogen and stored at −20°C.

HPLC-DAD and HPLC-MS Analysis. Phenolics in the bark were analyzed by high-performance liquid chromatography diode array detection (HPLC-DAD) by using 1-ml samples dissolved in water–methanol (1:1). HPLC conditions were as described in Julkunen-Tiitto et al. (1996), except that the injection volume was 15 μl. Identification of compounds was based on their retention times and UV spectra monitored at 220 and 320 nm. The concentrations of individual compounds were calculated using reference factors of the following commercial standards: picein (Extrasynthese, Geney, France) for 3,4′-dihydroxypropiophenone-3-glucoside (DHPPG); acacetin (Roth, Karlsruhe, Germany) for acacetin derivatives; apigenin-7-glucoside (Roth) for apigenin derivatives; (+)-catechin (Aldrich, Steinheim, Germany) for catechin derivatives; chrysoeriol (Roth) for chrysoeriol; chlorogenic acid (caffeoylquinic acid) (Aldrich) for neochlorogenic acid, chlorogenic acid derivatives, and cinnamic acid derivatives; gallic acid (Aldrich) for ellagic acid derivative; quercetin-3-galactoside (Roth) for quercetin derivatives; kaempferol-3-*O*-glucoside (Extrasynthese) for kaempferol-3-glucose; luteolin (Roth) for luteolin

derivatives; myricetin-3-rhamnoside (Roth) for myricetin derivative; naringenin for naringenin; pentagalloylglucose (provided by Prof. Ann Hagerman, Miami University, Miami, OH, USA) for pentagalloylglucose; platyphylloside (provided by Dr. Kerstin Sunnerheim-Sjöberg, Uppsala) for platyphylloside; protocatechuic acid for protocatechuic acid derivatives; salidroside (provided by Prof. H. Thieme, Berlin) for salidroside, lignan 1 (secoisolariciresinol 9-*O*-β-glucopyranoside), lignan 2 (lignan derivative 2), lignan 3 (lignan derivative 3) (Strack et al., 1989), and rhododendrin derivatives; and triandrin (Prof. H. Thieve, Berlin) for triandrin. In a few samples, some compounds were detected in only small amounts, so (+)-catechin is the sum of (+)-catechin and B3 (tentatively) (Figure 1); the quercetin derivative 1 is the sum of five different quercetin derivatives (Figure 2); the compound group of cinnamic acid

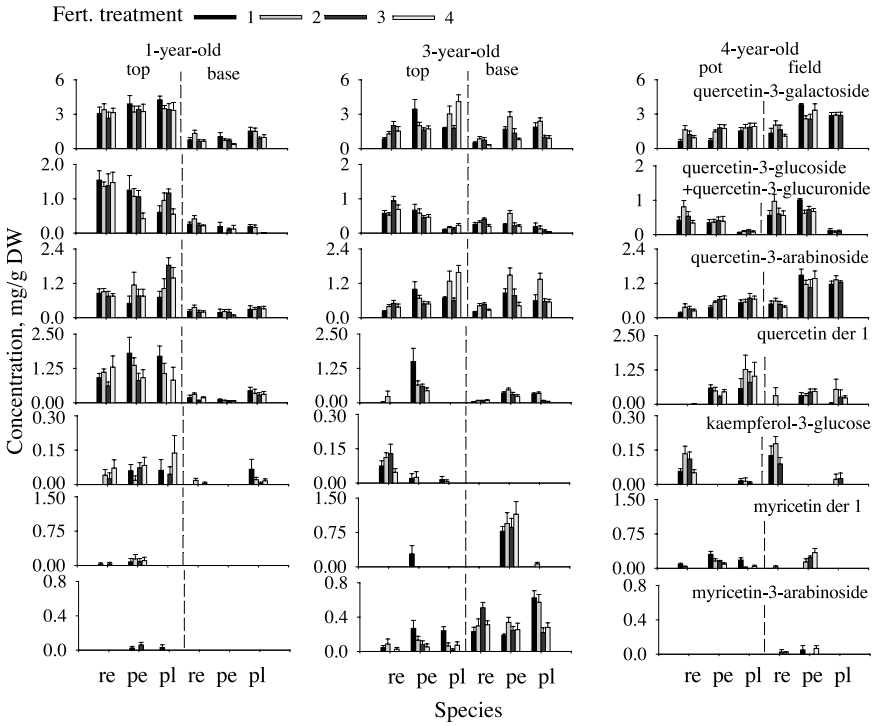

FIG. 2. Concentrations of flavonol glycosides (mean ± SE, mg/g of DW = dry weight) in seedlings and saplings (*B. resinifera* = re, *B. pendula* = pe, and *B. platyphylla* = pl) in top samples (at 1-, 3-, and 4-yr-old) and base samples (ages 1 and 3) in pot and field experiments with different fertilization treatments. The fertilization treatments are: 1 = lowest, 4 = highest. For N g/seedling and N kg/ha in each year, see Table 1.

derivatives is the sum of six cinnamic acid derivatives (Figure 7); and the chlorogenic acid derivative 1 is the sum of six different chlorogenic acid derivatives (Figure 3). Small concentrations of myricetin-3-galactoside, three myricetin derivatives, two apigenin derivatives, two luteolin derivatives, and two acacetin derivatives were also found in a few samples. These compounds are included in the sums of the respective compound groups.

The HPLC-DAD data for phenolics were verified by using HPLC/atmospheric pressure electrospray ionization (HPLC/API-ES), which produced the following molecular weights: chlorogenic acid 355 (M + 1) and 377 (M + 23); myricetin 3-galactoside 481 (M + 1) and 503 (M + 23); myricetin 3-arabinoside 451 (M + 1) and 473 (M + 23); quercetin 3-galactoside 465 (M + 1) and 487 (M + 23); quercetin 3-glucoside 465 (M + 1) and 487 (M + 23); quercetin 3-glucuronide 479 (M + 1) and 501 (M + 23); kaempferol 3-glucoside 449 (M + 1) and 471 (M + 23); salidroside 323 (M + 23); rhododendrin 351 (M + 23); platyphylloside 499 (M + 23), and 315 (M + 1); (+)-catechin 291 (M + 1); catechin xyloside 423 (M + 1); lignan 1 709 (M + 23).

Triterpenoids were analyzed quantitatively by HPLC–mass spectrometry (MS) by using 1-ml terpenoid samples dissolved in 8 ml 94% ethanol. The HPLC/API-ES conditions were as follows: the column was a Hypersil RP C-18, 2.1 mm ID × 10 cm; the ES fragmentor voltage was 100 V, and the flow rate

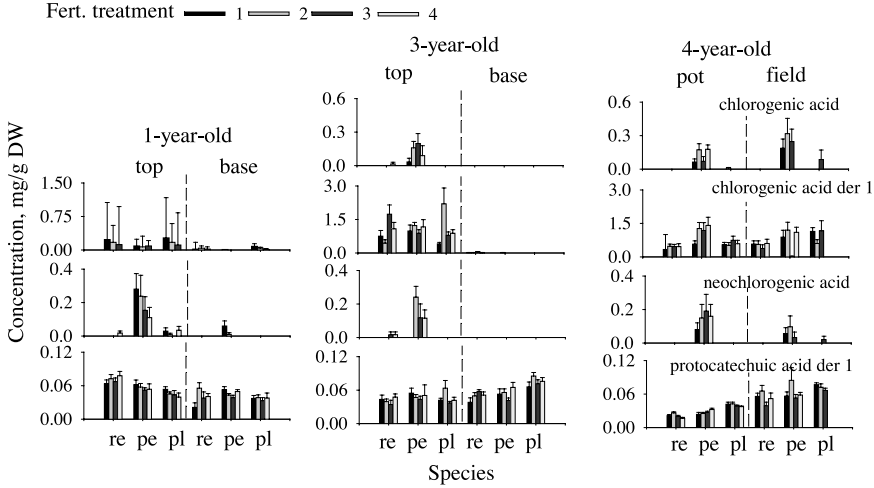

FIG. 3. Concentrations of caffeoylquinic and other acids (mean ± SE, mg/g of DW = dry weight) in seedlings and saplings (*B. resinifera* = re, *B. pendula* = pe, and *B. platyphylla* = pl) in top samples (at 1-, 3-, and 4-yr-old) and base samples (ages 1 and 3) in pot and field experiments with different fertilization treatments. The fertilization treatments are: 1 = lowest, 4 = highest. For N g/seedling and N kg/ha in each year, see Table 1.

was 0.2 ml/min. Isogratic elution was used with EtOH (94%): aqueous 1.5% tetrahydrofuran + 0.25% acetic acid (75:25, pH 5.4). The MS-SIM analyses were based on the following ions: papyriferic acid 627 (M + 23), 643 (M + 39), 423 (M + H-59-143); pendulic acid 611 (M + 23), 627 (M + 39), 511, 407; and deacetylpapyriferic acid 585 (M + 23), 601 (M + 39). Triterpenoid components were quantified by using purified papyriferic acid (provided by Prof. Paul Reichard, University of Alaska, USA). The concentrations of pendulic acid and deacetylpapyriferic acid are considered to be relative.

Concentrations of condensed tannins were determined from the dissolved methanol (1st, 3rd, and 4th years) and the extraction residue (4th year), using the butanol–HCl test according to Hagerman (1995). Quantification was based on purified tannin equivalents from *Betula nana* leaves.

Dry Weight and Nitrogen Analysis. To determine the amounts of phenolics and terpenoids, 5-cm samples were taken from just below (top samples) or above (base samples) those taken for chemical analyses. They were left to dry at room temperature for 2 d or dried at 105°C for 24 hr, after which their dry weight was determined. The amounts of nitrogen in the 3rd year bottom samples and 4th year top samples were measured with a nitrogen analyzer (Leco FP-528, Leco, St. Joseph, MI, USA).

Statistics. Effects of species, plant part, age, fertilization, and growing site on chemistry were analyzed using ANOVA. The model for individual compounds in 1- and 3-yr-old saplings and within the plant (top, base) was a full factorial model of fixed effect: species, fertilization, plant part, and age. For the age effect, the design was a split-plot design in which whole plot treatments were defined by species and fertilization, whole plots were defined by block, species, and fertilization, and split-plots were defined by block, species, fertilization, and age. The design for the plant part effect was also a split-plot design, in which split-plots were defined by block, species, fertilization, and plant part. The random effects of the ANOVA model were, therefore, block*species*fertilization, block*species*fertilization*age, block*species*fertilization*age*plant, and block*species*fertilization*age *plant. The analyses were performed by using the SPSS GLM procedure, which computes the degrees of freedom of the error terms by Satterthwaite's method (SPSS 11.0 for Windows 2001). The interpretation of high-order interactions is somewhat problematic, and to save space, only interactions up to the third degree are presented in the ANOVA tables in Appendices 1–3 (available online at http://www.springerlink.com; search for DOI: 10.1007/s10886-005-7100-5; Electronic Supplementary Material can be found at the end of the article). Concentrations of analyzed compounds were transformed with square root or natural logarithm transformations [$\ln(x + 1)$, $\ln(x + 0.5)$, or $\ln(x + 0.1)$]. The normality of the residuals was checked with probability plots, and the homogeneity of variances was checked by plotting residuals against fitted values. To evaluate the impact of species,

growing habitat, and age, the data for the compound groups were also analyzed by using principal component analyses (PCA). Correlations between different parameters were determined with Pearson's correlation or Spearman's nonparametric correlation test.

RESULTS

The concentrations of different secondary chemicals under different fertilization treatments within species, different species, and sites, and different plant parts and different years varied greatly and in many cases significantly (Appendices, Figures 1–9). Fifty-eight compounds were identified as phenolics and terpenoids. *B. pendula* contained all 58, *B. resinifera* 51, and *B. platyphylla* 52. Some compounds were present in both plant parts of the three species in all treatments. They included catechin-xyloside and (+)-catechin (Figure 1), quercetin-3-galactoside and quercetin-3-arabinoside (Figure 2), protocatechuic

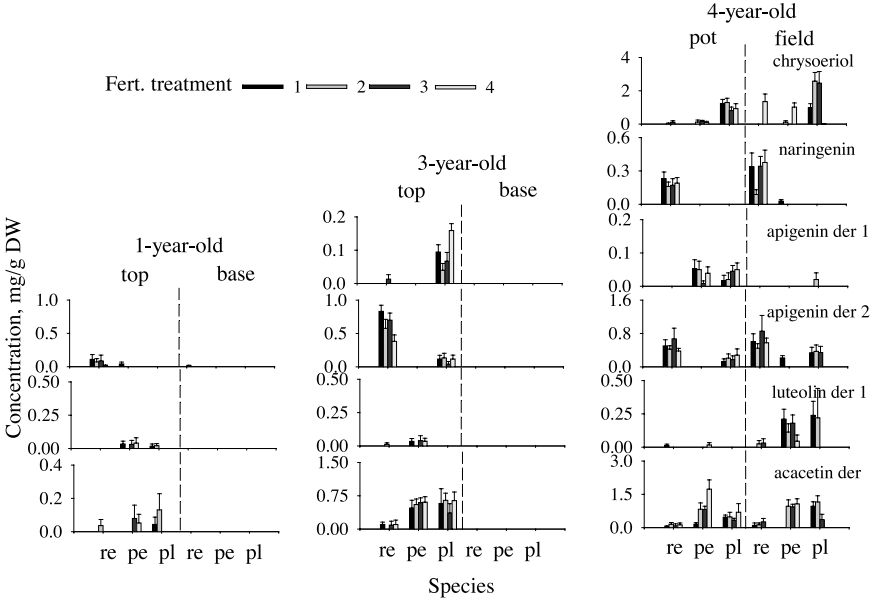

FIG. 4. Concentrations of flavonoid aglycones (mean ± SE, mg/g of DW = dry weight) in seedlings and saplings (*B. resinifera* = re, *B. pendula* = pe, and *B. platyphylla* = pl) in top samples (at 1-, 3-, and 4-yr-old) and base samples (ages 1 and 3) in pot and field experiments with different fertilization treatments. The fertilization treatments are: 1 = lowest, 4 = highest. For N g/seedling and N kg/ha in each year, see Table 1.

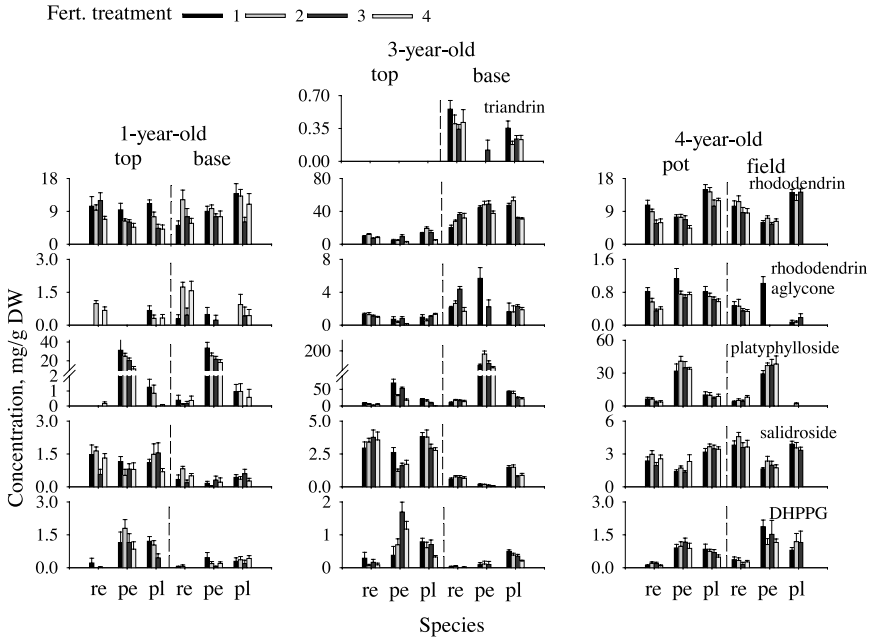

FIG. 5. Concentrations of other phenolic glycosides and small molecular weight derivatives (mean ± SE, mg/g of DW = dry weight) in seedlings and saplings (*B. resinifera* = re, *B. pendula* = pe, and *B. platyphylla* = pl) in top samples (at 1-, 3-, and 4-yr-old) and base samples (ages 1 and 3) in pot and field experiments with different fertilization treatments. The fertilization treatments are: 1 = lowest, 4 = highest. For N g/seedling and N kg/ha each year, see Table 1.

acid derivative 1 (Figure 3), rhododendrin and salidroside (Figure 5), and pendulic acid (Figure 8). There was also secoisolariciresinol 9-*O*-β-glucopyranoside and two of its derivatives, lignans 2 and 3, which have not previously been identified in birch bark (Figure 6).

Total concentrations of HPLC-phenolics in seedling base samples were much higher than those of total terpenoids (Figures 7 and 9). Particularly large concentrations of platyphylloside, a phenolic glycoside, were found in base samples from 3-yr-old saplings of *B. pendula* (Figure 5), and there were much higher concentrations of soluble condensed tannins in base samples from 3-yr-old saplings than in top samples (Figure 6). Concentrations of soluble tannins in 4-yr-old saplings were smaller than those of insoluble condensed tannins (Figure 6). Triandrin (Figure 5) and lignans 2 and 3 (Figure 6) were found only in base samples from seedlings and 3-yr-old saplings of *B. resinifera* and *B. platyphylla*. Flavonoid aglycones (Figure 4) and pentagalloylglucose (Figure 6) were found mostly in the top parts of the plants.

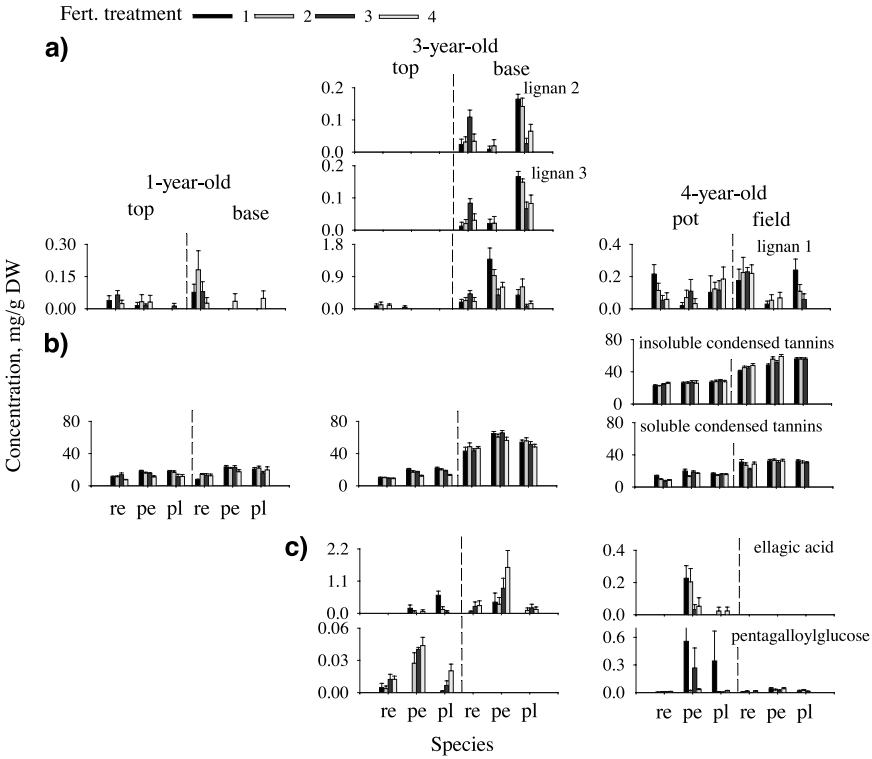

FIG. 6. Concentrations of (a) lignans, (b) condensed tannins, and (c) hydrolyzable tannins and their precursors (mean ± SE, mg/g of DW = dry weight) in seedlings and saplings (*B. resinifera* = re, *B. pendula* = pe, and *B. platyphylla* = pl) in top samples (at 1-, 3-, and 4-yr-old) and base samples (ages 1 and 3) in pot and field experiments with different fertilization treatments. The fertilization treatments are: 1 = lowest, 4 = highest. For N g/seedling and N kg/ha each year, see Table 1. Note the differences in scale on the vertical axes. Note also that insoluble tannins were determined only at the age of 4 yr.

Although the birches tested have fairly similar chemical profiles, the three species were clearly distinguished by PCA (Figure 10). Separation is greatest in fourth year field samples and hardly exists in seedlings (Figures 3–7).

The effect of fertilization on chemical profiles was dependent on age and species of plants (Appendices, Figures 1–9). Although there was considerable variation in responses to fertilization, the amounts of phenolics in 1-yr-old and 3-yr-old plants often fell with increasing fertilization (Figures 1–3 and 5–7). The effect of fertilization on terpenoids in 4-yr-old plants was variable (Figures 8 and 9).

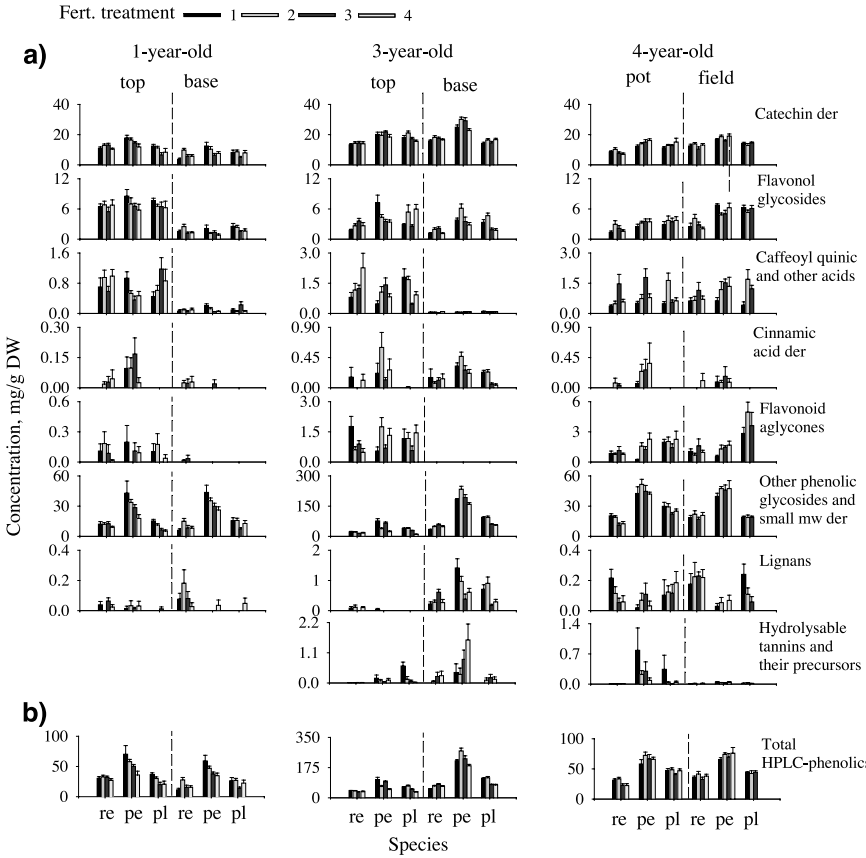

FIG. 7. Concentrations of (a) phenolic compound groups (mean ± SE, mg/g of DW = dry weight) and (b) total phenolics in seedlings and saplings of (*B. resinifera* = re, *B. pendula* = pe, and *B. platyphylla* = pl) in top samples (at 1-, 3-, and 4-yr-old) and base samples (ages 1 and 3) in pot and field experiments with different fertilization treatments. The fertilization treatments are: 1 = lowest, 4 = highest. For N g/seedling and N kg/ha each year, see Table 1. Note the differences in scale on the vertical axes. der = Derivative.

The effect of the growing site was dependent on species and compound (Appendices, Figures 1–9). When 4-yr-old saplings grown in pots were compared with those grown in the field, noticeably higher concentrations of condensed tannins were found in the latter (Figure 6). PCA also shows that, with regard to chemical profiles, 4-yr-old plants are differentiated by growing condition (Figure 10, Table 2).

Concentrations of some phenolic groups (catechin derivative, flavonoid aglycones, other phenolic glycosides, and small molecular weight phenolics;

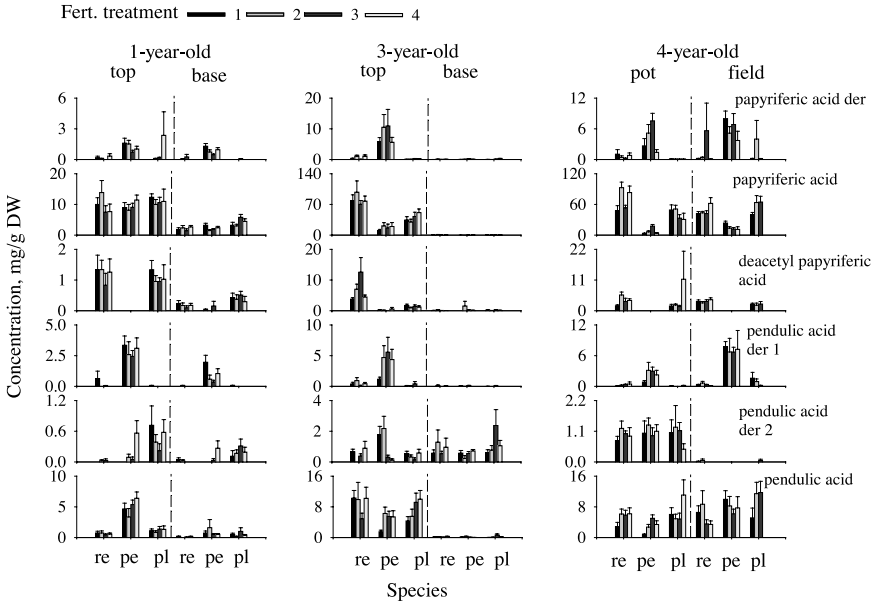

FIG. 8. Concentrations of terpenoids (mean ± SE, mg/g of DW = dry weight) in seedlings and saplings (*B. resinifera* = re, *B. pendula* = pe, and *B. platyphylla* = pl) in top samples (at 1-, 3-, and 4-yr-old) and base samples (ages 1 and 3) in pot and field experiments with different fertilization treatments. The fertilization treatments are: 1 = lowest, 4 = highest. For N g/seedling and N kg/ha each year, see Table 1. Note also the differences in scale on the vertical axes. der = Derivative.

Figure 7) were higher in 3-yr-old plants than in 1- and 4-yr-old ones. This was particularly the case in base samples from 3-yr-old saplings (Figures 1, 3, 5, and 6).

The highest concentrations of terpenoids were found in top samples from older saplings. However, except for pendulic acid derivative 2 (Figure 8), the concentrations of terpenoids in the base parts of plants declined with age (Figures 8 and 9). Correlations between resin droplets and individual terpenoid compounds or compound groups were positive but not statistically significant (Spearman rank correlation $P > 0.05$).

DISCUSSION

Species-specific quantitative and qualitative variation in secondary chemicals was considerably higher than that caused by site (pot vs. field), fertilization treatment, or ontogeny (Appendices, Figure 10). These genetic differences

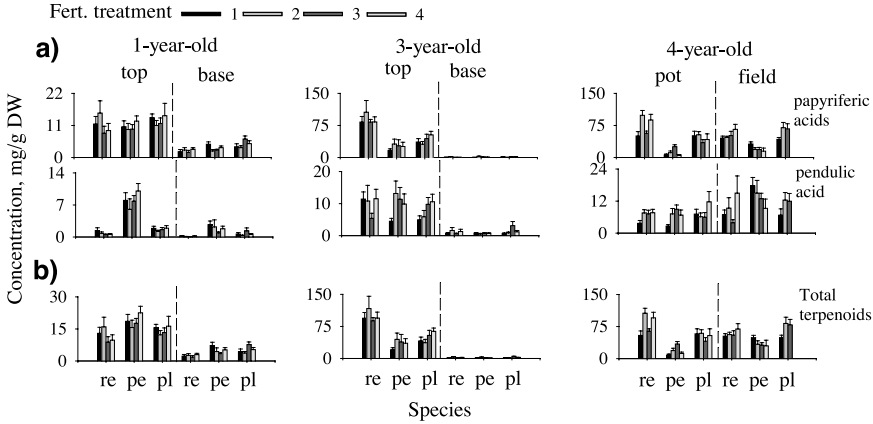

FIG. 9. Concentrations of (a) terpenoid compound groups (mean ± SE, mg/g of DW = dry weight) and (b) total terpenoids in seedlings and saplings (*B. resinifera* = re, *B. pendula* = pe, and *B. platyphylla* = pl) in top samples (at 1-, 3-, and 4-yr-old) and base samples (ages 1 and 3) in pot and field experiments with different fertilization treatments. The fertilization treatments are: 1 = lowest, 4 = highest. For N g/seedling and N kg/ha each year, see Table 1.

among different species are manifested in variations in survival, growth (Figure 11), and morphology found in research station experiments (Laitinen et al., 2004a). *B. pendula* particularly contained high concentrations (up to 20% dry weight) of platyphylloside, which is the most abundant individual compound in the bark of European white birch (Julkunen-Tiitto et al., 1996).

Many of the compounds analyzed [catechin-xyloside, (+)-catechin, quercetin-3-galactoside, quercetin-3-arabinoside, protocatechuic acid derivative 1, rhododendrin, salidroside, and pendulic acid] appeared in both seedlings and saplings. Others were found mainly in a specific species or at a particular developmental stage (e.g., chlorogenic acid, myricetin 3-arabinoside, neochlorogenic acid). We also detected three new components, secoisolariciresinol 9-*O*-β-glucopyranoside and two of its derivatives, which have not previously been reported for white birches. Two of these (lignans 2 and 3) appeared only in base samples from 3-yr-old saplings.

In our study, quantitative variation in secondary chemistry among age classes was specific to species (Appendices, Figures 1–10) and plant part. Quantitative variation in secondary metabolism is generally a reflection of site differences or seasonal and year-to-year variation in weather (Laitinen et al., 2000), whereas qualitative variation seems to be more affected by ontogeny. The considerable impact of growth conditions can be seen, for example, in the amounts of condensed tannins, which were higher in plants grown in the field

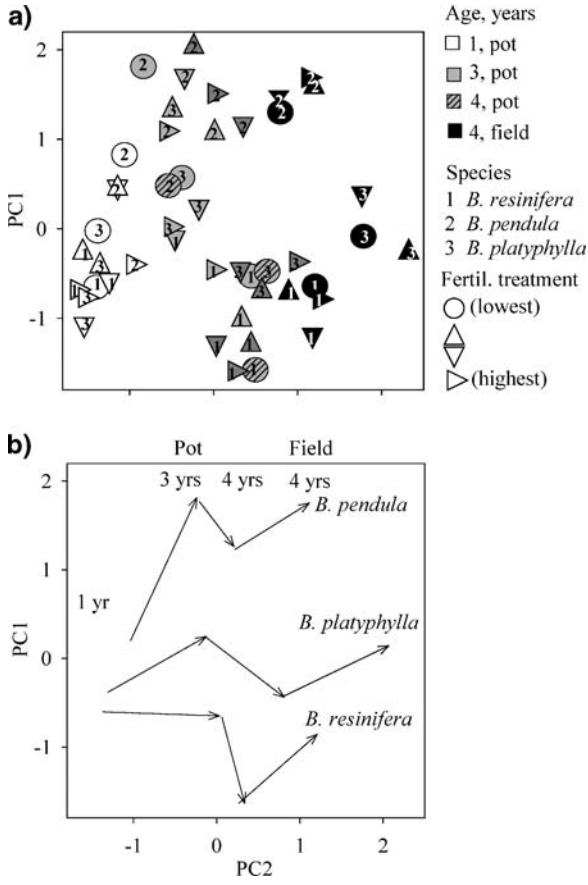

FIG. 10. Principal components analysis (PCA) on secondary compound groups of different species (*B. resinifera*, *B. pendula*, and *B. platyphylla*) in different fertilization treatments and years (a). (b) Simplified diagram of PCA.

than in those grown in pots (Figure 6). The poor growth (Figure 11) and survival rate of plants grown in the field indicate that the growth conditions there were not as favorable as those in the pots (Laitinen et al., 2004a). Poor growth may have led to an excess of carbon for the synthesis of condensed tannins, which are the end products of the phenolic biosynthetic pathway. On the other hand, in the pot experiment, both plant growth and the allocation of carbon to secondary chemicals may have been affected by increased root binding over 4 yr. Consequently, no general conclusions can be drawn from the pot experiment with respect to ontogenetic changes in the secondary chemistry.

TABLE 2. PRINCIPAL COMPONENT MATRIX OBTAINED BY PCA OF CHEMICAL COMPOUND GROUPS FOUND IN *B. resinifera*, *B. pendula*, AND *B. platyphylla*

	Component	
	1	2
Catechin der	0.872	
Other phenolic glycosides and small mw der	0.825	
Caffeoylquinic acids	0.712	
Cinnamic acid der	0.615	
Total pendulic acid	0.598	0.498
Soluble condensed tannins	0.563	0.302
Hydrolyzable tannins and their precursors	0.257	
Total papyriferic acids	−0.267	0.786
Lignans	−0.255	0.715
Flavonoid aglycones	0.346	0.696
Flavonol glycosides		−0.623

Insoluble condensed tannins are not included.
mw = molecular weight, der = derivative.

Although in a few cases fertilization reduced the total levels of phenolics, which accords with many earlier studies (e.g., Bryant, 1987; Price et al., 1989; Muzika and Pregitzer, 1992), individual compounds or compound groups reacted in a highly variable manner, and they were also affected by ontogeny. In general, with increasing fertilization, the amounts of phenolics seemed to decrease, especially in 1- and 3-yr-old birches. The concentrations of terpenoids were also affected by fertilization, but not as predictably as the concentrations of phenolics. Although it has been argued that terpenoids arising from the mevalonic acid pathway (Torssell, 1983; Seigler, 1998) do not directly compete with protein synthesis and should not be affected by changes in nutrient

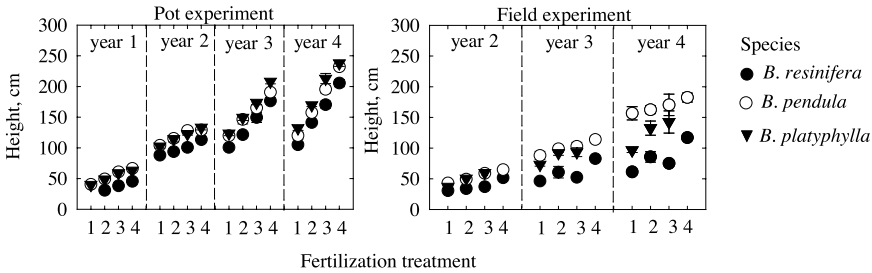

FIG. 11. Height of *B. resinifera*, *B. pendula*, and *B. platyphylla* seedlings and saplings grown in pots and in the field. The fertilization treatments are: 1 = lowest, 4 = highest. For N g/seedling and N kg/ha each year, see Table 1 (modified from Laitinen et al., 2004a).

availability (Muzika, 1993; Haukioja et al., 1998), earlier studies have also shown them to be affected by environment, in some cases even more so than phenolics (Laitinen et al., 2004c). It has been suggested that variation in the concentrations of individual phenolics in relation to the availability of nutrients is caused by a trade-off of substrates between different branches of the biosynthetic pathway of phenolic compounds (Haukioja et al., 1998), i.e., the concentration of one compound may increase at the expense of another (Keinänen et al., 1999; Koricheva et al., 1999).

The concentrations of terpenoids were markedly higher in base samples from 1-yr-old seedlings than from 3-yr-old saplings. Resin droplets, which contain terpenoids, are formed in current year shoots (Lapinjoki et al., 1991); as the plant ages, the bark gets thicker and the resin droplets tend to fall off, resulting in a decrease in the amount. Consequently, strong species × plant part interactions in secondary chemistry may be an indication of the varied abiotic and biotic pressures that the species have encountered in their original growing sites (Close and McArthur, 2002).

One-year-old seedlings of all these white birches are chemically similar, but at the sapling stage, the species are clearly differentiated (Figure 10). Alaskan *B. resinifera* and Japanese *B. platyphylla* seem to be similar chemically, whereas European *B. pendula* appears to be more distinct (Figure 10). Our results, thus, indicate (Figure 10) that secondary chemicals can be used for chemotaxonomic identification of older birches (see also Julkunen-Tiitto et al., 1996), as long as they are growing on sites to which they have adapted.

Secondary compounds in the bark of birches affect depredation by field voles (*Microtus agrestis*; e.g., Harju and Tahvanainen, 1991) and mountain hares (*Lepus timidus*; e.g., Reichardt et al., 1984; Laitinen et al., 2004b). When we compared the secondary chemistry of *B. resinifera*, *B. pendula*, and *B. platyphylla* with the feeding preference of field voles and mountain hares (Laitinen et al., 2004b) for the same experimental plants, we did not find any clear correlations. The effects of bark secondary compounds on vole feeding, in particular, varied and were dependent on the species and age of the plants.

The results of this study indicate that white birch species are differentiated by their secondary chemistry. They also show that secondary metabolism is considerably affected by ontogeny and environment, and that these effects are variable, depending on the properties of the growing sites and possibly also on seasonal and year-to-year variation in weather conditions.

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Appendix 1. Anova Table of Individual Compounds in 1- and 3- Year Old Birches Grown in Pots

	Sp.	Fe.	Part	Age	Sp.*Fe	Sp.*Part	Sp.*Age	Fe.*Part	Fe.*Age	Part*Age	Sp.* Fe.* Part	Sp.* Fe.* Age	Sp.* Part* Age	Fe.* Part* Age
df	2	3	1	1	6	2	2	3	3	1	6	6	2	3
df_{err}	25	25	25	24	25	25	24	25	24	25	25	24	25	25
Catechin Derivatives	74.240***	7.031**	5.964*	268.918***	2.623*	2.085^{NS}	7.829**	1.012^{NS}	2.092^{NS}	75.706***	0.618^{NS}	0.884^{NS}	21.954***	0.296^{NS}
gallocatechin	23.347***	1.118^{NS}	11.930**	0.032^{NS}	0.921^{NS}	4.270*	0.040^{NS}	1.606^{NS}	6.358**	11.618**	1.167^{NS}	5.898**	13.572***	3.669*
catechin–xyloside	51.120***	11.682***	1.476^{NS}	278.749***	5.061**	34.043***	15.030***	0.747^{NS}	4.315**	76.915***	1.330^{NS}	1.631^{NS}	40.889***	0.450^{NS}
(+)-catechin	75.773***	4.871**	0.146^{NS}	179.771***	1.289^{NS}	1.391^{NS}	3.779*	1.744^{NS}	0.403^{NS}	47.890***	0.437^{NS}	0.728^{NS}	1.569^{NS}	0.549^{NS}
epicatechin	8.476**	0.030^{NS}	225.349***	0.421^{NS}	2.186^{NS}	16.321***	8.373**	1.165^{NS}	1.998^{NS}	0.704^{NS}	5.322***	3.616*	7.058**	2.126^{NS}
Flavonol Glycosides	9.533**	4.443*	176.028***	8.300**	2.176^{NS}	0.002^{NS}	4.629*	1.537^{NS}	0.842^{NS}	110.417***	1.438^{NS}	1.301^{NS}	1.795^{NS}	1.535^{NS}
quercetin-3-galactoside	18.655***	5.553**	336.145***	4.473*	1.840^{NS}	0.607^{NS}	5.597*	8.698***	0.420^{NS}	74.823***	0.587^{NS}	1.365^{NS}	5.561*	3.580*
quercetin-3-glucoside + quercetin-3-glucuronide	43.5587***	3.324^{NS}	192.817***	7.585***	0.699^{NS}	1.951^{NS}	7.866**	1.264^{NS}	0.489^{NS}	77.911***	1.193^{NS}	1.707^{NS}	0.963^{NS}	2.576^{NS}
quercetin-3-arabinoside	15.251***	3.362*	73.562***	4.497*	1.800^{NS}	3.288^{NS}	5.800**	4.122*	0.797^{NS}	106.058***	0.954^{NS}	1.702^{NS}	1.478^{NS}	3.643^{NS}
quercetin der 1	24.914***	14.432***	73.562***	61.913***	2.902*	41.033***	24.761***	4.432*	2.548^{NS}	105.099***	1.218^{NS}	0.919^{NS}	0.833^{NS}	4.573*
kaempferol-3-glucose[a]														
myricetin der 1	89.453***	0.688^{NS}	50.506***	17.637***	0.974^{NS}	50.257***	17.540***	0.278^{NS}	0.092^{NS}	101.610***	0.351^{NS}	0.035^{NS}	87.254***	3.531*
myricetin-3-arabinoside[b]	3.512*	3.595*	176.857***		4.349**	11.546***		2.515^{NS}			5.840**			
Caffeoyl Quinic and Other Acids	0.135^{NS}	1.394^{NS}	902.894***	5.890**	1.653^{NS}	1.499^{NS}	3.177^{NS}	1.551^{NS}	3.906*	27.173***	2.508*	3.251*	3.852*	1.287^{NS}
chlorogenic acid[c]														
chlorogenic acid der 1[a]														
neochlorogenic acid[a]														
protocatechuic acid der	0.079^{NS}	2.156^{NS}	0.419^{NS}	0.883^{NS}	1.484^{NS}	8.569**	7.990**	1.166^{NS}	0.130^{NS}	58.589***	0.908^{NS}	0.622^{NS}	3.040^{NS}	3.110^{NS}
Cinnamic Acid Der	47.714***	2.939^{NS}	41.202***	105.701***	3.382*	18.272***	15.885***	1.411^{NS}	3.770*	141.433***	5.346**	2.262^{NS}	7.570**	3.876*
Flavonoid Aglycones[d]														
Other Phenolic Glycosides and Small MW der	391.295***	31.039***	347.742***	569.883***	8.048***	40.625***	20.312***	7.133**	1.534^{NS}	346.948***	1.908^{NS}	0.965^{NS}	28.389***	1.212^{NS}
triandrin[e]														
rhododendrin	2.010^{NS}	4.603**	198.768***	172.160***	1.920^{NS}	19.090***	4.622*	2.744*	2.508^{NS}	185.212***	0.913^{NS}	1.037^{NS}	12.472***	2.389^{NS}
rhododendron aglycone	32.807***	0.401^{NS}	12.960**	52.863***	7.044***	3.089^{NS}	0.796^{NS}	1.135^{NS}	5.168**	0.461^{NS}	3.132*	1.786^{NS}	0.200^{NS}	0.475^{NS}
platyphylloside	509.986***	19.592***	177.941***	581.452***	2.573*	0.013^{NS}	25.979***	10.065***	4.926**	103.271***	3.419*	2.072^{NS}	1.022^{NS}	6.645***
salidroside	86.213***	3.579*	325.108***	162.280***	7.529***	1.675^{NS}	10.038**	0.712^{NS}	0.037^{NS}	62.341***	0.604^{NS}	2.967*	3.507*	1.806^{NS}

APPENDIX 1. CONTINUED

	Sp.	Fe.	Part	Age	Sp.*Fe	Sp.*Part	Sp.*Age	Fe.*Part	Fe.*Age	Part*Age	Sp.* Fe.* Part	Sp.* Fe.* Age	Sp.* Part* Age	Fe.* Part* Age
df	2	3	1	1	6	2	2	3	3	1	6	6	2	3
df_{err}	25	25	25	24	25	25	24	25	24	25	25	24	25	25
DHPPG	62.445***	2.071^{NS}	100.205***	0.855^{NS}	2.626*	29.353***	3.162^{NS}	3.221*	2.765^{NS}	0.432^{NS}	6.515^{NS}	0.659^{NS}	1.670^{NS}	6.303**
Lignans	2.330^{NS}	1.596^{NS}	249.608***	271.516***	1.758^{NS}	3.725*	7.509**	3.438*	6.376**	151.842***	3.769**	3.883**	8.557**	1.134^{NS}
lignan 1	7.525**	1.011^{NS}	98.252***	96.762***	1.235^{NS}	6.498**	11.072***	1.826^{NS}	3.371*	56.904***	2.073^{NS}	2.216^{NS}	9.504**	0.487^{NS}
lignans 2 and 3[e]														
Condensed Tannins														
insoluble[f]														
soluble	57.139***	8.838***	1245.588***	261.026***	2.895*	5.673**	0.373^{NS}	4.740**	0.196^{NS}	607.241***	0.544^{NS}	0.565^{NS}	7.389**	0.105^{NS}
Hydrolysable Tannins and their Precursors														
ellagic acid[b]	8.820***	1.776^{NS}	18.923**		2.369^{NS}	14.728***		10.482***			1.221^{NS}			
pentagalloylglucose[g]	18.763***	12.356***			3.034**									
Total HPLC Phenolics	312.429***	25.545***	96.959***	561.178***	7.611***	30.911***	24.306***	3.978*	0.988^{NS}	326.666***	1.120^{NS}	0.823^{NS}	24.566***	0.501^{NS}
Total Papyriferic Acids	3.977*	0.260^{NS}	980.695***	1.338^{NS}	0.500^{NS}	13.066***	13.281***	0.471^{NS}	0.228^{NS}	176.222***	0.332^{NS}	1.062^{NS}	5.353*	1.197^{NS}
papyriferic acid	21.452***	0.346^{NS}	727.357***	39.337***	0.803^{NS}	31.946***	23.449***	0.266^{NS}	0.144^{NS}	174.689***	0.616^{NS}	0.320^{NS}	21.616***	1.009^{NS}
papyriferic acid der	69.657***	0.872^{NS}	59.132***	12.045**	0.621^{NS}	24.756***	2.985^{NS}	0.740^{NS}	0.612^{NS}	29.052***	1.022^{NS}	0.928^{NS}	25.057***	0.264^{NS}
deacetyl papyriferic acid	62.533***	0.479^{NS}	159.607***	17.682***	0.803^{NS}	73.955***	13.035***	0.053^{NS}	0.717^{NS}	32.389***	1.078^{NS}	1.322^{NS}	21.438***	1.184^{NS}
Total Pendulic Acids	17.971***	1.433^{NS}	329.629***	81.811***	1.686^{NS}	8.353**	30.907***	0.031^{NS}	1.208^{NS}	22.657***	1.594^{NS}	1.286^{NS}	1.526^{NS}	0.144^{NS}
pendulic acid	6.771**	0.853^{NS}	1.288^{NS}	83.147***	0.441^{NS}	3.182^{NS}	1.541^{NS}	4.515*	1.397^{NS}	9.479**	3.066*	1.893^{NS}	5.080*	3.136*
pendulic acid der 1	78.124***	0.045^{NS}	69.321***	0.002^{NS}	0.432^{NS}	41.458***	7.259**	0.688^{NS}	5.546**	13.496**	1.597^{NS}	3.070*	4.965*	0.624^{NS}
pendulic acid der 2	2.138^{NS}	1.202^{NS}	390.420***	39.067***	1.903^{NS}	1.292^{NS}	24.766***	0.769^{NS}	0.977^{NS}	66.912***	1.219^{NS}	1.205^{NS}	9.709**	0.180^{NS}
Total Terpenoids	4.641*	0.745^{NS}	625.1077***	70.076***	0.977^{NS}	15.811***	27.419***	0.178^{NS}	0.661^{NS}	135.444***	0.500^{NS}	0.653^{NS}	13.028***	0.505^{NS}
Nitrogen[f]														

F and *df* and df_{err} values are presented, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Sp. = Species; Fe. = Fertilization; der = derivative. DHPPG = 3.4 dihydroksi-propiophenoneglucoside; der = derivative.

[a] See Appendix 3.

[b] Analyzed only between top and base of 3-year-old saplings.

[c] Identified only in 3 and 4-year old plants.

[d] Not identified in the base of plants.

[e] Identified only at the base of 3-year-old saplings.

[f] Not detected.

[g] Analyzed between top parts of 3-year-old saplings.

APPENDIX 2. ANOVA TABLE OF INDIVIDUAL COMPOUNDS IN TOP SAMPLES FROM 1-, 3- AND 4-YEAR-OLD BIRCHES GROWN IN POTS

	Sp.	Fe.	Age	Sp.*Fe.	Sp.*Age	Fe.*Age	Sp.*Fe.*Age
df	2	3	2	6	4	6	12
Catechin derivates	47.183***	2.825*	66.568***	1.124^{NS}	8.499***	2.918**	1.925*
gallocatechin	140.523***	1.421^{NS}	1.969^{NS}	0.636^{NS}	1.921^{NS}	4.901***	4.449***
catechin–xyloside	1.608^{NS}	4.452**	93.270***	1.746^{NS}	9.584***	3.284**	2.433**
(+)-catechin	105.824***	4.529**	38.231***	0.773^{NS}	5.113**	2.242*	1.599^{NS}
epicatechin	18.295***	2.043^{NS}	11.654***	1.934^{NS}	9.617***	1.806^{NS}	0.503^{NS}
Flavonol Glycosides	9.234***	0.957^{NS}	249.071***	4.114***	8.830***	1.395^{NS}	2.158*
quercetin-3-galactoside	27.460***	4.149**	3.923*	1.373^{NS}	3.799**	1.030^{NS}	1.345^{NS}
quercetin-3-glucoside + quercetin-3-glucuronide	59.761***	2.635*	7.852***	0.511^{NS}	7.524***	0.271^{NS}	1.323^{NS}
quercetin-3-arabinoside	30.240***	4.403**	8.405***	0.750^{NS}	9.418***	1.053^{NS}	2.333**
quercetin der 1	29.122***	5.945**	24.422***	1.182^{NS}	12.983***	2.733*	1.061^{NS}
kaempferol-3-glucose[a]	1.901^{NS}	0.509^{NS}	1.866^{NS}	0.973^{NS}	12.842***	4.760**	0.672^{NS}
myricetin der 1	32.799***	8.584***	28.272***	0.579^{NS}	5.136**	3.702**	1.623^{NS}
myricetin-3-arabinoside[b]							
Caffeoyl Quinic and Other Acids	5.799**	1.023^{NS}	33.011***	0.557^{NS}	6.975***	1.014^{NS}	1.809*
chlorogenic acid	46.423***	2.028^{NS}	13.422***	1.702^{NS}	12.319***	1.665^{NS}	1.979*
chlorogenic acid der	14.343***	1.326^{NS}	7.317**	0.608^{NS}	5.246***	1.245^{NS}	1.470^{NS}
neochlorogenic acid	53.142***	0.484^{NS}	1.991^{NS}	0.664^{NS}	1.897^{NS}	1.451^{NS}	1.451^{NS}
protocatechuic acid der	0.004^{NS}	1.428^{NS}	54.137***	0.774^{NS}	12.118***	0.381^{NS}	0.940^{NS}
Cinnamic Acid Derivatives	51.631***	1.621^{NS}	5.511**	2.085^{NS}	1.969^{NS}	1.101^{NS}	1.344^{NS}
Flavonoid Aglycones[c]	7.404**	2.116^{NS}	52.605***	3.661**	5.636**	1.877^{NS}	0.844^{NS}
chrysoeriol[d]							
naringenin[d]							
apigenin der 1[c]	17.793***	1.345^{NS}	2.241^{NS}	2.195*	8.352***	0.356^{NS}	1.174^{NS}
apigenin der 2[c]	3.089^{NS}	0.183^{NS}	0.225^{NS}	1.900^{NS}	5.294*	0.635^{NS}	0.098^{NS}

APPENDIX 2. CONTINUED

	Sp.	Fe.	Age	Sp.*Fe.	Sp.*Age	Fe.*Age	Sp.*Fe.*Age
df	2	3	2	6	4	6	12
luteolin der 1	5.941**	0.496^{NS}	1.659^{NS}	1.335^{NS}	1.325^{NS}	0.094^{NS}	0.396^{NS}
acacetin der[c]	2.157^{NS}	3.454*	0.336^{NS}	0.733^{NS}	3.252*	0.965^{NS}	1.239^{NS}
Other Phenolic Glycosides and Small MW Derivatives	171.527***	12.988***	220.627***	2.500*	3.059*	2.587*	1.568^{NS}
triandrin[e]							
rhododendrin	40.376***	19.407***	4.510*	3.025**	14.202***	3.713**	3.109***
rhododendrinaglykone	35.951***	1.208^{NS}	86.803***	11.573***	9.125***	10.555***	3.406***
platyphylloside	457.582***	5.807**	233.366***	0.722^{NS}	16.54***	6.636***	1.969*
salidroside	43.596***	1.099^{NS}	91.586***	2.350*	7.582***	1.389^{NS}	1.517^{NS}
DHPPG	99.932***	3.597*	38.610***	1.860^{NS}	4.248**	1.779^{NS}	1.116^{NS}
Lignans	0.833^{NS}	2.188***	51.192***	1.092^{NS}	6.038***	1.644^{NS}	1.502^{NS}
ignan 1	6.304**	0.124^{NS}	16.895**	1.144^{NS}	2.416*	0.837^{NS}	1.598^{NS}
lignans 2 and 3[e]							

Condensed Tannins							
insoluble[d]							
soluble	86.216***	21.168***	3.106*	1.691^{NS}	4.953**	3.286**	2.532**
Hydrolysable Tannins and their Precursors	17.437***	6.823***	21.911***	3.003**	10.832***	1.892^{NS}	2.864**
Total HPLC Phenolics	145.011***	17.444***	44.256***	4.087**	6.453***	5.071***	3.886***
Total Papyriferic Acids	18.119***	0.215^{NS}	89.441***	0.612^{NS}	9.470***	0.586^{NS}	0.663^{NS}
papyriferic acid	27.072***	0.352^{NS}	83.080***	0.759^{NS}	11.060***	0.628^{NS}	0.635^{NS}
papyriferic acid der	173.083***	0.708^{NS}	19.649***	1.683^{NS}	8.340***	2.101^{NS}	1.197^{NS}
deacetyl papyriferic acid	147.716***	0.146^{NS}	37.342***	0.616^{NS}	17.747***	0.462^{NS}	0.564^{NS}
Total Pendulic Acids	17.214***	0.606^{NS}	105.358***	1.062^{NS}	9.907***	1.069^{NS}	0.541^{NS}
pendulic acid	0.963^{NS}	1.348^{NS}	13.004***	2.347*	0.366^{NS}	1.775^{NS}	1.709^{NS}
pendulic acid der 1	183.179***	0.309^{NS}	22.031***	0.485^{NS}	8.588***	1.454^{NS}	1.048^{NS}
pendulic acid der 2	2.326^{NS}	1.322^{NS}	86.466***	1.134^{NS}	8.039***	0.835^{NS}	0.454^{NS}
Total Terpenoids	18.192***	0.463^{NS}	111.077***	0.601^{NS}	12.595***	0.576^{NS}	0.706^{NS}

F and *df* values are presented; df_{err} = 252; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; NS = not significant. Sp. = Species; Fe. = Fertilization; DHPPG = 3'4 dihydroksi-propiophenoneglucoside; der = derivative.

[a] Analyzed only between top parts of 1- and 3-year-old saplings.

[b] Not identified in 4-year-old saplings grown in pots, see Appendix 1.

[c] Analyzed only between 3- and-4-year-old saplings.

[d] Only identified in 4-year-old saplings.

[e] Only identified at base of 3-year-old saplings.

Appendix 3. Anova Table of Individual Compounds in 4-Year-Old Birches Grown in Pots and in the Field

	Sp.	Fe.	Site	Sp.*Fe.	Sp.*Site	Fe.*Site	Sp.*Fe.*Site
df	2	3	1	6	2	3	5
Catechin Derivatives	50.506***	3.378*	42.246***	2.418*	1.914^{NS}	1.095^{NS}	1.016^{NS}
gallocatechin	164.417***	2.259^{NS}	21.213***	2.233*	4.548*	2.044^{NS}	2.889*
catechin–xyloside	4.230*	3.696*	62.569***	2.313*	0.580^{NS}	1.743^{NS}	0.595^{NS}
(+)-catechin	81.338***	1.347^{NS}	0.069^{NS}	1.267^{NS}	8.711***	0.467^{NS}	1.299^{NS}
epicatechin	59.059***	3.811*	100.999***	3.158**	3.325*	1.210^{NS}	1.095^{NS}
Flavonol Glycosides	35.384***	1.103^{NS}	53.276***	1.977^{NS}	2.540^{NS}	1.932^{NS}	0.580^{NS}
quercetin-3-galactoside	28.815***	2.195^{NS}	58.995***	1.825^{NS}	5.254**	3.441*	1.701^{NS}
quercetin-3-glucoside + quercetin-3-glucuronide	53.684***	0.855^{NS}	15.875***	1.807^{NS}	2.381^{NS}	1.206^{NS}	0.750^{NS}
quercetin-3-arabinoside	52.609***	0.742^{NS}	85.093***	0.725^{NS}	3.635*	2.304^{NS}	0.989^{NS}
quercetin der 1	35.090***	0.892^{NS}	3.966*	0.471^{NS}	3.126*	0.361^{NS}	0.623^{NS}
kaempferol-3-glucose[a]	64.028***	5.423**	0.484^{NS}	1.518^{NS}	0.861^{NS}	0.624^{NS}	1.847^{NS}
myricetin der 1	75.018***	1.492^{NS}	9.001**	4.976***	0.007^{NS}	22.997***	3.846**
myricetin-3-arabinoside[b]							
Caffeoyl Quinic and Other Acids	21.355***	0.901^{NS}	2.719^{NS}	1.626^{NS}	1.415^{NS}	1.190^{NS}	0.156^{NS}
chlorogenic acid[c]	3.722^{NS}	0.831^{NS}				0.365^{NS}	
chlorogenic acid der	14.653***	0.420^{NS}	0.005^{NS}	1.424^{NS}	1.581^{NS}	1.440^{NS}	0.735^{NS}
;neochlorogenic acid	25.773***	0.687^{NS}	0.812^{NS}	0.497^{NS}	1.938^{NS}	0.277^{NS}	0.430^{NS}
protocatechuic acid der	13.050***	3.319*	127.394***	0.721^{NS}	0.374^{NS}	2.006^{NS}	1.067^{NS}
Cinnamic Acid Derivatives	18.653***	1.060^{NS}	2.132^{NS}	0.490^{NS}	1.920^{NS}	0.526^{NS}	0.450^{NS}
Flavonoid Aglycones	25.322***	3.058*	4.882*	4.243**	3.029^{NS}	0.382^{NS}	0.965^{NS}
chrysoeriol	228.984***	2.714*	2.259^{NS}	0.923^{NS}	7.515**	1.000^{NS}	3.184**
naringenin[c]		1.929^{NS}	2.965^{NS}			1.130^{NS}	
apigenin der 1[d]							
apigenin der 2[a]	13.577***	0.183^{NS}	3.410^{NS}	0.551^{NS}	0.738^{NS}	0.131^{NS}	0.036^{NS}
luteolin der 1[d]							
acacetin der	38.111***	5.530**	0.600^{NS}	8.480***	1.592^{NS}	1.234^{NS}	1.179^{NS}

Other Phenolic Glycosides and Small MW Derivatives	125.681***	3.094*	0.008^{NS}	1.698^{NS}	7.553**	2.347^{NS}	0.699^{NS}
triandrin[e]							
rhododendrin	70.658***	6.258***	2.649^{NS}	1.053^{NS}	2.305^{NS}	2.393^{NS}	1.569^{NS}
rhododendrinaglykone	6.442**	23.769^{NS}	218.366***	8.483***	30.526***	2.301^{NS}	8.760***
platyphylloside	143.330***	2.241^{NS}	20.590***	0.432^{NS}	25.480***	0.723^{NS}	1.699^{NS}
salidroside	60.969***	2.741*	13.963***	0.950^{NS}	7.932**	1.495^{NS}	0.500^{NS}
DHPPG	80.922***	0.921^{NS}	8.316**	0.779^{NS}	0.083^{NS}	1.058^{NS}	1.043^{NS}
Lignans							
lignan 1	11.9532***	0.518^{NS}	3.714^{NS}	0.914^{NS}	2.568^{NS}	0.791^{NS}	2.305^{NS}
lignans 2 and 3[e]							
Condensed Tannins							
insoluble	32.227***	3.777*	570.411***	1.331^{NS}	12.188***	1.870^{NS}	1.384^{NS}
soluble	25.163***	5.468**	110.758***	1.818^{NS}	0.471^{NS}	1.239^{NS}	3.107**
Total HPLC Phenolics	141.969***	2.4189^{NS}	6.881**	1.405^{NS}	1.817^{NS}	0.964^{NS}	0.286^{NS}
Total Papyriferic Acids	107.157***	3.555*	5.271*	3.860**	9.467***	2.048^{NS}	4.544**
papyriferic acid	121.609***	3.618*	3.382***	3.499**	9.659***	2.197^{NS}	4.465**
papyriferic acid der	107.377***	3.341*	4.349*	2.188*	2.079^{NS}	0.385^{NS}	3.129*
deacetyl papyriferic acid	150.581***	0.834^{NS}	0.483^{NS}	0.742^{NS}	0.133^{NS}	2.494^{NS}	0.838^{NS}
Total Pendulic Acids	4.077*	1.752^{NS}	3.284^{NS}	0.513^{NS}	7.338**	2.324^{NS}	3.052*
pendulic acid	1.452^{NS}	0.904^{NS}	172.328***	0.808^{NS}	0.509^{NS}	0.222^{NS}	0.435^{NS}
pendulic acid der 1	116.160***	0.638^{NS}	28.187***	1.030^{NS}	8.673***	2.462^{NS}	1.090^{NS}
pendulic acid der 2	1.420^{NS}	2.882*	5.954*	0.548^{NS}	5.648**	2.136^{NS}	4.552**
Total Terpenoids	58.229***	2.903*	5.717*	2.768*	9.949***	0.703^{NS}	4.022**
Nitrogen	3.801*	1.150^{NS}	37.798***	1.048^{NS}	0.516^{NS}	3.567^{NS}	1.178^{NS}

F and *df* values are presented; df_{err} = 161; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; NS = not significant. Sp. = Species; Fe. = Fertilization, DHPPG = 3'4 dihydroksipropiophenoneglucoside; der = derivative.

[a] Analyzed between saplings of *B. resinifera* and *B. platyphylla.*

[b] Not identified in the 4-year-old saplings grown in pot.

[c] Analyzed between saplings of *B. pendula.*

[d] Analyzed only between 3- and 4-year-old saplings.

[e] Not identified in the 4-year-old saplings

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FERTILITY, ROOT RESERVES AND THE COST OF INDUCIBLE DEFENSES IN THE PERENNIAL PLANT *Solanum carolinense*

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Abstract—We examined the relationship between internal resources (root reserves), external resources (soil fertility), and allocation to defense vs. growth in the clonal, perennial herb *Solanum carolinense*. In a short-term (9 d) greenhouse experiment, plants were treated once with jasmonic acid (JA) to determine if polyphenols and glycoalkaloids were inducible by simulated herbivory. In a longer-term (4 wk) greenhouse experiment, we measured the cost, in terms of growth, of treatment with JA every 3 d, to determine if the induced response was due more to carbon limitation or nitrogen limitation. We manipulated the resources available to the plants by varying soil fertility and the size of root cuttings from which plants were grown, and assessed how different resource levels affected the growth and production of polyphenols and alkaloids under JA treatment or control conditions. In the short term, JA increased the concentration of polyphenols in both above- and belowground plant parts, as well as alkaloid concentrations in the roots. In the long term, the only significant secondary chemistry response to JA was an increased polyphenol concentration in above ground tissues. The total amount of polyphenols produced was the same for JA and control plants, indicating that the higher concentration was a result of the lower biomass of treated plants. In contrast, alkaloid concentrations in plants treated with JA for 4 wk did not differ from controls, but JA-treated plants contained lower total amounts of alkaloids in above ground tissues, as a result of decreased growth. Fertilizer level and root cutting size had effects on growth and the production of secondary compounds and influenced the cost of induction. Plants grown under high

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fertility had a greater reduction in growth in response to JA than plants grown under low fertility, indicating a greater trade-off between growth and defense for high fertility plants. Plants from larger root cuttings grew bigger without any reduction in the concentration of polyphenols and alkaloids. We demonstrated that the phenotype of *S. carolinense* was plastic in response to simulated herbivory, fertility level, and root cutting size, and that there was a significant growth cost to induction that varied with the environment and appears to be due in large part to the allocation of limited carbon reserves.

Key Words—*Solanum carolinense*, horsenettle, inducible defenses, herbivory, nitrogen, carbon, nutrients, root reserves, polyphenols, alkaloids.

INTRODUCTION

Herbivore attack is nearly universal for plants; as a result, all plants possess some form of defense against herbivory (Strong et al., 1984). Despite the clear benefits of defenses against herbivores, natural selection does not always maintain constitutively high levels of defensive traits (Karban and Baldwin, 1997). If constitutive defense traits have costs that outweigh their benefits in some environments, contrasting selection pressures in different environments can promote selection for plasticity in responses to herbivores (Sultan, 1987; Via et al., 1995; De Witt et al., 1998; Kliebenstein et al., 2002). In theory, there should be selection for inducible defense traits when herbivore pressure is variable and when defense traits have a high cost in the absence of herbivores (Givnish, 1986; Karban and Baldwin, 1997). The ultimate measure of the cost of any trait is an associated reduction in fitness. For plants, the accumulation of biomass during growth is often correlated with survival and reproduction and can be used as a surrogate for fitness. Costs of constitutive or inducible defense production may arise by many mechanisms, including allocation trade-offs, ecological interactions, and genetic effects such as pleiotropy (Heil and Balwin, 2002). There may also be a cost of plasticity *per se* (De Witt et al., 1998; Agrawal et al., 2002; Relyea, 2002).

Many studies have quantified the cost of resistance manifested as a reduction in growth or reproduction (see reviews in Bergelson and Purrington, 1996 and Strauss et al., 2002), but the results are equivocal. In the survey by Bergelson and Purrington (1996), a cost was detected in only 29% of the cases. Some studies have demonstrated a cost (e.g., Baldwin, 1998; Redman et al., 2001), while others have not (e.g., Gianoli and Niemeyer, 1997), and still others found a cost in some situations but not in others (Agrawal, 1999; Siemens et al., 2002). It is becoming clear that the costs of a particular defensive phenotype are dependent upon the environment in which they are measured, including the resources available to the plant and the ecological interactions of the community

(Heil, 2002; Koricheva, 2002; Strauss et al., 2002). Inclusion of ecological costs and consideration of the conditions under which they are measured has improved the ability to identify them (Koricheva, 2002; Strauss et al., 2002), yet some theoretical predictions of when costs should be present lack strong empirical support. For example, Bergelson and Purrington (1996) suggested that costs of defense should increase in stressful environments because of resource limitation. This prediction is supported by some studies (Bergelson, 1994; Cipollini and Bergelson, 2001), but not by others (Karban, 1993; Siemens et al., 2002).

Allocation models, in which plants divide limited resources among growth, reproduction, and defense, are one of the more widely used theoretical approaches to studying phenotypic costs (reviewed in Stamp, 2003). How much a plant allocates to a particular function is assumed to depend on how much of the resource is available (Bryant et al., 1983; Maschinski and Whitham, 1989; Herms and Mattson, 1992; Hunter and Schultz, 1995), which in turn depends on the external environment (e.g., edaphic conditions or light levels) and on the internal environment (e.g., maternal effects or stored reserves). Correlated effects can complicate the measurement of allocation to inducible defensive traits; for example, plant hormones involved in responses to herbivory, such as jasmonic acid (JA) and abscisic acid, may also have effects on plant growth (Sembdner and Parthier, 1993; León et al., 2001; Heil and Balwin, 2002). Although there are models to explain the evolution of inducible defense responses that do not depend directly on an analysis of costs and benefits (e.g., Adler and Karban 1994; Karban et al., 1997; Jokela et al., 2000), we use the cost/benefit model here because it remains central to the study of inducible responses to herbivory.

The majority of studies that measure costs of inducible defenses use annual herbs because of the obvious advantage of being able to more accurately assess lifetime fitness; however, this has also limited the generality of the results. In this study, we used the clonal herb *Solanum carolinense* L. (Solanaceae) to look for a trade-off between the production of inducible secondary compounds and growth, and to gain an understanding of how root reserves and soil fertility influence that trade-off. *S. carolinense* is a long-lived perennial plant native to eastern North America, common in old fields and as an agricultural weed.

Our first objective was to determine if the levels of two classes of putative resistance compounds, polyphenols and alkaloids, were plastic in response to simulated herbivory in *S. carolinense*. Polyphenols can be directly toxic, or can reduce the digestibility of plant tissue, slowing the growth of herbivores and reducing feeding damage (Herms and Mattson, 1992; Appel, 1993; Karban and Baldwin, 1997). Polyphenol content in plants is a plastic trait that can increase with actual or simulated herbivory (Baldwin, 1998; Tscharntke et al., 2001; Francheschi et al., 2002; Rudell et al., 2002). Alkaloids contribute to plant resistance through toxicity. Work in species closely related to *S. carolinense* has

demonstrated that alkaloid levels can increase under actual and simulated herbivory, and that this can increase a plant's resistance (Baldwin et al., 1990; Baldwin, 1998; Baldwin and Hamilton, 2000). However, increased alkaloid content may not increase resistance to all herbivores, because many insects are specialists that can tolerate high levels of toxic compounds or use them for their own defense (Mullin et al., 1997; Bolser and Hay, 1998; Cipollini et al., 2002; Marcel et al., 2002; Siemens et al., 2002). The evolution of an increased production of alkaloids as a plastic response to herbivory will depend on the ratio of the levels of damage by specialist vs. generalist herbivores.

Our second objective was to determine if induction has a fitness cost in the absence of herbivores. JA is an endogenous plant-signaling molecule, which increases in plants under herbivore attack. When applied externally, it can stimulate plant defenses in the absence of herbivores (Zhang and Baldwin, 1997), allowing defense costs to be measured without the confounding affects of tissue loss. JA has been used successfully to induce defense responses in other members of the Solanaceae (Thaler et al., 1996; Baldwin, 1998; Cipollini and Redman, 1999; Baldwin and Hamilton, 2000; Redman et al., 2001). We used growth as the sole measure of fitness, because lack of pollinators in the greenhouse prevented seed production. We believe growth accurately represents fitness in *S. carolinense*, because its indeterminate growth form leads to a strong correlation between growth and flower and fruit production (Cipollini et al., 2002; Cipollini et al., 2004). Furthermore, vegetative reproduction is often the most important means of population growth and maintenance for clonal plants, while establishment by seed is used primarily for dealing with severe disturbance or for long distance dispersal (Cook, 1985; Erickson, 1993; Bazzaz, 1996; Pan and Price, 2002). Allocation to root growth in particular is an important component of fitness in clonal plants (Wijesinghe and Whigham, 1997), especially in weeds such as *S. carolinense* that survive tillage by sprouting new plants from severed root pieces (Tisdell, 1961; Rhode Island Agriculture Experimental Station, 1962).

Finally, we sought to understand how external and internal resource supply influenced the potential cost of exhibiting the induced phenotype in the absence of herbivores. We examined the influence of root reserves and soil fertility on patterns of allocation to growth and to the putative defense compounds polyphenols and glycoalkaloids. We expected soil fertility to influence allocation to growth vs. defense because of its influence on the C:N ratio (Bryant et al., 1983). Likewise, root reserves could influence allocation to growth vs. defense if root reserves are a significant source of C or N. We determined whether C and/or N limitation was responsible for the cost of producing inducible defenses in *S. carolinense*, that is, if there is a resource-based trade-off. If it is limited by N, then supplying excess N should reduce or eliminate the trade-off between growth and defense. If the production of an inducible defense is limited by C, then there should be less of a trade-off between growth and defense when C is abundant relative to N, such as under low fertility.

METHODS AND MATERIALS

Plant Material. In May 2001, fleshy roots of *S. carolinense* were excavated from an old field in Julian, PA, USA. Roots were collected from 24 locations, with each at least 2 m apart. Although *S. carolinense* has been known to produce lateral roots up to 15 m long (Rhode Island Agriculture Experimental Station, 1962), excavation at the field site did not reveal any longer than 1.5 m (R. Walls, personal observation), so the 24 plants used should represent a mix of genotypes.

Roots were rinsed with tap water and cut into small, medium, or large segments. Small root cuttings had a fresh mass of 0.400–0.500 g, medium cuttings had a mass of 1.0–1.199 g, and large root cuttings had a mass of 2.0–2.199 g. A mixture of small, medium, and large cuttings was taken from each genotype. To insure viability, all cuttings were sown in flats in Metro-Mix 250 (Scott's Corporation, Marysville, IN, USA). Two wk after sowing, all cuttings had sprouted shoots, except approximately 5% of the small cuttings. Root cuttings with sprouts were transferred into individual pots, 18 cm diam and 25 cm tall. The growth medium was Pro-Mix BX (Premier Horticulture Inc., Red Hill, PA, USA), a peat-based medium with no added nutrients. The pots were placed on a bench in a greenhouse, supplied with supplemental lighting from 8:00 A.M. to 8:00 P.M. daily, and watered as necessary with tap water. The placement of pots on the benches was randomized every 3 d to reduce microclimatic variation.

Experiment 1: Short-term Induction Experiment. A short-term experiment was conducted to determine if exogenous application of JA would increase the concentrations of glycoalkaloids (GA) and polyphenolics (PP) in *S. carolinense*. Twenty-four plants grown from medium and large root cuttings, as described above, were raised in the greenhouse for 2 wk after sprouting. They were fertilized weekly with approximately 1 l 18-18-21 Miracle-Gro for Tomatoes (Stern's Miracle-Gro, Port Washington, NY, USA), diluted to approximately 300 ppm N. Plants were treated on July 2, 2001 (day 0). One half were sprayed until saturation with 1 mM JA (JA treatment), and the other half with a solution of 0.8% ethanol in distilled water (control treatment). JA solution was made by dissolving 250 mg JA (Sigma, St. Louis, MO, USA) in 10 ml ethanol, then dissolving the JA/ethanol solution in 1.189 l water. To prevent contamination of control plants, JA-treated plants were sprayed in the hallway of the greenhouse, downwind of the airflow through the bays, and allowed to dry before being returned to the bench.

Three, 6, and 9 d after treatment, four plants from each treatment were harvested. Each had a main shoot and one to three side shoots. Young leaves were harvested by cutting the distal portion of each stem containing the terminal bud and two semiexpanded leaves. Old leaves were harvested by cutting all fully expanded leaves (except those touching the soil surface) at the petiole. After leaves were harvested, the stem was clipped at soil level, and the roots were rinsed

with tap water to remove growth medium. All tissues were placed in envelopes immediately after harvest, flash-frozen in liquid nitrogen, and maintained at −20°C until being freeze-dried. After freeze-drying, all tissues were ground in a UDY Cyclone Mill to prevent heating. The lyophilized powder was stored in sealed plastic vials at −20°C. Subsamples of young leaf tissue, old leaf tissue, and root tissue from each plant were analyzed for PP concentration (see below). Because there was insufficient young leaf tissue, GA concentration was analyzed in old leaves and roots only (see below). The data were analyzed using the MANOVA option of general linear model (GLM) in SAS 6.12 (SAS Institute, Cary, NC, USA), with JA treatment as the independent variable and PP and GA concentration in the leaves and roots as response variables. *Post hoc* contrasts were conducted for the response variables that showed a significant effect due to JA, with α adjusted by stepwise Bonferroni correction (Rice, 1989).

Experiment 2: Factorial Long-term Induction Experiment. One hundred and twenty plants were assigned to 12 different treatments. Treatments were full-factorial combinations of three cutting sizes (small, medium, and large), two fertility levels (low and high), and two hormone levels (1 mM JA and control). Different genotypes were assigned randomly to each treatment. High fertility treatments were watered every 3 d with approximately 1 L 18-18-21 Miracle-Gro for Tomatoes (Stern's Miracle-Gro, NY USA), diluted to approximately 300 ppm N. Low fertility plants were watered every 6 d with approximately 1 l 18-18-21 Miracle-Gro for Tomatoes, diluted to approximately 100 ppm N. Additional tap water was supplied as needed. JA and ethanol solutions for the hormone treatments were the same as those described for the short-term induction experiment. Plants were sprayed until saturation with JA or ethanol solution every 3 d for approximately 4 wk.

After 4 wk, plants were harvested for analysis. Each was cut at soil level and all aboveground tissues, including stems, leaves, and flowers, were placed into an envelope, flash-frozen in liquid nitrogen, and maintained at −20°C until freeze-drying. Roots were rinsed with tap water and handled as aboveground plant parts. Because the growth medium was peat-based, there was some contamination of the roots, but the same person rinsed all roots, so the level of contamination (approximately 2–4% peat fibers) was consistent for all plants (R. Walls, personal observation). Plant tissues were freeze-dried, ground and stored as described above, and weighed to the nearest 0.1 g.

Data were collected on growth parameters and levels of secondary compounds. Growth was measured as shoot mass, root mass, total plant mass (all dry masses), and relative growth. Relative growth was calculated as whole plant mass divided by the cutting size (0.45, 1.1, or 2.1 g). The concentrations of PP and GA in both roots and aboveground tissues were determined as described below. Total amount of PP and GA in roots and aboveground tissues was calculated as the product of concentration × dry mass. Data were analyzed

with the MANOVA option of the GLM in SAS 6.12, using all of the above response variables, with cutting size, fertility, and JA, plus first-order interactions, as effects. To control for possible correlations between the concentration of secondary compounds and plant mass, we conducted an analysis of covariance in the GLM using PP and GA concentrations as response variables, cutting size, fertility, and spray treatments, plus interactions, as effects, and total plant mass as a covariate. As no significant effect of total plant mass was found for any of the variables, PP and GA concentrations were included in the main MANOVA. *Post hoc* contrasts were conducted for all response variables that showed a significant effect as a result of any of the treatments, with α adjusted by stepwise Bonferroni correction (Rice, 1989).

Polyphenol Analysis. We used purified PP from the plant tissues collected from each experiment as standards. The use of self standards takes into consideration the variability in PP composition that occurs with different treatments and harvest dates, and provides a more accurate measurement of PP concentration than the use of commercial standards (Appel et al., 2001). Bulk samples were prepared for use as a standard by mixing together approximately 0.2 g of lyophilized tissue from each plant. Separate standards were prepared for each tissue type (roots, young leaves, and old leaves for experiment 1, and roots and aboveground tissues for experiment 2). For experiment 1, separate standards were prepared for each harvest date. Purified PP for standards was extracted using the method of Hagerman and Klucher (1986). Five g of tissue were washed with 75 ml ether $\times$ 3 to remove pigments and waxes, then extracted under sonication once with 50 ml 70% acetone and $\times$ 3 with 25 ml 75% acetone. Acetone was removed by evaporation under reduced pressure. Nonphenolics were removed by thoroughly mixing the extract solution with a slurry of 50 g Sephadex (Pharmacia, Piscataway, NJ, USA) in 1 l of 95% ethanol then centrifuging. This process was repeated until the eluant tested negative for the presence of PP (Appel et al., 2001). PPs were removed from the Sephadex by mixing with 150 ml of 70% acetone and shaking for 5 min. The acetone rinse was repeated until the eluant tested negative for the presence of PP with Prussian blue. Acetone was removed by evaporation under reduced pressure, and the resulting solution was freeze-dried to yield dried, powdered tannins for use as standards.

PP concentration of plant tissues was analyzed using the Folin Denis assay (Swain and Hillis, 1959; Swain and Goldstein, 1964; Appel et al., 2001). PPs were extracted from 0.01 g freeze-dried tissue with 2 ml of 50% methanol then rinsed with 2 ml hexane. The concentration of PP in each extraction was calculated by measuring absorbance at 725 nm after reaction with Folin Denis reagent and converting absorbance to g total polyphenols/g dry tissue.

Glycoalkaloid Analysis. GA concentration in tissue samples was analyzed with the method of Birner (1969). In this technique, GAs of *S. carolinense*

(primarily solasonine and solamargine) are simultaneously extracted and hydrolyzed to their steroidal base solasodine. An advantage of this technique is that it uses fairly large quantities of tissue and is sensitive in the analysis of plant tissues with low alkaloid concentrations. Because GAs in *S. carolinense* hydrolyze to the single compound, solasodine, this technique is adequate to estimate allocation to glycoalkaloids as a group. Freeze-dried tissue (0.2 g) was first dissolved in 5 ml 1 N HCl, hydrolyzed for 2 hr at 100°C under reflux conditions, then neutralized with 5 ml 1 N NaOH. Five ml glacial acetic acid were added, and the hydrolyzed extract was filtered and diluted to 25 ml with ddH2O. Commercial solasodine (Sigma, MO, USA) was used as a standard. Duplicate 5-ml aliquots of samples and standards were added to reaction tubes followed by 5 ml acetate buffer (pH 4.7), 1 ml 5% methyl orange solution, and 5 ml methylene dichloride. The tubes were vortexed, contents allowed to separate, and the methylene dichloride layer was removed to a spec tube. Absorbance was read at 420 nm and converted to mg solasodine/g dry tissue.

RESULTS

Experiment 1: Short-term Induction Experiment. The MANOVA showed a multivariate effect for JA and date of harvest on PP concentration and GA concentration (JA: $F = 10.458$, $P < 0.001$; date: $F = 4.676$, $P < 0.001$). Application of JA resulted in a significant increase in PP concentration in young leaves, old leaves, and roots (Tables 1 and 2, Figure 1A). Date of harvest did not affect PP concentration in leaf tissue, but there was a rapid and significant decline in PP level in the roots of both JA and control plants over 9 d, independent of the effect of JA. GA concentration in roots, but not old leaves, was significantly higher in JA-treated plants than controls during the short-term

TABLE 1. RESULTS OF THE UNIVARIATE ANALYSES OF VARIANCE FOR THE EFFECTS OF JASMONIC ACID (JA) AND DATE OF HARVEST ON POLYPHENOL (PP) CONCENTRATION AND GLYCOALKALOID (GA) CONCENTRATION OF *S. carolinense* FOR THE SHORT-TERM INDUCTION EXPERIMENT

		PP concentration				GA concentration					
		Old leaves		Young leaves		Roots		Old leaves		Roots	
Source	*df*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
JA	1	11.46	0.004	22.41	>0.001	6.03	0.026	0.05	0.833	16.17	0.001
Date	2	0.19	0.832	0.82	0.457	26.50	>0.001	2.22	0.141	1.17	0.336
JA*Date	2	0.44	0.651	0.39	0.681	1.80	0.197	3.01	0.078	0.56	0.584

TABLE 2. LEAST-SQUARE MEANS AND *P* VALUES OF CONTRASTS BETWEEN JASMONIC ACID (JA) AND CONTROL TREATMENTS, FOR RESPONSE VARIABLES THAT SHOWED A SIGNIFICANT EFFECT OF JA IN THE ANOVAS OF TABLE 1

Response variable	JA	Control	*P*
PP old leaves (mg/mg)	0.034	0.029	0.004
PP young leaves (mg/mg)	0.045	0.035	<0.001
PP roots (mg/mg)	0.183	0.150	0.026
GA roots (mg/g)	4.915	3.548	0.001

PP = polyphenols, GA = glycoalkaloids.

experiment (Tables 1 and 2; Figure 1B). There was no effect of date on GA concentration. Both PP and GA were 1 order of magnitude lower in leaves than in roots.

Experiment 2: Factorial Long-term Induction Experiment. There was a multivariate effect for JA, cutting size, and fertilizer on growth and secondary chemistry response variables (MANOVA results for JA: $F = 3.251$, $P < 0.001$; cutting size: $F = 25.923$, $P < 0.001$; fertilizer: $F = 27.939$, $P < 0.001$).

Experiment 2: JA Effects. Unlike experiment 1, treatment with JA led to significantly increased PP concentration in aboveground tissues but not in roots (Tables 3 and 4; Figure 2). Concentration of PP in aboveground tissues after 4 wk was 1 order of magnitude higher than that in young and old leaves in experiment 1 after 9 d, while the concentration of PP in root tissue after 4 wk was similar to that in roots after 9 d (Figures 1A and 2B). In contrast to concentration, JA had no significant effect on the total amount of PP in either the roots or the aboveground tissues (Table 3, Figure 2). Application of JA had no significant effect on GA concentration in either the aboveground tissues or the roots of plants in experiment 2 (Table 3, Figure 3). However, treatment with JA significantly lowered the total amount of GA in aboveground tissues (Tables 3 and 4, Figure 3C). JA treatment also had an effect on growth of *S. carolinense*. Plants treated with JA had lower relative growth, aboveground mass, root mass, and total plant mass (Tables 3 and 4, Figure 4).

Experiment 2: Fertilizer Effects. The application of fertilizer significantly increased plant growth (Table 3 and Figure 4). Although belowground mass was not influenced by fertility level, aboveground mass, total plant mass, and relative growth all increased under high fertility (Table 4). There was a significant JA $\times$ fertilizer interaction for total mass and relative growth, indicating that fertilized plants had a growth response to JA treatment and unfertilized plants did not (Figure 4). Fertilized plants had lower PP concentrations in both aboveground tissues and the roots, while the amount of PP in the roots was lower for fertilized plants, but the same in aboveground tissues (Tables 3 and 4, Figure 2). There were no interactions between fertilizer and cutting size or JA for PP

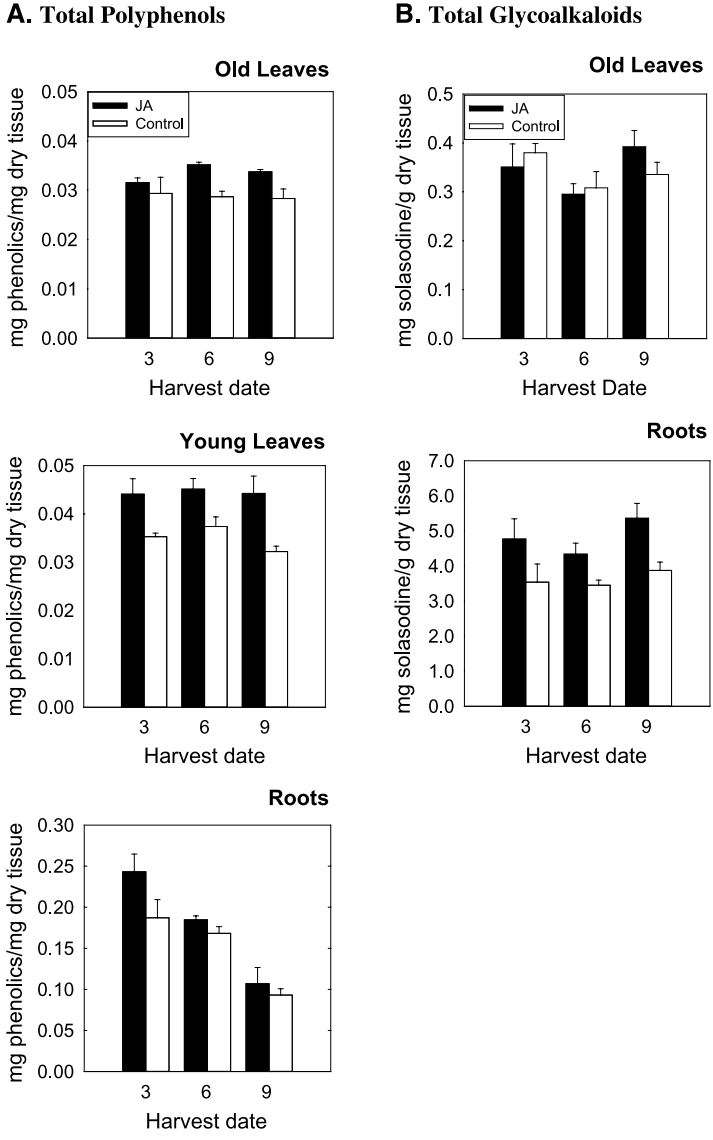

FIG. 1. (A) Concentration of total polyphenols in young leaves, old leaves, and roots of *S. carolinense* 3, 6, and 9 d after treatment with jasmonic acid or control solution, measured by reaction with the Folin Denis reagent. (B) Concentration of total glycoalkaloids in young and old leaves of *S. carolinense* 3, 6, and 9 d after treatment with jasmonic acid or control solution, measured as the steroidal base solasodine. Bars represent the mean of four plants ± SE.

TABLE 3. RESULTS OF THE UNIVARIATE ANALYSES OF VARIANCE FOR THE EFFECTS OF JASMONIC ACID, FERTILIZER, CUTTING SIZE AND FIRST-ORDER INTERACTIONS ON GROWTH AND SECONDARY CHEMISTRY RESPONSE VARIABLES IN *S. carolinense*

	df	*F*	*P*
Aboveground dry mass			
JA	1	9.18	0.003
Cutting size	2	13.49	<0.001
Fertilizer	1	87.85	<0.001
JA*Cutting size	2	2.16	0.122
JA*Fertilizer	1	2.97	0.089
Cutting size*Fertilizer	2	2.90	0.060
Root dry mass			
JA	1	7.58	0.007
Cutting size	2	25.26	<0.001
Fertilizer	1	1.04	0.311
JA*Cutting size	2	1.12	0.330
JA*Fertilizer	1	3.13	0.081
Cutting size*Fertilizer	2	0.83	0.438
Total dry mass			
JA	1	10.07	0.002
Cutting size	2	18.00	<0.001
Fertilizer	1	64.90	<0.001
JA*Cutting size	2	2.05	0.135
JA*Fertilizer	1	3.43	0.068
Cutting size*Fertilizer	2	2.60	0.079
Relative growth			
JA	1	8.85	0.004
Cutting size	2	67.66	<0.001
Fertilizer	1	34.87	<0.001
JA*Cutting size	2	4.01	0.022
JA*Fertilizer	1	5.37	0.023
Cutting size*Fertilizer	2	1.73	0.184
Aboveground PP concentration			
JA	1	13.21	<0.001
Cutting size	2	2.86	0.063
Fertilizer	1	126.51	<0.001
JA*Cutting size	2	0.82	0.443
JA*Fertilizer	1	0.09	0.764
Cutting size*Fertilizer	2	2.80	0.066
Root PP concentration			
JA	1	2.47	0.120
Cutting size	2	10.14	<0.001
Fertilizer	1	39.35	<0.001
JA*Cutting size	2	0.83	0.438
JA*Fertilizer	1	0.00	0.994
Cutting size*Fertilizer	2	0.37	0.691
Aboveground PP amount			
JA	1	0.29	0.593

TABLE 3. CONTINUED

	df	*F*	*P*
Cutting size	2	15.4	<0.001
Fertilizer	1	0.01	0.926
JA*Cutting size	2	0.45	0.639
JA*Fertilizer	1	0.10	0.749
Cutting size*Fertilizer	2	0.93	0.397
Root PP amount			
JA	1	1.33	0.253
Cutting size	2	24.95	<0.001
Fertilizer	1	5.69	0.019
JA*Cutting size	2	0.22	0.804
JA*Fertilizer	1	0.87	0.352
Cutting size*Fertilizer	2	0.65	0.523
Aboveground GA concentration			
JA	1	1.81	0.182
Cutting size	2	3.25	0.044
Fertilizer	1	2.52	0.116
JA*Cutting size	2	4.11	0.020
JA*Fertilizer	1	2.72	0.103
Cutting size*Fertilizer	2	0.07	0.928
Root GA concentration			
JA	1	1.24	0.270
Cutting size	2	8.69	<0.001
Fertilizer	1	44.56	<0.001
JA*Cutting size	2	0.26	0.775
JA*Fertilizer	1	0.03	0.855
Cutting size*Fertilizer	2	1.38	0.257
Aboveground GA amount			
JA	1	10.41	0.002
Cutting size	2	1.14	0.324
Fertilizer	1	67.93	<0.001
JA*Cutting size	2	4.45	0.015
JA*Fertilizer	1	9.20	0.003
Cutting size*Fertilizer	2	0.35	0.703
Root GA amount			
JA	1	0.33	0.569
Cutting size	2	17.10	<0.001
Fertilizer	1	16.92	<0.001
JA*Cutting size	2	0.39	0.680
JA*Fertilizer	1	0.13	0.721
Cutting size*Fertilizer	2	1.80	0.172

PP = polyphenols, GA = glycoalkaloids, JA = jasmonic acid.

TABLE 4. LEAST SQUARE MEANS FOR RESPONSE VARIABLES THAT SHOWED A SIGNIFICANT TREATMENT AFFECT IN THE ANOVAS IN TABLE 3[a]

	JA treatments		Significance level[b]
Response variable	JA	Control	JA vs. Control
AG dry mass (g)	13.21 a	16.03 b	*
Root dry mass (g)	3.59 a	4.31 b	*
Total dry mass (g)	16.81 a	20.35 b	**
Relative growth (g/g)	17.72 a	22.13 b	*
AG PP concentration (mg/mg)	0.175 a	0.146 b	**
AG GA amount (mg)	7.65 a	10.27 b	**
	Fertilizer treatments		
	High	Low	High vs. Low
AG dry mass (g)	19.00 a	10.25 b	**
Total dry mass (g)	23.09 a	14.07 b	**
Relative growth (g/g)	24.32 a	15.53 b	**
AG PP concentration (mg/mg)	0.115 a	0.206 b	**
Root PP concentration (mg/mg)	0.120 a	0.157 b	**
Root PP amount (g)	0.501 a	0.611 b	*
Root GA concentration (mg/g)	3.27 a	5.72 b	**
AG GA amount (mg)	12.32 a	5.60 b	**
Root GA amount (mg)	13.67 a	22.80 b	**
	Cutting size		
	Small	Medium	Large	S vs. M/S vs. L/M vs. L
AG dry mass (g)	11.57 a	14.76 b	17.55 c	**/**/*
Root dry mass (g)	2.75 a	4.06 b	5.06 c	**/**/**
Total dry mass (g)	14.32 a	18.81 b	22.60 c	**/**/*
Relative growth (g/g)	31.82 a	17.22 b	10.73 c	**/**/**
Root PP concentration (mg/mg)	0.120 a	0.148 b	0.149 b	**/**/ns
AG PP amount (g)	1.58 a	2.12 b	2.64 c	*/**/*
Root PP amount (g)	0.337 a	0.594 b	0.737 c	**/**/*
AG GA concentration (mg/g)	0.686 a	0.618 a,b	0.529 b	ns/*/ns
Root GA concentration (mg/g)	3.42 a	4.82 b	5.24 b	**/**/ns
Root GA amount (mg)	9.77 a	19.17 b	25.76 c	**/**/**

[a] Different letters within one row indicates that the means are significantly different from one another.

[b] There were a total of 45 unplanned contrasts. Four of the contrasts were nonsignificant (ns). Of the remaining 41 contrasts, all but 10 were significant at an experiment-wide error rate of $\alpha = 0.05$ adjusted by stepwise Bonferroni correction (**), and the remaining 10 contrasts were significant at an experiment-wide error rate of $\alpha = 0.08$ (*).

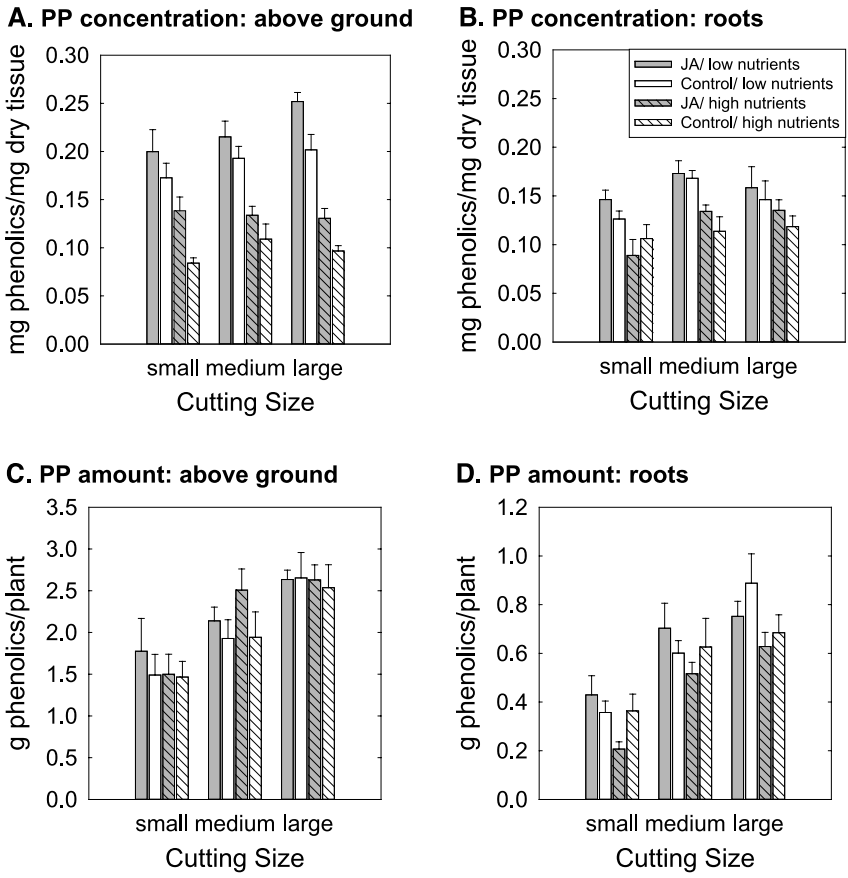

FIG. 2. Mean levels of total polyphenols (PP) ± standard error in above ground and below ground plant tissues of *S. carolinense*, measured by reaction with the Folin Denis reagent.

concentration or amount (Table 3). Fertilizer influenced GA concentration in the roots, but not in aboveground tissues, with fertilized plants having significantly lower GA concentrations in the roots (Tables 3 and 4, Figure 3B). Fertilizer affected the total amount of GA in both the roots and the shoots, with fertilized plants having higher amounts of GA in aboveground tissues, but lower amounts of GA in the roots (Tables 3 and 4, Figure 3C and D). There were no interactions between fertilizer and cutting size or JA for GA concentration, but there was a significant JA × fertilizer interaction for the amount of GA in aboveground tissues, with high fertility plants responding to JA while low fertility plants did not (Table 3, Figure 3C).

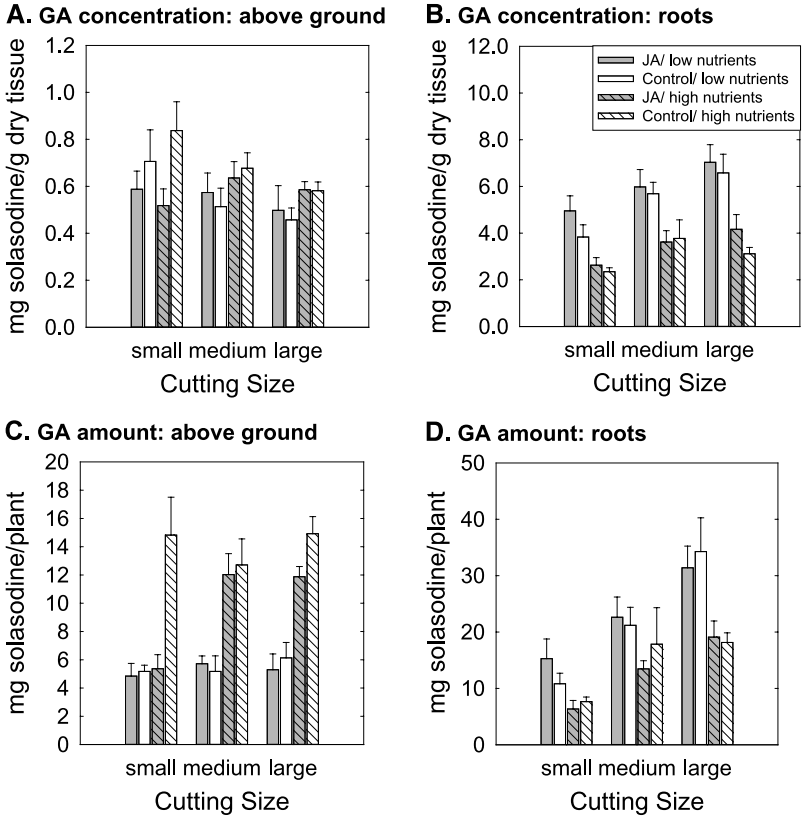

FIG. 3. Mean levels of total glycoalkaloids (GA) ± standard error in above- and belowground plant tissues of *S. carolinense*, measured as the steroidal base solasodine.

Experiment 2: Root Reserve Effects. Cutting size had a significant effect on the growth of *S. carolinense* in experiment 2 (Table 3, Figure 4). Plants from larger cuttings had greater root and aboveground mass, whereas plants from smaller cuttings had higher relative growth (Table 4). There was no significant JA × cutting size interaction for plant mass, but there was an interaction between JA and cutting size for relative growth. Plants from small and large cuttings had more of a reduction in relative growth when sprayed with JA than did plants from medium cuttings, a response due primarily to low fertility plants (Figure 4). Plants from larger cuttings had higher concentrations of PP in the roots but not in aboveground tissues, while the amount of PP in plants from larger cuttings was higher in both the roots and aboveground tissues (Tables 3 and 4, Figure 2). There were no interactions between cutting size and JA or

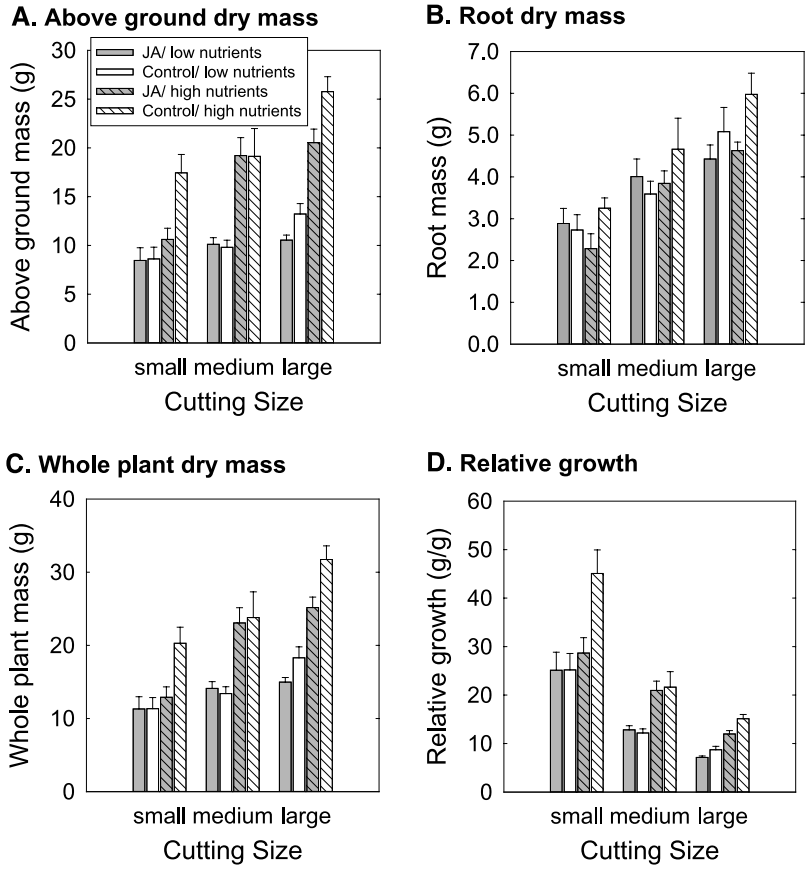

FIG. 4. Mean growth responses of *S. carolinense* ± standard error.

fertilizer for PP concentration or PP amount. Cutting size had a significant effect on GA concentration and amount (Table 3). GA concentration in the roots increased with cutting size. Although the ANOVA indicated an effect of cutting size on GA concentration in aboveground tissues ($P = 0.044$), the decrease in GA concentration in aboveground tissues between plants from small vs. large cuttings was only marginally significant (Table 4). GA amount increased with cutting size in the roots but not the aboveground tissues (Table 4). There was a significant interaction between JA and cutting size for both the concentration and amount of GA in the shoots (Table 3), showing that plants from smaller cuttings responded more to JA than plants from medium and large cuttings. This response was a decrease in GA concentration and amount under JA treatment for small plants (Figure 3).

DISCUSSION

Induction of Secondary Compounds. The first goal of this study was to determine if the levels of PP and GA would increase in response to the application of JA. We found a clear pattern of induction for PP concentration in the above ground tissues of *S. carolinense*, in both experiments 1 and 2 (dark vs. light stippled or solid bars in Figures 1A and 2A). PP concentration in above ground tissues after 4 wk in experiment 2 was approximately 5 times higher than PP concentration in leaves after 9 d in experiment 1. This difference in PP concentration between experiments may be attributable to the use of different tissues: leaves in experiment 1 vs. all aboveground tissue in experiment 2. However, it is unlikely that stems would have a much higher PP concentration than leaves. Alternatively, the increase may have resulted from an accumulation of PP in *S. carolinense* during the growing season that was not detectable during the 9-d time course of experiment 1. It could also be the result of the JA doses applied in each experiment. Plants in experiment 1 received only one JA application, whereas those in experiment 2 were sprayed every 3 d for 4 wk. Similar increases in the level of response variables with increasing jasmonate application have been shown in other studies (Thaler et al., 1996; Redman et al., 2001).

Unlike in shoots, application of JA increased PP concentration in roots only during the short-term experiment. At the end experiment 2, there was no significant difference in PP levels in the roots between JA and control plants, measured as either concentration or amount. However, PP levels in roots did change over the course of the growing season, independent of JA treatment. PP levels in roots declined during the 9 d of experiment 1 (Figure 1A), and the levels after 4 wk were similarly low in experiment 2 (Figure 2B). During experiment 1, PP concentrations in roots were almost 10 times higher than in leaves (~10–25% in roots vs. ~2.9–4.5% in shoots), whereas in experiment 2, the concentrations were similar in roots and aboveground tissues (~10–25%). The differences may relate to the functional role of PP in different tissues at different times, as well as how and when PPs are manufactured and stored by the plant. During the growing season, PPs are manufactured in the leaves, where they contribute to herbivore resistance. In the earliest part of the growing season, much of the carbon acquired by plants is shunted into growth. Later, as growth slows down, more carbon becomes available for secondary compounds, so levels of PP can be expected to increase. If the roots act as overwinter storage organs for PP, then levels in roots should be high early in the season (i.e., at day 3 in experiment 1) and decline as reserves are transferred to growing shoots (i.e., at day 9 in experiment 1 or at the end of experiment 2). High PP levels in the roots over winter and in the spring, and the plastic levels of PP in roots in the earliest part of the growing season may serve to protect roots at a time when there is little photosynthetic activity to replace lost root tissue, and

when belowground herbivores and pathogens may be more active because of higher soil moisture.

Induction of GA in response to JA was less consistent than induction of PP. The only significant effect of JA on GA concentration was in the roots of plants during the short-term experiment. Although average GA concentrations were roughly the same in experiments 1 and 2, there was more variability in experiment 2 (~3–5.5 mg/g in experiment 1 vs. ~2.5–7 mg/g in 2). The greater environmental variability of the factorial experiment may have masked the effects of JA that were detectable in the less complex short-term experiment. The lack of induction in experiment 2 may also have been a result of pot-bound plants. Baldwin (1988) demonstrated that pot-bound tobacco plants were not inducible for alkaloid production. Alternatively, like PP levels, GA levels in roots may respond to JA only early in the growing season. This temporal change in inducibility could arise if belowground herbivore pressure is more variable in the spring than summer, or if the production of GA and PP is more costly in the spring than summer (because, for example, plants need resources for rapid growth during the spring).

GA concentrations in aboveground tissues were low and did not respond significantly to JA treatment in either experiment. Although previous studies in the Solanaceae have demonstrated induction of alkaloids, most have concerned plants such as *Nicotiana* that produce tropane alkaloids (e.g., Baldwin 1988), which differ from the steroidal glycoalkaloids produced by *Solanum* in structure, activity, and the sites of biosynthesis. Furthermore, Cipollini et al. (2001) have shown that *S. carolinense* has relatively low levels of GA compared to congeners. Increasing concentration of GA in response to herbivory may be of little value against the primarily specialist herbivores of *S. carolinense* (Solomon, 1983; Wise and Sacchi, 1996), because studies have shown that specialists are unlikely to be negatively affected by increased alkaloids levels (Mullin et al., 1997; Bolser and Hay, 1998; Marcel et al., 2002). In experiment 2, the total amount of GA in the aboveground tissues of JA-treated plants was lower, especially for the smallest plants. (Table 4, dark vs. light bars in Figure 3C), but this was primarily due to the greatly reduced growth of high fertility plants in to response JA, especially for plants from small root cuttings (Figure 4).

The Cost of Induction. The second goal was to determine if there was a cost of possessing the putatively defensive phenotype (with higher JA levels) in the absence of herbivores. The factorial greenhouse experiment demonstrated a growth cost. Plants treated with JA had lower relative growth, lower root mass, and lower aboveground mass than controls. Stimulation of inducible defenses by JA can lead to reduced growth either through a direct trade-off of the resources allocated to defense or through inhibition of growth, irrespective of the allocated resources (Sembdner and Parthier, 1993; León et al., 2001). In a study of plant growth in response to JA and herbivory, Moore et al. (2003a,b)

found that both treatments caused similar decreases in expansion rates of subsequent leaves, suggesting that growth reduction is a feature characteristic of plant response to herbivory and not simply an artifact of JA. Although JA treatment increased the concentration of PP in aboveground tissues, it had no effect on the total amount of PP per plant (dark bars are higher than light bars in Figure 2A but not different in Figure 2C, Table 3). PP concentration did not covary with mass, but since concentrations are a function of the amount of chemical produced and plant biomass, changes in concentrations may be a result of either changes in production of the secondary compound or changes in biomass (Koricheva, 1999). Because we found an increased concentration of PP under JA treatment, a decrease in the aboveground mass, and no difference in the total amount of PP produced by JA-treated plants, it appears that *S. carolinense* did not necessarily allocate more resources to defense *per plant*, but instead allocated fewer resources to growth and more resources to defense *per gram of plant*. This allocation pattern does not conclusively distinguish between direct inhibition of growth and a trade-off between growth and defense, but much of the cost of induction appears to be associated with a trade-off in the allocation of limited carbon at the whole plant level (see below). Regardless of the mechanisms by which JA treatment reduced growth, turning on the defense response in *S. carolinense* in the absence of herbivores had an associated cost. This is consistent with the theory of the evolution of inducible defenses, and with theories of the evolution of plastic traits in general (Karban and Baldwin, 1997; De Witt et al., 1998).

The Trade-off Between Growth and Secondary Compounds. We found a clear pattern of reduced growth for plants treated with JA, which we interpret as a cost of induction. However, the cost was not independent of the environment. By examining how resource levels influenced the relative allocation to growth or defense, we were able to address our third goal of determining whether or not this cost was associated with carbon or nitrogen limitation. We hypothesized that if nitrogen limitation were largely responsible for the cost of induction, then under low nitrogen conditions there should be a trade-off between growth and defense, while under high nitrogen conditions there should be no (or less of a) trade-off. If carbon limitation were largely responsible, then under low carbon conditions (high fertility) there should be a trade-off between growth and defense, whereas under high carbon conditions (low fertility) there should be less of a trade-off or no trade-off. For this analysis, we focus on PP concentration as our measure of defense response, because PP concentration responded more consistently to JA than did GA concentration.

The pattern we found was consistent with carbon limitation and inconsistent with nitrogen limitation. Induction occurred in both low- and high nutrient environments, as is clearly seen by separately comparing the PP concentration in either roots or aboveground tissues for JA vs. control plants under

high and low fertility (compare dark vs. light solid bars or dark vs. light stippled bars in Figure 2A and B). This is supported by the lack of a significant JA × fertilizer interaction for PP concentration ($P = 0.76$ for aboveground tissues, $P = 0.99$ for roots). When comparing plants treated with JA to controls, high fertility (putatively high N and low C) plants had increased PP concentration but decreased growth (compare dark vs. light stippled bars in Figures 2A and 4D). Low fertility plants (putatively low N and high C) had increased PP concentration and the same growth when treated with JA or control solution (compare dark vs. light solid bars in Figures 2A and 4D). While JA treatment led to a strong reduction in growth for plants in the high nutrient environment, it did not affect growth in the low nutrient environment (Figure 4D). This result is supported by a significant JA × fertilizer interaction for relative growth ($P = 0.02$) and a marginally significant JA × fertilizer interaction for total plant mass ($P = 0.07$), and suggests that there was a trade-off between growth and defense under low C/N conditions and no trade-off under high C/N conditions.

The Effects of Fertilizer on Growth and Secondary Compounds. Independent of induction or cutting size, fertilizer had significant effects on growth and the production of secondary compounds. Fertilized plants were larger aboveground and had higher relative growth, as well as lower PP concentrations in both the roots and the aboveground tissue. Increased growth is a typical and expected reaction to fertilizer application. Lower PP concentration under high fertility is consistent with a number of plant defense models (e.g., Grime, 1979; Bryant et al., 1983; Coley et al., 1985; Herms and Mattson, 1992) proposing that when plants have access to unlimited nutrients, they are able to grow quickly and need not be so well defended against herbivores. Our results appear to contradict a recent study by Cipollini et al. (2002, 2004), who found an increase in PP concentration under high fertility for *S. carolinense*. Cipollini et al. (2002, 2004) analyzed PP concentration in leaf tissue only, whereas we analyzed it in the entire aboveground biomass. If fertilization changes the ratio of support tissue to leaf tissue, and if support tissue has substantially lower levels of extractable PP than leaf tissue, then increasing the proportion of support tissue under high fertility could lower the whole plant concentration of PP while maintaining high levels of PP in the leaves. This allocation pattern could help to offset a C-based trade-off between growth and defense under high N.

In experiment 2, high and low fertility plants had the same concentrations of GA in their aboveground tissues, consistent with the results of Cipollini et al. (2002). High fertility plants had higher total amounts of GA in their aboveground tissues, but this was due to the higher mass of fertilized plants. Similar to PP, fertilization led to *lower* concentrations of GA in the roots. Although other studies with plants in the Solanaceae have demonstrated an increase in alkaloid concentration under high fertility (e.g., Al-Humaid, 2003;

Armer et al., 2004), or an increased trade-off between reproduction and alkaloid production under low fertility (Baldwin et al., 1998), this study indicates that the production of GA in *S. carolinense* is limited more by C than by N. GAs are large molecules compared to most alkaloids, with a high C/N ratio, increasing the likelihood that C availability would limit their production. The much higher concentration of GA in the roots than in the aboveground tissues, and its plasticity in roots early in the short-term experiment (Figures 1 and 3) suggest that GAs play a more important role in defense belowground than in aboveground vegetative tissues. We were not able to measure GA levels or induction in fruits, but GAs are known to protect the fruits of *S. carolinense* from frugivores and fungal pathogens (Cipollini and Levey, 1997), and *S. carolinense* seems to maintain fairly constant GA concentration in its fruits (Cipollini et al., 2004).

The Effects of Root Cutting Size on Growth and Secondary Compounds. Like fertility, the size of the root cutting from which plants were grown had effects on growth and the production of secondary compounds. Although plants grown from smaller cuttings had higher (but more variable) relative growth (Figure 4D), plants from larger cuttings were larger after 4 wk (Figure 4C). In contrast to the fertility treatment, where larger plants had a decrease in the concentration of defense chemicals, plants from larger cuttings had higher concentrations of PP and GA in their roots and the same PP concentrations in the aboveground tissues (Table 4). Because of their larger size, plants from larger cuttings also had higher total amounts of PP in both their roots and their shoots and GA in their roots (Figures 3C–D and 4C–D). The contrasts between the effects of fertilizer and cutting size probably arise from their different effects on growth: high fertility leads to larger plants with higher relative growth, whereas larger cutting size leads to larger plants with lower relative growth. Lower relative growth appears to allow plants to accumulate higher concentrations of secondary compounds. This pattern was less clear for alkaloids in the aboveground tissues. Plants from larger cuttings had lower concentrations of GA in their aboveground tissues than plants from smaller cuttings, but the significance was marginal. Plants from larger cuttings still had greater total amounts of GA because of their greater shoot mass (Table 4).

Based on these results, larger roots in the beginning of the growing season could provide protection from herbivores in two ways: plants from larger cuttings have greater vegetative growth and have higher or equal production of putative defense chemicals. If the effects we observed in the greenhouse hold true for in the field, this advantage will be passed on and possibly magnified in the following season, as plants that started from larger roots one year will begin the following year with even larger roots. This prediction is based on the assumption that root mass in the middle of the growing season, which we measured, is directly related to the mass of the roots that survive the winter.

We analyzed our data to determine if larger root cuttings offset the trade-off between growth and secondary compound production in *S. carolinense*. The only significant interactions between JA and cutting size were for alkaloid concentration in the shoots and relative growth (Table 3). The JA × cutting size interaction for relative growth was uninformative. The interaction showed that plants from small and large cuttings had more of a reduction in growth when sprayed with JA than plants from medium cuttings, which does not lead to any conclusions regarding any allocation trade-off. Plants from smaller cuttings responded more to JA than plants from larger cuttings in aboveground GA concentration. However, as the overall results suggest that shoot alkaloid concentration does not respond to JA treatment, this result has little meaning. Overall, cutting size did not have an affect on the response to JA, so that plants from different size cuttings had the same proportional reduction in growth and the same proportional increase in PP level when treated with JA. Nonetheless, our data support the hypothesis that cutting size will affect the trade-off between growth and defense because of the positive effects of cutting size on both plant mass and levels of secondary compounds (Figures 2 and 4). Although the response was not qualitatively different than that of plants from small cuttings, plants from larger cuttings were better equipped to deal with herbivory, because they were bigger, had higher levels of PP, and had no reduction in GA levels.

Conclusions. In summary, the concentration of PP is an inducible trait in *S. carolinense* that responds not only to simulated herbivory, but also to exogenous and endogenous environmental conditions. However, the total amount of PP a plant produces is less variable. For example, plants have lower PP concentrations in aboveground tissues when fertilized and higher PP concentrations in aboveground tissues when treated with JA, but have the same total amount of PP under either treatment. Rather than change the total amount of resources allocated to PP production, *S. carolinense* plants appear to adjust allocation to growth in response to external environmental changes. This suggests a trade-off between PP production and growth in response to both JA and fertilizer. Larger root reserves, on the other hand, allowed plants to grow larger without reducing PP concentrations or amounts. Total GA production appears to be less plastic in response to JA, but does vary in response to resource supply (fertilizer and cutting size).

The application of JA resulted in reduced growth, demonstrating a cost of carrying the induced phenotype. This cost depended on the edaphic environment. In a low nutrient environment, there was no reduction in growth for treated plants, so induction with JA was not costly. In a high nutrient environment, induction carried a large cost in terms of growth. Based on this analysis, it appears that much of the cost was associated with PP production. However, other unmeasured defense responses, such as enzymes and trichomes, could also represent significant material investments for induced plants. Three

lines of evidence suggest that the cost of induction of a putatively defensive phenotype is due at least in part to carbon limitation and a resource trade-off. First, fertilized plants, which presumably had lower C/N ratios, had lower concentrations of PP and equal or lower concentrations of GA, suggesting that C was more limiting than N to PP and GA production. Second, there was a trade-off between induced defenses and growth, for which fertilizer (excess nitrogen) did not compensate. Although fertilized, induced plants were able to grow more than unfertilized, induced plants, they had lower concentrations of defense chemicals. Finally, large root reserves, which are likely a significant source of carbon, allowed plants to reduce the trade-off between growth and inducible defenses.

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INFLUENCE OF SEASONAL NITROGEN NUTRITION FLUCTUATIONS IN ORANGE AND LEMON TREES ON POPULATION DYNAMICS OF THE GLASSY-WINGED SHARPSHOOTER (*Homalodisca coagulata*)

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Abstract—The glassy-winged sharpshooter (GWSS) is a xylem feeder that develops conspicuous, year-round populations in many citrus-producing regions of California. Field studies were conducted in a combined lemon and orange orchard to determine the influence of changing amino acid concentrations on relative densities of GWSS. Nineteen protein amino acids were detected in xylem fluid of both lemon and orange trees. Although annual profiles of total and essential amino acids were similar for each citrus species, mean concentrations were consistently higher in lemons than in oranges for most of the year, except for one critical period in late winter–early spring when concentrations were higher in oranges. Principal component analysis followed by factor analysis was performed individually on lemon and orange data sets to identify a reduced number of orthogonal factors composed of amino acids having similar seasonal profiles. Four factors were identified for each citrus species that accounted for 85% and 79% of the total variation in the orange and lemon analyses, respectively. These were then examined with respect to shifts in GWSS numbers that occurred asynchronously in lemons and oranges over the annual population cycle. Three distinctive number shifts were identified that included a peak in adult numbers in lemons during August 2001, significantly higher numbers in lemons relative to oranges during midwinter, and finally an increase in oranges of both adults and nymphs during spring 2002. Various groups of amino acids, i.e., factors, displayed peak annual or elevated levels during the intervals when shifts in GWSS numbers were occurring. Soluble protein levels in oranges and lemons did not correspond to shifting GWSS numbers as certain amino acids. However,

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soluble protein levels were higher in oranges during late winter/early spring when GWSS adults were sexually active. Potential roles of these amino acids and proteins in GWSS host selection are discussed.

Key Words—*Homalodisca coagulata*, GWSS numbers, *citrus*, free amino acids, soluble proteins, citrus–GWSS interactions.

INTRODUCTION

The glassy-winged sharpshooter (GWSS) *Homalodisca coagulata* (Say) is an exotic insect in California and important vector of *Xylella fastidiosa*, a xylem-limited, plant pathogenic bacterium that causes Pierce's disease (PD) in grapes (Purcell, 1981; Blua et al., 1999). Although it is unknown when and how GWSS first arrived in the state, the earliest recorded specimen was collected in 1989 near Irvine (Sorensen and Gill, 1996). During the 1990s, populations expanded throughout much of southern California and became conspicuous in citrus orchards and vineyards as well as in much of the urban landscape (Blua et al., 1999). In Temecula valley, a major wine grape production area in southern California, wineries have lost 20–30% of their vines to PD since 1997 (Hix, 2001). Although the disease has been in the state since the 1880s and outbreaks have occurred periodically over the last century, PD was apparently limited by the fact that native sharpshooters do not fly far from their preferred native habitats to develop large populations (Varela, 1996). With the introduction of the GWSS, which flies farther and feeds on a greater range of plants than California's native sharpshooters, PD problems in vineyards have been increasing dramatically (Blua et al., 1999). The threat from the GWSS is not limited to the grape industry. Since becoming established in California, the GWSS has also been responsible for vectoring oleander leaf scorch and almond leaf scorch diseases (Blua et al., 1999; Almeida and Purcell, 2003). In addition, citrus growers have been hampered by restrictions upon shipments of citrus fruits across county lines imposed by the State of California in an attempt to prevent spread of GWSS to new areas of the state. Less certain at this time is the potential impact on fruit quality and yield in some citrus orchards that have sustained heavy infestations of GWSS over the past decade.

GWSS is highly polyphagous and may feed on over 100 plant species (Turner and Pollard, 1959; Adlerz, 1980). One of the most important hosts in California is citrus and the proximity of citrus groves to vineyards has influenced the incidence and severity of PD in grapes (Perring et al., 2001). It is imperative that effective control strategies be implemented to curb the spread of the vector—vital to this effort would be establishing the host plant range of the GWSS and determining the physiological and biochemical mechanisms for host selection.

Dietary nitrogen is an important nutritional index impacting survival, growth, and reproduction of phytophagous insects (White, 1984; Bi et al., 1994, 1997, 2001, 2003; Simpson et al., 1995; Joern and Behmer, 1997; Blackmer and Byrne, 1999). Nitrogen nutrients are particularly limited for xylophagous insects, such as GWSS, because xylem fluid consists of over 95% water and is the most dilute food source for herbivores (Anderson et al., 1989, 1992). The primary nitrogen nutrients in xylem fluid are amino acids and soluble proteins (Anderson et al., 1989, 1992; Bi and Toscano, unpublished data). There are 19 amino acids detectable in the xylem fluid of most host plants, and amides (glutamine and asparagine) are predominant in most woody host plant species investigated (e.g., Anderson et al., 1989, 1992; Brodbeck et al., 1993, 1996, 1999). Adult GWSS prefers and performs best on host plants containing high contents of amides in the xylem fluid (Brodbeck et al., 1990; Anderson et al., 1992), whereas immatures require lower levels of amides and higher levels of many other amino acids (Brodbeck et al., 1996, 1999). Most of those results were derived from studies of GWSS interaction with grape, soybean, peach, or some wild host plant species (e.g., Anderson et al., 2003; Brodbeck et al., 2004).

GWSS host selection and utilization among citrus plant species and the related nutritional mechanisms have not been reported. As a year-round host in southern California, citrus plays a critical role in the feeding and reproductive ecology of GWSS. The present research was initiated to determine the influence of seasonal nitrogen nutrition fluctuations in xylem fluid on GWSS population dynamics on lemon and orange trees.

METHODS AND MATERIALS

Experimental Plots. A lemon and orange mix-planted orchard at the Agricultural Experimental Station, University of California, Riverside was used for the experiment. Three blocks of 30 orange (*Citrus orange* L. var. Frost Valencia grafted on Troyer Citrange) and 30 lemon (*C. lemon* L. var. Lupe grafted on Cook) trees adjacent to one another were used. Both orange and lemon trees were 30 yr old. Five trees of lemon or orange were randomly selected from each block to monitor the GWSS population dynamics and to extract xylem fluid.

Collection of Xylem Fluid. Xylem fluid was used for determination of nutritional quality because GWSS exclusively feeds on xylem fluid (Anderson et al., 1989). Collections of xylem fluid were done between 6:00 A.M. and 10:00 A.M. on biweekly sampling dates using a pressure bomb apparatus (Anderson et al., 1989, 1992). One to three terminal shoots from each tree were used for xylem fluid extraction because these shoots are the preferred feeding sites for GWSS on citrus plants (Mizell and French, 1987). Upon collection, the xylem fluid was immediately placed on dry ice before final storage in a −80°C freezer.

The samples were used for chemical analyses of free amino acids and soluble proteins. Sampling was initiated on June 8, 2001 and terminated on May 30, 2002.

Determination of Free Amino Acids and Soluble Proteins. Free amino acids were quantified with a Perkin Elmer Applied Biosystem Model 420A PTC derivatizer with an on-line Perkin Elmer Applied Biosystem Model 130A PTC Amino Acid Analyzer. After filtration with a 0.45-μm syringe filter, 20 μl of xylem fluid were reacted with phenylisothiocynate (PITC) to produce phenylthiocarbamyl amino acid derivatives. Following derivatization, a methanol solution containing the PTC amino acids was transferred to an HPLC system for separation. PTC amino acids were separated on a Spheri-5 PTC column (220 × 2.1 mm), and the PTC chromophore was detected at 263 nm. The buffer system used for separation was 50 mM sodium acetate pH 5.45 as buffer A and 70% acetonitrile/32 mM sodium phosphate pH 6.1 as buffer B. The program was run using a gradient of buffer A and buffer B with an initial 7% buffer B concentration and ending with a 60% buffer B concentration at the end of the gradient. A standard mixture of known quantities of individual amino acids (Sigma) was derivatized as described above and used for quantification and identification of individual amino acids.

Protein content was determined by the Bradford method (Bradford, 1976). Three 20-μl aliquots of each sample were mixed with 180 μl of Bio-Rad (Bio-Rad, Richmond, CA, USA) protein assay reagent. Absorbance of the reaction mixture was read at 595 nm with a spectrophotometer, and protein content was determined from a standard curve established using known quantities of bovine serum albumin (from Sigma) and the above reagent.

Sampling of GWSS. A bucket-sampling device (5-gal plastic bucket, with a funneling collector in the bottom, attached to an extendable pole), as described by Castle et al. (2005), was used to sample both immature and adult GWSS in orange and lemon trees. Seven beats were processed with the bucket in each of the five directions (360°/5) around the trees. Numbers of adults and nymphs in the collector from each tree were then counted. Population dynamics of both adult and immature GWSS were monitored on a weekly basis. The same 15 trees used for xylem fluid extraction from each citrus species were used for monitoring GWSS population dynamics.

Statistical Analyses. Multivariate statistical methods were used to identify groups of amino acids that exhibited similar dynamical behavior. Principal component analysis followed by factor analysis (SAS Institute Inc., 1989) was run separately for lemon and orange data sets. Outlier analysis using Mahalanobis distances was applied prior to factor analysis to eliminate outlying points from the analysis. Individual amino acids with loading values $> |0.6|$ within each factor were grouped and related to shifts in GWSS adult populations as characterized by increasing numbers in one citrus species while remaining static or decreasing in the other. Analysis of variance (ANOVA) and the least significant

difference (LSD) test in one-way randomized complete block general linear models (GLM) (SAS Institute Inc., 1989) were used to analyze the data and separate the means of protein data in each sampling date. A repeated-measures analysis of variance (SAS Institute Inc., 1989) was applied to time segments in which visible shifts in GWSS numbers were apparent by graphical analysis. Numbers of GWSS adults and nymphs from bucket-collected samples were $\log(y + 1)$-transformed before ANOVA to normalize the data (Little and Hill, 1978).

RESULTS

Free Amino Acids and Soluble Proteins. Annual amino acid profiles were similar for each citrus species, but levels were consistently higher in lemons than oranges for much of the year, save for one critical period in late winter–early spring when concentrations were higher in oranges (Figure 1). Levels of total amino acids fluctuated by >3-fold for lemons and >4-fold for

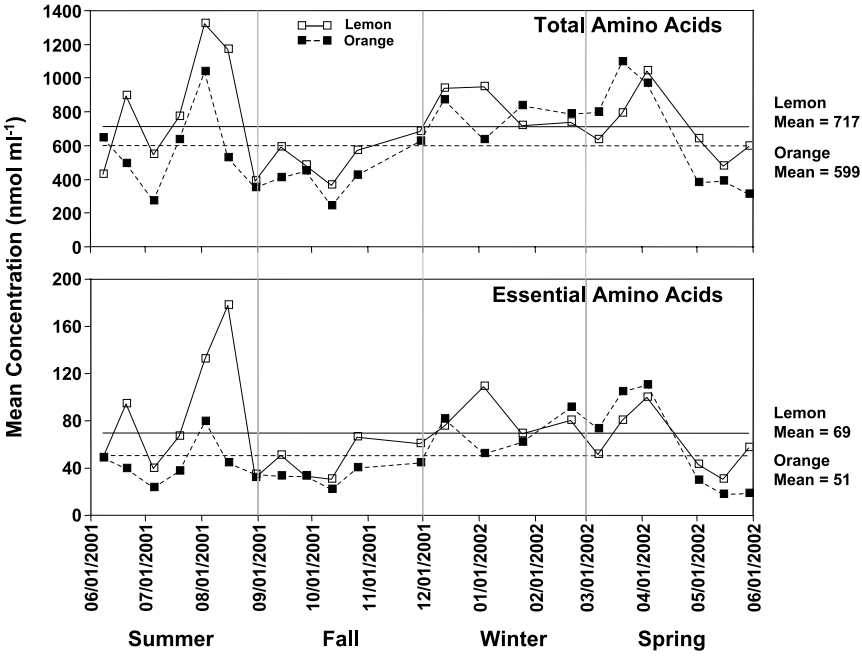

FIG. 1. Mean concentrations of total amino acids (top) and essential amino acids (bottom) from June 2001 through May 2002 in lemons and oranges. The solid horizontal line in each figure represents the annual mean for lemons, the dashed horizontal line is the annual mean for oranges.

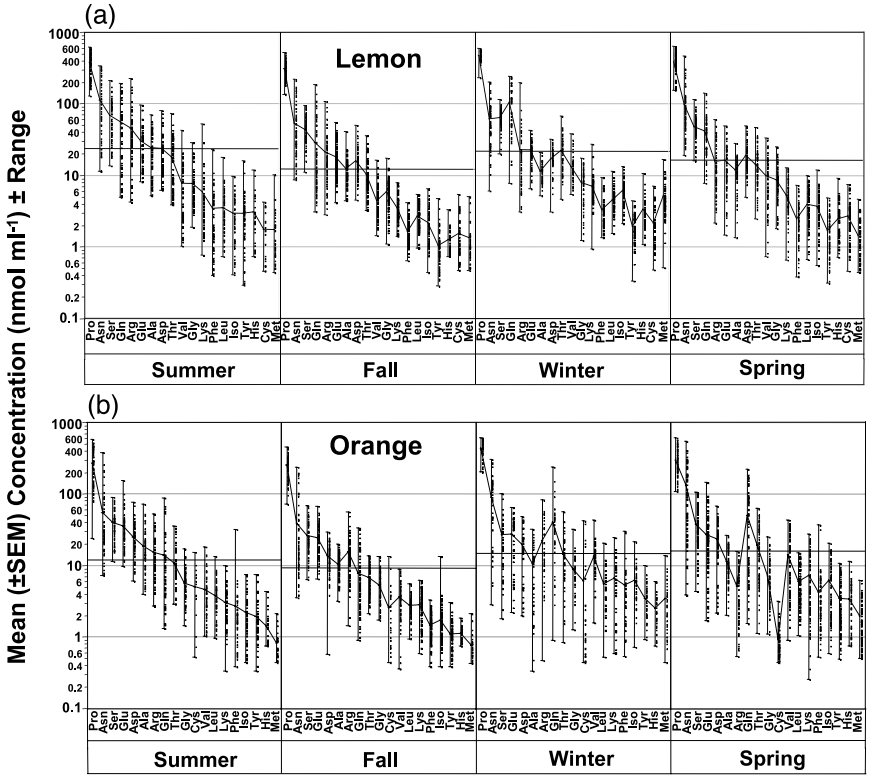

FIG. 2. Mean concentrations of 19 amino acids in xylem fluid of (a) lemons and (b) oranges collected over a 1-yr period and represented for each season. The amino acids for each citrus species were arranged in descending order for the June–August period and maintained in the same order for each subsequent season despite changes in rank. Each point represents the mean of all samples collected for a tree during the respective seasons. Vertical lines represent the range of mean concentrations for each amino acid, whereas the intersection with the traversing lines indicates the mean concentration. Seasonal means for 18 amino acids (excluding proline) are represented by the horizontal line in each panel.

oranges between the lowest and highest mean concentrations during the year-long evaluation with particularly large changes observed within summer and spring intervals. The annual mean levels of total amino acids in lemons and oranges were 717 and 599 nmol/ml, respectively (Figure 1). The subset of essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, arginine, and histidine) for insect growth and development (Taylor and Medici, 1966; Brodbeck and Strong, 1987) showed a similar annual

profile for each species with higher levels in oranges occurring notably during the late winter–early spring period (Figure 1). Overall, however, the annual mean level of essential amino acids in lemons (69 nmol/ml) was higher than in oranges (51 nmol/ml).

Nineteen protein amino acids were detected in both lemon and orange xylem fluid samples. The proportion of total amino acids made up by individual amino acids varied more than 3 orders of magnitude in lemons and oranges. By far, the highest level of any amino acids was proline, varying between 52% and 62% of the total depending on species and time of year (Figure 2a, b). The mean level of asparagine was the next highest in both species during three out of four seasons, accounting for 7–17% of the total. Other amino acids in relative high levels were glutamine (1–12% of the total), serine (2–9%), and aspartate (1–6%). The rest of amino acids were less than 5% of the total. Mean levels for

TABLE 1. FACTOR LOADING VALUES FOR AMINO ACIDS IN XYLEM FLUID FROM ORANGE AND LEMON TREES COLLECTED OVER A 1-YR PERIOD

	Factor							
	Orange				Lemon			
Amino acids	1	2	3	4	1	2	3	4
Aspartic acid	−0.390	**0.836**[a]	−0.003	0.073	0.099	**0.826**	0.350	−0.143
Glutamic acid	−0.218	**0.805**	0.140	0.193	0.223	**0.872**	0.023	0.070
Asparagine	**−0.819**	0.283	0.203	0.019	0.375	0.259	−0.113	−0.600
Serine	−0.410	**0.822**	−0.005	−0.009	0.496	**0.695**	0.182	−0.269
Glutamine	**−0.870**	0.241	0.045	0.015	**0.899**	0.126	−0.154	−0.288
Glycine	**−0.617**	0.481	−0.017	0.153	−0.002	0.350	**0.715**	−0.233
Histidine	**−0.911**	0.262	0.087	0.082	**0.613**	0.084	0.270	−0.558
Arginine	0.022	0.200	−0.009	**0.941**	−0.059	**0.742**	−0.076	−0.328
Threonine	**−0.840**	0.447	0.079	−0.080	**0.757**	0.265	0.089	−0.487
Alanine	−0.187	**0.811**	−0.049	0.116	0.008	**0.0760**	−0.054	−0.419
Proline	−0.554	0.505	−0.100	0.431	**0.619**	0.448	0.390	−0.281
Tyrosine	**−0.860**	0.402	−0.086	−0.041	0.181	0.240	0.054	**−0.779**
Valine	**−0.950**	0.248	0.052	0.004	**0.647**	0.017	0.263	**−0.624**
Methionine	**−0.832**	0.036	−0.221	0.214	**0.901**	0.053	0.123	−0.049
Cysteine	−0.014	−0.032	**−0.954**	0.019	0.126	−0.103	**0.708**	−0.001
Isoleucine	**−0.949**	0.238	0.024	−0.009	**0.795**	0.019	0.276	−0.476
Leucine	**−0.863**	0.374	−0.156	−0.014	0.365	0.181	0.527	**−0.654**
Phenylalanine	**−0.870**	0.307	−0.222	0.027	0.397	0.331	0.206	**−0.718**
Lysine	**−0.881**	0.339	0.039	0.096	0.436	0.301	0.417	**−0.651**
Eigenvalue	12.089	1.982	1.187	0.910	0.920	2.579	1.443	1.077
Percent	63.6	10.4	6.2	4.8	52.2	13.6	7.6	5.7
Cumulative %	63.6	74.1	80.3	85.1	52.2	65.8	73.4	79.0

[a] Loading values > |0.6| are in bold typeface.

individual amino acids ranged 10-fold or greater both within and among trees over each season (Figure 2a, b). Seasonal mean levels for all amino acids (exclusive of proline) varied by 1.8- and 2.2-fold for lemons and oranges, respectively, with fall representing the lowest mean concentrations for both species. Thus, less variation in mean levels of all amino acids was observed among seasons compared to the 3- to 4-fold shifts that occurred within particular seasons.

Distinctive groups of amino acids between lemons and oranges were apparent following factor analysis. Separate analysis on each species produced a rotated loading matrix that had four factors (eigenvalues >1) for lemons and three factors for oranges, but a fourth factor was subsequently included for orange based on an eigenvalue close to 1 (0.91) (Table 1). Over 63% of the variance in the solution for oranges was accounted for by factor 1 that contained 12 variables (asparagine, glutamine, glycine, histidine, threonine, tyrosine, valine, methionine, isoleucine, leucine, phenylalanine, and lysine) with loading values $>|0.6|$, but all the amino acids with the exception of glycine having values $>|0.8|$. Factor 2 accounted for 10.4% of the solution for orange and contained four variables (aspartic acid, glutamic acid, serine, and alanine) with loading values $>|0.6|$ (Table 1). A single variable (cysteine or arginine) was accounted for in factors 3 and 4 for oranges, bringing the total number of amino acids grouped by factor analysis to 18, the lone exception being proline. The cumulative variance explained by the solution for orange was 85.1% compared to 79% explained by the four factors in the lemon solution. Only seven amino acids (glutamine, histidine, threonine, proline, valine, methionine, and isoleucine) met the criterion for factor 1 in the lemon solution, but at a lower level of

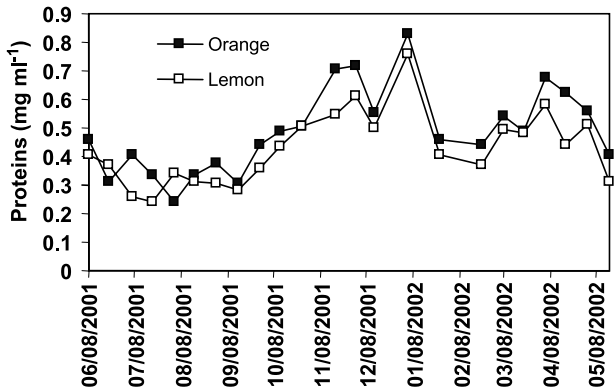

FIG. 3. Mean concentrations of soluble proteins from June 2001 through May 2002 in lemons and oranges.

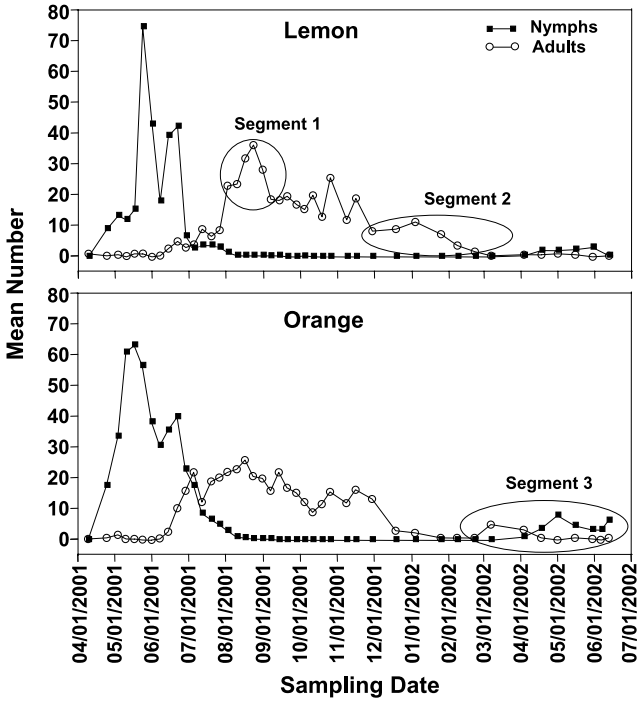

FIG. 4. Mean numbers of GWSS nymphs and adults in lemons (top) and oranges (bottom) between April 2001 and June 2002. Encircled areas in each chart are examined in greater detail in subsequent figures.

correlation than observed for factor 1 in the orange solution. Factor 2 for lemons contained all four amino acids that grouped in factor 2 for oranges, but also included alanine (Table 1). Factor 3 contained two amino acids (glycine and cysteine), but factor 4 for lemons contained six amino acids (asparagine, tyrosine, valine, leucine, phenylalanine, and lysine) compared to one for oranges. Only one amino acid (valine) in both analyses correlated with more than one factor (factors 1 and 4) in the lemon analysis.

Soluble protein contents in orange and lemon xylem fluid samples ($P > 0.05$) were similar throughout much of the experimental period with the exception of five sampling dates (September 28, 2001; November 17 and 30, 2001; April 18, 2002; and May 30, 2002, respectively) when the content was 18–40% higher ($P < 0.05$) in orange xylem fluid than in lemon xylem samples (Figure 3).

GWSS Numbers and Their Responses to the Nitrogen Nutrients. The annual profiles of GWSS nymphs and adults are quite similar for lemons and oranges

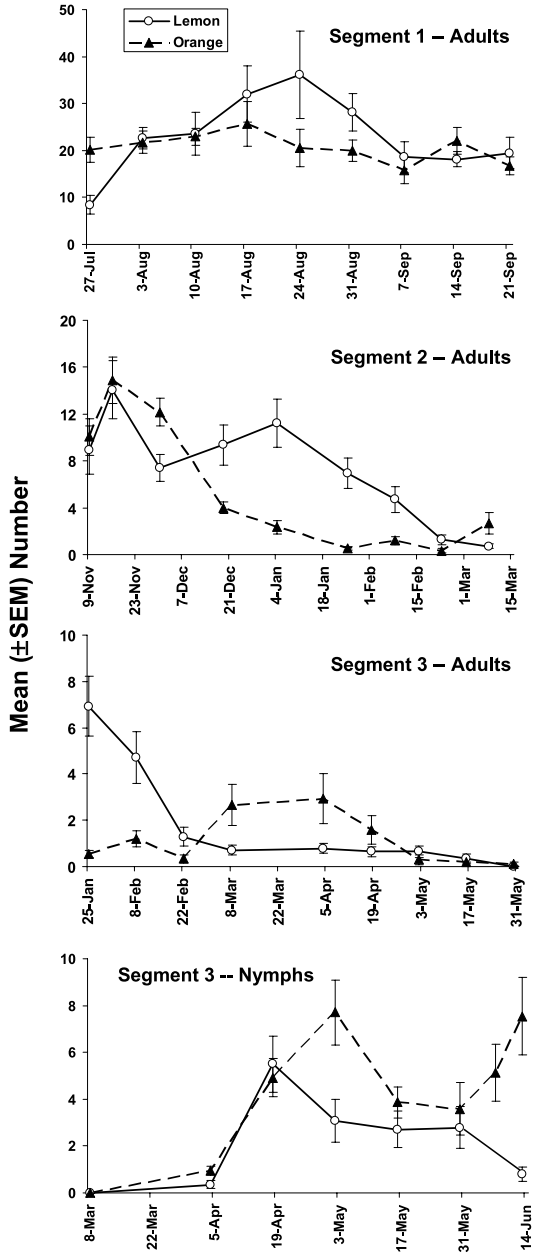

FIG. 5. Comparative numbers of GWSS adults and nymphs in lemons and oranges at three different periods during the annual cycle (see Fig. 4).

(Figure 4), but with notable fluctuations in GWSS numbers occurring at specific times on either lemons or oranges. We identified three intervals during the annual cycle that GWSS numbers increased on one species while remaining static or declining in the other. Two of these intervals occurred as increases in GWSS adults in lemons (Figure 4) while the third interval occurred with both adults and nymphs increasing in oranges (Figure 4).

To examine the changes in numbers on lemons or oranges more closely, the circled and numbered areas in Figure 4 were expanded to provide greater detail on GWSS numerical differences between lemons and oranges and to focus attention on near-synchronous changes that occurred in certain amino acids. The first departure of relative numbers of adult GWSS occurred on July 27–September 7, 2001 (Figure 5, Segment 1). Mean adult numbers on lemon trees had been depressed over three consecutive sampling dates from July 13 through July 27, but by August 3 had more than doubled to a level equivalent to the numbers on oranges (Figure 5, Segment 1). One wk later (August 10), adult numbers were again equal on lemons and oranges, but then increased over the next 2 wk on lemons while remaining fairly constant on oranges. After peaking on August 24, adult numbers on lemons and oranges declined over the next 2 wk with near parity between the two citrus species on September 7. A repeated-measures ANOVA conducted on the data from the seven sampling dates between July 27 and September 7 yielded a nearly significant result ($F_{6,12} = 2.86$, $P = 0.057$) of higher adult GWSS numbers in lemons than oranges. In the second example, adult numbers in lemons were significantly greater ($F_{5,13} = 6.03$, $P = 0.004$) than in oranges during the period November 30, 2001–February 22, 2002 (Figure 5, Segment 2). Adult numbers in oranges dropped quite drastically after November 30, 2001, while remaining stable in lemons and even gaining slightly (Figure 5, Segment 2). By early spring 2002, few GWSS adults were present on lemons or oranges, their numbers apparently having declined due to winter mortality. However, this is a critical time of the year as adults become sexually active and soon begin laying egg clutches that eventually emerge as adults beginning in June each year. A significant shift ($F_{4,33} = 3.03$, $P = 0.031$) in GWSS adult numbers to oranges from lemons occurred between February 22 and May 2, 2002 (Figure 5, Segment 3—Adults). Higher adult numbers in oranges during early spring translated into significantly higher nymphal numbers ($F_{4,33} = 2.98$, $P = 0.033$) in oranges between April 18 and June 13 (Figure 5, Segment 3—Nymphs).

The peak in GWSS adults reached on August 24 (Figure 5, Segment 1) corresponds closely to the rise and fall in levels of 10 amino acids that were grouped into two different factors in the lemon factor analysis (Table 1). The five amino acids that grouped into factor 2, including aspartic acid, glutamic acid, serine, alanine, and arginine (Figure 6a), had only one significant peak over the entire year that occurred in August. In contrast, the five amino acids grouped into factor 4, including tyrosine, lysine, asparagine, phenylalanine, and

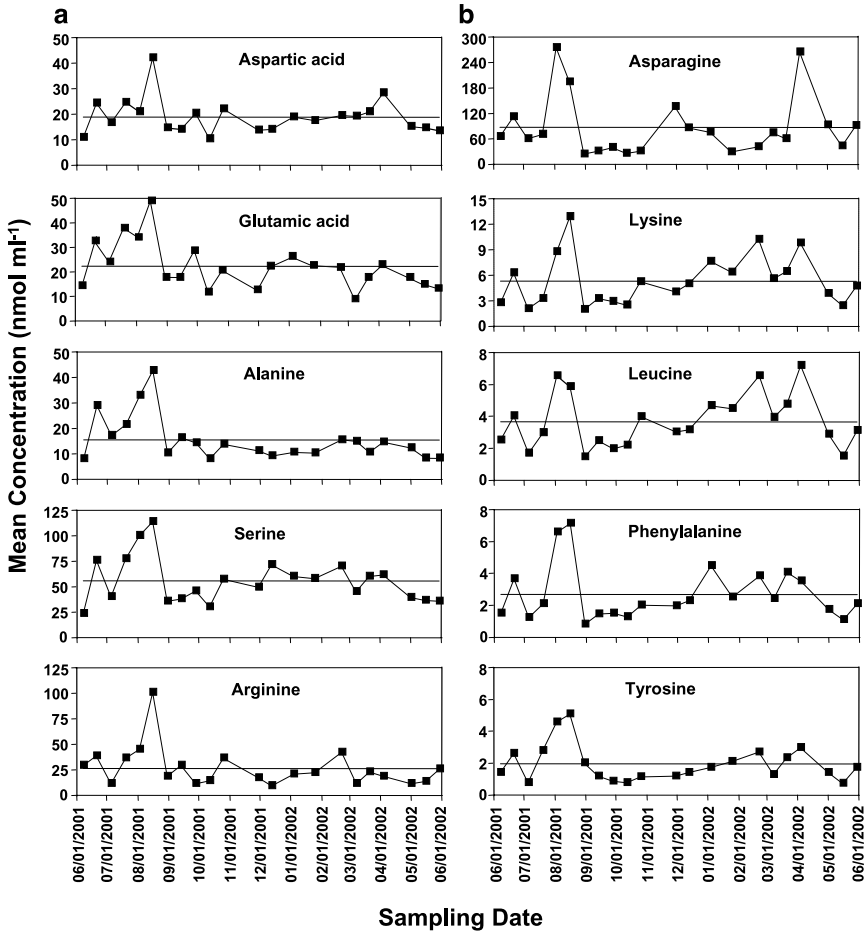

FIG. 6. Annual profiles of five amino acids that loaded onto (a) factor 2 in the lemon analysis, and another five amino acids that loaded onto (b) factor 4. The horizontal line in each chart is the annual mean concentration.

leucine (Figure 6b), had their highest peaks in August, but also showed secondary peaks in levels during the following spring. In addition to these 10 amino acids, six out of the seven amino acids that grouped into factor 1, including threonine, valine, isoleucine, glutamine, proline, and histidine (Figure 7), also displayed an early August peak in their levels that likely contributed to the increase in numbers of GWSS adults recorded on lemons relative to oranges.

A more critical role for the seven amino acids grouped in factor 1 of the lemon analysis (Figure 7) may have occurred in midwinter at a time that GWSS

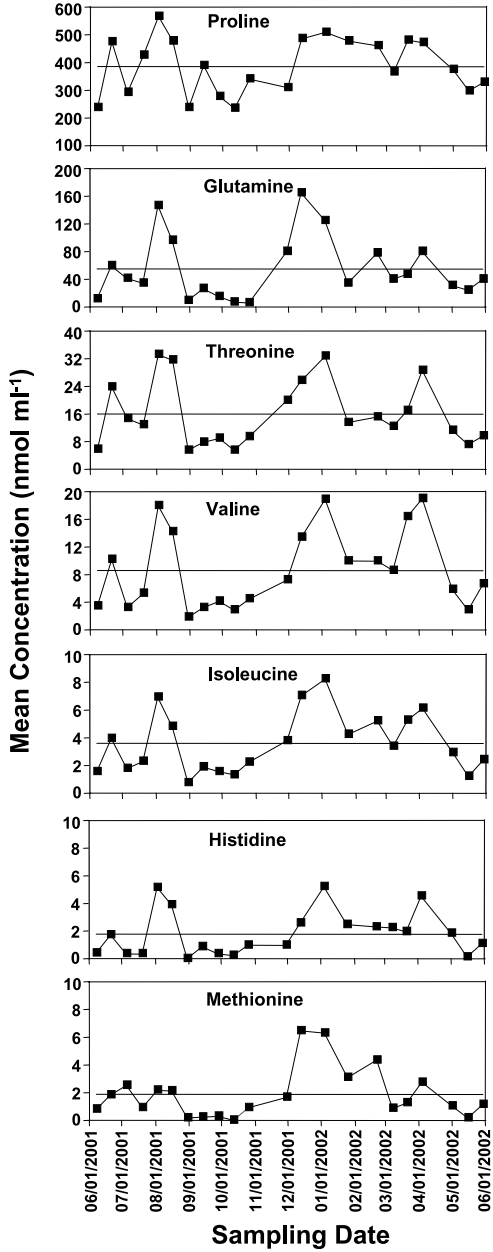

FIG. 7. Annual profiles for seven amino acids from xylem fluid of lemons that loaded onto factor 1 of the lemon analysis. The horizontal line in each chart is the annual mean concentration.

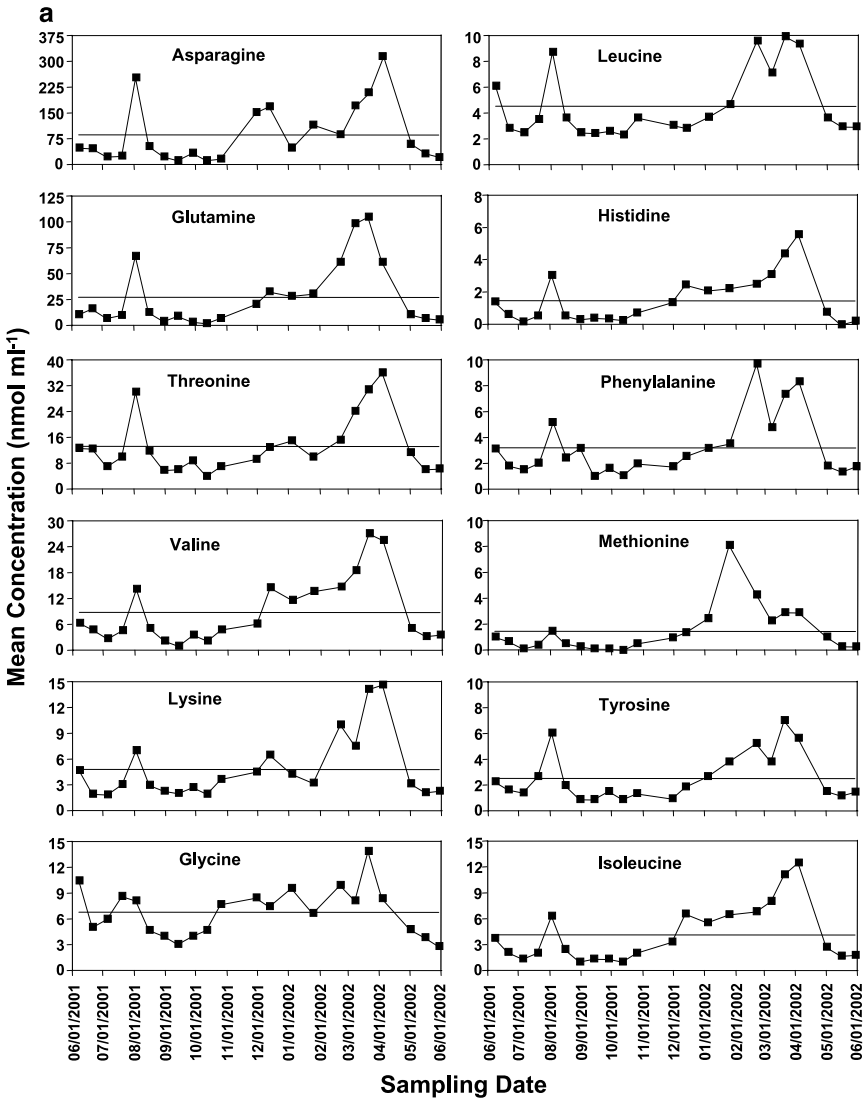

FIG. 8. Annual profiles for 12 amino acids that loaded onto (a) factor 1 of the orange analysis, and another four amino acids that loaded onto (b) factor 2. The horizontal line in each chart is the annual mean concentration.

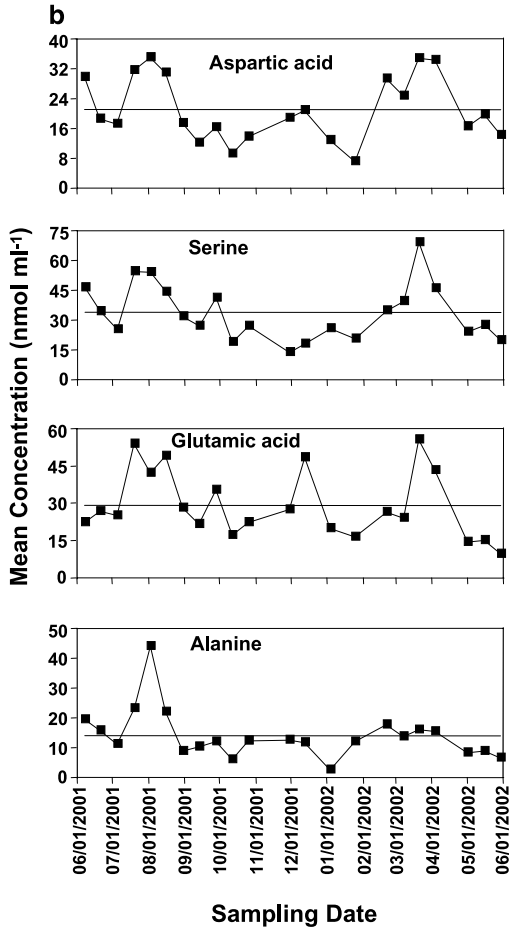

FIG. 8. Continued.

numbers in lemons consistently exceeded those in oranges (Figure 5, Segment 2). The levels of threonine, valine, histidine, and isoleucine all reached peaks on January 4, 2002 during this interval. Methionine and glutamine were also at near peak levels during this interval, reaching their peak levels one date earlier on December 19, 2001. In fact, annual peaks were attained for each of these amino acids with the exception of valine and threonine, whose concentrations during this period were just slightly below annual peak levels.

Amino acid concentrations in oranges also showed fluctuations from season to season, but on a different time frame than lemons. The most coherent group of amino acids in either the lemon or orange factor analyses occurred in

the orange analysis with 12 amino acids grouped into factor 1. These include valine, leucine, isoleucine, histidine, threonine, lysine, tyrosine, asparagine, glutamine, methionine, phenylalanine, and glycine (Figure 8a). Concurrent with the early spring peak in these 12 amino acids was a shift in GWSS adults (Figure 5, Segment 3—Adults) from lemons to oranges that subsequently resulted in a greater number of nymphs on orange (Figure 5, Segment 3—Nymphs). The levels of all 12 amino acids in factor 1 of the orange analysis had significantly increased by February 22, 2002, with most reaching their annual peak levels in late March or early April. This closely corresponds to the time in late winter and early spring when GWSS adults are reproductively active as evidenced by pairs in copula and the presence of brochosomes on adult females. In addition to the 12 amino acids in factor 1, three out of the four amino acids that grouped into factor 2 of the orange analysis also displayed peak levels during spring (Figure 8b). However, these three amino acids, aspartic acid, glutamic acid, and serine, also had other periods of peak levels during the year in contrast to the 12 amino acids in factor 1 that displayed a prolonged rise in levels peaking in early spring.

DISCUSSION

Throughout the experimental season, 19 protein amino acids were detected in both lemon and orange xylem fluid in this study. These results are consistent with previous reports on amino acids in xylem fluid of GWSS hosts such as *Baccharis halimifolia* (L.), *Lagerstroemia indica* (L.), *Prunus salicina* (Lindl.), *Prunus persica* (L.), *Pyrus communis* (L.), *Vitis hybrid*, *Catharanthus roseus* (L.), and *Glycine max* (L.) (Brodbeck et al., 1990, 1993, 1999, 2004; Anderson et al., 1992). The predominant amino acids detected in this study were proline and asparagine. Our results agree with a previous report in which proline was the most abundant amino acid in *C. sinensis* (L.) (Moreno and Garcia-Martinez, 1983). In contrast, asparagine and glutamine were predominant (over 50% of the total) in other GWSS hosts (Brodbeck et al., 1990, 1993, 1999, 2004; Anderson et al., 1992). Apparently, those differences may be due to the differences in host plant families and/or species.

Although GWSS is capable of feeding on over 100 plant species, well-defined host preferences vary seasonally and successful development often requires host switching (Mizell and French, 1987; Brodbeck et al., 1999; Redak et al., 2004). In many instances, host switching may represent multiway traffic as GWSS adults are highly mobile and often conspicuous in their movements to and from among various hosts. At times, however, population levels on particular host species are apparently higher over the others, presumably due to nutritional requirements being better satisfied by these host species. In the present study, only the two citrus species were available as hosts to the GWSS

population as the study area was centrally located within a 12-ha orchard split evenly between lemons and oranges. Despite the close relationship between lemon and orange and the apparent similarity in annual amino acid profiles (Figure 1), there were clear instances when the GWSS population was more concentrated in one species than the other (Figure 5). Factor analysis of each data set, lemon and orange, revealed different groups of amino acids that varied from one another in terms of their seasonal peak concentrations as well as the number of peaks they exhibited over the annual cycle. By aligning the three distinctive intervals of host switching by GWSS between lemons and oranges with amino acid groups from the lemon and orange factor analyses, it was possible to infer nutritional shifts that were occurring concomitantly to the GWSS population shifts.

This study revealed that adult GWSS numbers responded positively to higher levels of aspartic acid, glutamic acid, serine, alanine, aspargine, tyrosine, lysine, asparagine, phenylalanine, leucine, methionine, threonine, valine, histidine, isoleucine, glutamine, and proline in xylem fluid of lemons, and valine, isoleucine, threonine, tyrosine, glutamine, phenylalanine, leucine, histidine, lysine, asparagine, methionine, glycine, aspartic acid, glutamic acid, and serine in xylem fluid of oranges. These amino acids may serve as important nutrients to determine host preference for GWSS. Indeed, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, arginine, and histidine are considered as essential amino acids for insect growth and development (Taylor and Medici, 1966; Brodbeck and Strong, 1987). In previous studies, the levels of asparagine and glutamine in xylem fluid of four host species (*L. indica*, *B. halimifolia*, *P. persica*, *and P. salinicia*) have been positively correlated with GWSS host selection (Brodbeck et al., 1990). The fact that glutamine and asparagine have high nitrogen/carbon ratios and account for high caloric value may make them an ideal host selection cue for generalist feeder GWSS (Brodbeck et al., 1990), especially adult GWSS in their sexually active status. Both of these amides are also considered phagostimulants for GWSS (Anderson et al., 1992). It is noted that adult GWSS females produce protein-containing brochosomes just prior to egg laying (Hix, 2001). Higher levels of these amino acids may be critical for the brochosome production.

Previous research indicated that adult GWSS prefers and performs best on host plants containing high contents of amides in the xylem fluid (Brodbeck et al., 1990; Anderson et al., 1992), while immatures require lower levels of amides and higher levels of many other amino acids, thus the more balanced amino acid profiles (Brodbeck et al., 1995, 1996, 1999). Our results showed that a majority of GWSS eggs are laid on oranges, and the immatures then grow and fully develop from April to July on oranges when amino acid (especially glutamine and asparagine, the second and third most predominant amino acids in the xylem fluid) fluctuations drop to minimal level to make the profiles more

balanced. During this period, levels of isoleucine, tyrosine, phenylalanine, and lysine are particularly higher in oranges than in lemons. It is known that tyrosine and phenylalanine are essential for insect cuticle formation (Bernays and Woodhead, 1984). Thus, immature GWSS may need higher levels of these two amino acids for their successful development of cuticle.

Increased protein quantity is frequently associated with enhanced insect survival, growth, and fecundity (McNeill and Southwood, 1978; Mattson, 1980; Ohgushi, 1992; Slansky, 1993). Protein quality also affects insect performance (Bi et al., 1994; Bi and Felton, 1995; Felton, 1996; Bi et al., 1997). Our results indicated that substantial amounts of soluble proteins exists in xylem fluid of GWSS host plants. Soluble protein levels in xylem fluid of oranges and lemons were similar except on a few of the sampling dates (e.g., mid-April) when levels were significantly higher in xylem fluid of oranges than in lemons. Although it is unknown how GWSS utilizes host proteins, soluble proteins may help GWSS female to produce brochosomes for the egg laying (Hix, 2001). Our results clearly indicated that the soluble protein levels in April and May were higher in oranges. The effect of protein quality in host xylem fluid on GWSS performance and host selection warrants further investigation.

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ANALYSIS OF BIOGENIC VOLATILE ORGANIC COMPOUNDS IN ZUCCHINI FLOWERS: IDENTIFICATION OF SCENT SOURCES

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Abstract—An analytical method has been applied to determine volatile organic compounds in zucchini flowers. In a first step, the analytical method was applied to characterize the main scents emitted by whole male and female living flowers of three main commercial cultivars of zucchini (Tosca, Chapin, and Consul). In a second step, the compounds were quantified in different parts of the living flowers to identify the contribution of nectar, petals, anther, and stigma to the aroma profile of the flower. The analytical method is based on headspace solid-phase microextraction coupled on-line with GC and tandem MS detection (HS-SPME-GC-MS/MS). A reference compound is added to samples as part of the field quality control procedure to check for likely analyte losses or sample decomposition. The reference compound also acts as an internal standard for quantification purposes. Results have been statistically studied applying principal component analysis (PCA), which shows that three components explain more than 91% of the variance. PCA emphasizes the great importance of nectar as being the main source of 1,4-dimethoxybenzene and 1,2,4-trimethoxybenzene, which influence the aroma profile of flowers. The remaining components can be accounted for by emissions from petals and sexual organs (adroecium and gynoecium anthers or stigmas).

Key Words—zucchini, nectar, headspace solid-phase microextraction, tandem mass spectrometry, biogenic volatile organic compounds, flower aroma, PCA.

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INTRODUCTION

Regulations and consumer attitudes lead agricultural practices toward an ever more environmentally friendly production with an optimization of agrochemical use. There is an increasing interest of growers in using beneficial insects in integrated crop management (ICM), with reduced chemical input, for example, the use of pollinators as an alternative to parthenocarpy in zucchini crops (Roldán Serrano et al., 2002; Roldán Serrano and Guerra Sanz, 2005), or the use of pheromones in traps, which act as attractants or repellents. From an ecological point of view, this requires a knowledge of the environment in which crops are grown and their interactions with the rest of the ecosystem. In this aspect, color and aromas play an essential role. For example, visual cues and floral scents are important in attracting pollinators; they can act over long distances as attraction cues and over short distances as orientation cues between different parts of the flower or between different flowers (Williams, 1983; Dobson et al., 1990). Scents are more complex, as floral fragrances vary widely among species in terms of the number, identity, and relative amounts of constituent volatile compounds. Insects are able to distinguish between complex floral scent mixtures and discriminate flower visits depending on floral scent content. In addition, different parts of the flower (e.g., pollen and petals) contribute to volatile production (Dobson, 1991; Flamini et al., 2002). The apparently ubiquitous occurrence of odor in pollen, its varying chemical composition, including insect repellents and antimicrobial compounds, suggests that pollen odor chemicals may have a range of purposes that include pollinator attraction, defense against destructive pollen-feeding animals and pathogens, as well as defense of the plant against competition from other species through the phenomenon of pollen allelopathy (Dobson, 1991; Dobson and Bergström, 2000; Stephen, 2000).

A knowledge of the composition and dynamic emission of aromas and, in general, of biogenic volatile organic compounds (BVOC) is fundamental because these play a role in processes of reproduction, defense, and communication between species (Ware and Compton, 1994; Dudareva and Pichersky, 2000; Augusto et al., 2003). Studies need to be based on data obtained using validated analytical methods, with special emphasis on sampling and identification steps (Egea et al., 2004), and on statistical interpretation of results (Martínez Vidal et al., 1998; Díaz-Maroto et al., 2002; Hernández Torres et al., 2002).

Choice of methods for sampling of volatile compounds depends partly on the availability of equipment, type of flower material (e.g., whole flowers vs. flower parts or pollen, laboratory vs. field plants), and the amount and general chemical composition of emitted volatiles. Methods involving separation steps to isolate volatile compounds for analysis run a greater risk of the loss of volatiles, and, although no method gives a complete picture of the actual

mixture emitted by a flower, headspace sorption appears to be an appropriate technique (Dobson, 1991).

Solid-phase microextraction (SPME) has demonstrated the utility of this technique for analysis of volatile compounds in several matrices, for example, environmental samples, food and beverages, etc. (Pawliszyn 1995, 1999; Stefen and Pawliszyn, 1996; Scheppers, 1999). For volatiles determination, the SPME fiber can be used as a passive sampler by exposing it to air containing compounds or to the headspace (HS) of vials containing the sample. SPME presents many advantages over other methods allowing sampling and preconcentration in a single step (Pawliszyn, 1997; Prosen and Zupancic-Kral, 1999; Sides et al., 2000; Kim and Lee, 2002; Augusto et al., 2003). Considering an optimum sampling time, compounds are concentrated in the fiber to amounts greater than detection limits; after that, the fiber is desorbed directly into the injection port of the GC, transferring all compounds to the column and detection system. The above steps can be performed with an automatic SPME injector, avoiding handling the sample, which is important to reduce analyte losses. In addition, this technique does not require the use of solvents, which minimizes coelution with volatile compounds of interest and has environmental and economic advantages. Finally, the use of MS/MS detection allows the mass spectral identification of compounds from two fragmentations, resulting in an analytical method with high selectivity and sensitivity.

In this work, we characterize the BVOC of three *Cucurbita* cultivars, with the aim of identifying differences in the volatile compounds emitted from the whole living male and female flowers and from different parts. The studies were conducted applying a previously validated analytical method (Mena et al., 2004). Initially, the fragrances emitted by whole living flowers were identified without disrupting the aroma profiles. Once the compounds had been identified, they were quantified in each of the individual parts of excised flowers. The objectives were to identify the likely influence of cultivar and flower gender on the aromas emitted by flowers, and, to identify the sources of each compound contributing to the whole aroma profile of flowers.

METHODS AND MATERIALS

Plant Material and Growing Conditions. Three cultivars of *Cucurbita pepo* (L.), Tosca (Clause Iberica, Vicar, Spain), Consul (Seminis Seeds, Aguadulce, Spain), and Chapin (Fitó, S.L., El Ejido, Spain) were cultured under plastic greenhouse conditions in the experimental station of CIFA La Mojonera (Almería, Spain). The crop was grown in Perlite sacks irrigated and nourished by drip irrigation. Sampling of nectar flowers was made in October 2003, when the blooming and crop were at their maximum level.

Flower Sampling. Ten whole living flower samples (five of each gender) of each cultivar were selected at random in the greenhouse and enclosed on the plant in a glass receptacle. The enclosed atmosphere was sampled with SPME fibers for 1 hr for qualitative analysis.

Target volatile compounds were identified and quantified in nectar, petals, anthers, and stigmas, which were sampled at the same time, from male and female flowers of each cultivar during flower anthesis hours (from 6:00 A.M. to noon time). Nectar samples consisted of 80 μl of nectar that were collected from nectaries using a micropipette. They were placed directly in 10-ml headspace (HS) vials, spiked with 5 μl of the internal standard (IS, *p*-xylene-d10) at 10-μg/ml concentration, and fitted with Teflon-lined septa.

Petal, stigma, and anther samples were taken in preweighed 10-ml HS vials and treated the same as nectar. Samples were transported to the laboratory within a period not greater than 2 hr in a portable refrigerator. Each vial was reweighed to determine the amount of floral tissue in each sample (results are referred to as amount of compound per gram of fresh mass of anthers, styles, and petals and per milliliter of nectar).

A field quality control was planned for ensuring that results were under statistical control. Three, 10-ml HS vials containing filter paper were spiked in the field with a standard mixture of all the target compounds, in the field and treated in the same way as flower samples. The control vials were transported, stored, and analyzed in the same batch as the rest of samples.

Aroma Analysis. Reference standards of volatiles known to be present in the flowers of cucurbits (Metcalf and Lampman, 1991; Metcalf et al., 1998), benzene, toluene, *o*-xylene, *m*-xylene, *p*-xylene, ethylbenzene, α-(+)-pinene, myrcene, (*R*)-(+)-limonene, eucalyptol, ocimene, linalool, 1,4-dimethoxybenzene, *p*-anisaldehyde, cinnamaldehyde, indole, cinnamyl alcohol, dibuthyl phthalate, eugenol, 1,2,4-trimethoxybenzene, and *p*-xylene-d10, were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Tokyo Kasei (Nihonbashi, Tokyo, Japan). A reference standard solution was prepared for each compound using acetone as the solvent at 200-μg/ml concentration, and a multicompound working standard solution (2-μg/ml concentration) was prepared from the above by appropriate dilution with acetone and stored under refrigeration (4°C).

For SPME analysis, fibers of 65 μm polydimethylsiloxane–divinylbenzene (PDMS-DVB; Supelco, Bellefonte, PA, USA) were used to sample the headspace of nectar, petal, stigma, and anther samples. Samples were equilibrated in a thermostatic carrousel for 10 min at 60°C, known to be enough time to achieve transfer of the analytes to the fiber. After adsorption, the fiber was injected into the GC SPI/1079 split/splitless programmed-temperature injector operating at 250°C in splitless mode. The desorption time of analytes from the fiber was set at 9 min, which is enough to desorb and transfer the analytes to the analytical column (Mena et al., 2004). In fact, the desorption of

most analytes took place during the first minute, but a longer period avoided carryover between samples.

GC analysis was accomplished with a Varian 3800 gas chromatograph with electronic flow control (EFC) and fitted with a Saturn 2000 ion-trap mass spectrometer (Varian Instruments, Sunnyvale, CA, USA). High-speed analysis was carried out by connecting a fused silica untreated capillary column (2 m × 0.25 mm i.d. from Supelco) used as guard column, to a Rapid-MS (WCOT fused silica CP-Sil 8 CB low bleed of 10 m × 0.53 mm i.d. × 0.25 μm film thickness) analytical column, both obtained from Varian Instruments. The controlling computer system had an EI-MS/MS library that was specially created for the target analytes under our experimental conditions. Analytical conditions were as follows: oven temperature set to 35°C during injection, 9 min hold, increased at 1.5°C/min to 55°C, at 5°C/min to 65°C, and finally raised to 300°C at 100°C/min that was held for 5 min. Helium (99.999%) at a flow rate of 1 ml/min was used as carrier and collision gas.

The mass spectrometer was operated in the electron impact mode (EI) with electron energy of 70 eV. The transfer line, manifold, and trap temperatures were 280, 50, and 200°C, respectively.

Analyses were performed in MS/MS detection mode. The main parameters to be optimized were excitation storage level (González Rodríguez et al., 2002) and excitation amplitude (or resonance excitation voltage). To assure an appropriate definition of the chromatographic peaks, we selected two microscans, adjusting the m/z range to each compound (González Rodríguez et al., 2002).

Identification of the target compounds was based on the retention time windows (RTW) and on MS/MS spectra. Each MS/MS spectrum was compared with spectra stored in an in-house MS/MS spectral library prepared from standards under our working conditions. The results of the comparison (FIT parameter), obtained using the match algorithm of the Saturn software, are scaled, setting 1000 for the best match (identical spectra).

The procedure was validated (Mena et al., 2004), establishing the performance parameters that show the fitness for purpose of the method. For quantification, a reference compound (*p*-xylene-d10), acting as a surrogate analyte and also as internal standard, was included at the same concentration either in field samples, quality control samples, and calibration solutions. Such calibration is performed with each batch of samples, four standard solutions containing the target compounds at different concentration levels, and plotting the ratio analyte/internal standard vs. analyte concentration.

Data Analysis. Statgraphics 5.1 was used for data treatment. The statistic applied consisted of a multifactor analysis of variance and a principal component analysis. These studies allow classifying the samples, obtaining the influence of factors in the presence of a given compound, and to approach likely sources of aromas to the whole aroma profile of the flowers.

RESULTS AND DISCUSSION

The method allowed the quantification of volatile compounds in relation to the mass of sample analyzed. The field quality control carried out was satisfactory because the results are under statistical control. Most of the selected compounds were identified in living flower samples so they were targeted for quantification in each excised part.

The use of the reference compound *p*-xylene-d10 in this work had a double function. On one hand, it allowed monitoring of the volatilization of compounds and potential analyte losses during sample transport and analysis. The ratio analyte/reference peak area did not differ in field samples, spiked samples, and calibration curves. On the other hand, it acted as an internal standard for calibration purposes on the basis of signal ratio analyte/reference. The quality control performed showed that recovery rates of the field spiked samples ranged between 87 and 103%, calibration plots fit to straight lines with a relative standard deviation less than 12%, and a determination coefficient greater than 0.98.

Tables 1–3 show the concentrations of volatiles in petals, nectar, anthers, and stigmas of the three cultivars. The main compounds found in nectar were 1,4-dimethoxybenzene and 1,2,4-trimethoxybenzene, at concentrations around 60 mg/l in male flowers and 40 mg/l in female flowers. These compounds were almost exclusive to nectar, as they were found only at trace levels in anthers and stigmas and at the lowest concentration in petals (around 2 ppm). Terpenes were found in petals in a greater concentration than in nectar samples. In all cases, the dominant odor from zucchini petals was a blend of linalool, α-pinene, limonene, indole, and eucalyptol. The concentration of these compounds was between 3 and 25 mg/kg, with male flowers having a higher content of volatile compounds than female flowers. Indole was found exclusively in petal samples, at concentrations around 4 mg/kg. Linalool, eucalyptol, and ocimene were found in both anthers and stigmas, with linalool being the predominant compound encountered, at concentrations around 25 mg/kg in male samples and 5 mg/kg in female samples. Eugenol, myrcene, and 1,4-dimethoxybenzene were present at lower concentrations, but only in anther samples. Ocimene was the only compound found in greater concentrations in stigmas than in anthers.

Principal component analysis (PCA) was performed for compounds found in concentrations greater than 0.2 ppm. Three components explained 92% of the variance. The first component (PC1) explained the predominant odor of the whole flower, which was related to two different groups: the petals profile and the presence at high concentrations of two volatiles from nectar. Figure 1 shows that the factors with more weight in the negative axis of PC1 were compounds with a relative petals/nectar concentration of greater than 1, such as α-pinene, toluene, indole, limonene, linalool, eugenol, and *o*-xylene that are compounds principally found in flower petals. Less weight in PC1 was given to those

TABLE 1. COMPOSITION OF THE VOLATILES (IN PPM) OF PETALS, NECTAR, ANTHER, AND STIGMA OF FEMALE AND MALE FLOWERS OF CULTIVAR TOSCA

Compound	Petals[a] (mg/kg)		Nectar[b] (mg/l)		Anther[a] (mg/kg)	Stigma[a] (mg/kg)
	Male	Female	Male	Female		
Toluene	1.61 ± 0.26	1.12 ± 0.08	0.09 ± 0.02	0.3 ± 0.12	0.05 ± 0.01	0.02 ± 0.01
Ethylbenzene, *p*,*m*,-xylene	2.70 ± 0.34	2.35 ± 0.18	1.5 ± 0.33	2.28 ± 0.28	1.11 ± 0.16	<LOQ
o-Xylene	2.22 ± 0.34	1.18 ± 0.21	0.05 ± 0.02	0.04 ± 0.01	0.82 ± 0.12	<LOQ
α-Pinene	10.38 ± 0.57	7.39 ± 0.37	1.66 ± 0.27	0.80 ± 0.22	1.74 ± 0.50	0.28 ± 0.23
(*R*)-(+)-Limonene	9.49 ± 0.83	4.52 ± 0.65	<LOQ	0.05 ± 0.01	1.25 ± 0.86	0.91 ± 0.18
Myrcene	1.74 ± 0.43	2.38 ± 0.42	1.7 ± 0.16	2.16 ± 0.24	1.18 ± 0.29	<LOQ
Eucalyptol	4.51 ± 0.56	3.41 ± 0.14	4.56 ± 0.42	3.42 ± 0.56	7.44 ± 1.18	0.74 ± 0.1
Ocimene	2.12 ± 0.18	1.8 ± 0.17	1.52 ± 0.24	0.54 ± 0.13	1.65 ± 0.57	5.04 ± 0.78
Linalool	22.24 ± 0.18	15.69 ± 0.89	2.80 ± 0.22	1.36 ± 0.2	24.58 ± 5.10	6.85 ± 0.98
1,4-Dimethoxybenzene	2.01 ± 0.22	1.04 ± 0.06	63.54 ± 3.33	49.02 ± 2.32	0.16 ± 0.10	<LOQ
Indole	4.42 ± 0.39	3.62 ± 0.39	<LOQ	<LOQ	<LOQ	<LOQ
Eugenol	1.2 ± 0.13	0.99 ± 0.1	<LOQ	0.02 ± 0.01	0.57 ± 0.32	<LOQ
1,2,4-Trimethoxybenzene	0.03 ± 0.01	<LOQ	61.26 ± 1.77	52.98 ± 2.72	<LOQ	<LOQ

[a]The data shown are the averages ± SD from three measures.
[b]The data shown are the averages ± SD from five measures.
LOQ: Limit of quantification.

TABLE 2. COMPOSITION OF THE VOLATILES (IN PPM) OF PETALS, NECTAR, ANTHER, AND STIGMA OF FEMALE AND MALE FLOWER OF CULTIVAR CONSUL

	Petals[a] (mg/kg)		Nectar[b] (mg/l)			
Compound	Male	Female	Male	Female	Anther[a] (mg/kg)	Stigma[a] (mg/kg)
Toluene	1.77 ± 0.20	1.23 ± 0.08	0.03 ± 0.01	0.18 ± 0.08	0.05 ± 0.03	<LOQ
Ethylbenzene, *p*,*m*,-xylene	2.76 ± 0.47	2.14 ± 0.13	1.64 ± 0.16	2.44 ± 0.23	1.33 ± 0.26	<LOQ
o-Xylene	2.10 ± 0.12	1.15 ± 0.02	0.072 ± 0.01	0.04 ± 0.02	1.01 ± 0.32	<LOQ
α-Pinene	9.53 ± 0,48	7.88 ± 0.27	1.74 ± 0.19	0.92 ± 0.21	1.45 ± 0.47	0.95 ± 0.15
(*R*)-(+)-Limonene	8.83 ± 1.12	3.80 ± 0.35	<LOQ	0.048 ± 0.02	1.29 ± 0.16	0.75 ± 0.12
Myrcene	1.57 ± 0.18	2.45 ± 0.30	1.72 ± 0.13	2.08 ± 0.20	1.26 ± 0.20	<LOQ
Eucalyptol	4.23 ± 0.22	3.93 ± 0.33	4.3 ± 0.56	3.5 ± 0.46	7.53 ± 0.26	0.61 ± 0.1
Ocimene	2.04 ± 0.24	1.66 ± 0.14	1.48 ± 0.30	0.56 ± 0.25	1.59 ± 0.67	1.78 ± 0.56
Linalool	22.04 ± 0.88	15.59 ± 0.54	2.82 ± 0.56	1.96 ± 0.25	25.98 ± 1.60	5.26 ± 0.24
1,4-Dimethoxybenzene	1.46 ± 0.31	1.07 ± 0.14	61.44 ± 2.12	46.98 ± 2.45	0.11 ± 0.03	<LOQ
Indole	3.96 ± 0.45	3.77 ± 0.44	<LOQ	<LOQ	<LOQ	<LOQ
Eugenol	1.05 ± 0.12	0.95 ± 0.051	<LOQ	0.03 ± 0,01	0.48 ± 0.15	<LOQ
1,2,4-Trimethoxybenzene	0.04[b] ± 0.01	<LOQ	59.94 ± 2.80	51.42 ± 2.95	<LOQ	<LOQ

[a]The data shown are the averages ± SD from three measures.
[b]The data shown are the averages ± SD from five measures.
LOQ: Limit of quantification.

TABLE 3. COMPOSITION OF THE VOLATILES (IN PPM) OF PETALS, NECTAR, ANTHER, AND STIGMA OF FEMALE AND MALE FLOWER OF CULTIVAR CHAPIN

Compound	Petals[a] (mg/kg)		Nectar[b] (mg/l)		Anther[a] (mg/kg)	Stigma[a] *(mg/kg)*
	Male	Female	Male	Female		
Toluene	1.22[a] ± 0.10	0.83 ± 0.05	0.05 ± 0.04	0.32 ± 0.16	0.04 ± 0.01	<LOQ
Ethylbenzene, *p*,*m*,-xylene	2.61 ± 0.32	1.76 ± 0.10	1.50 ± 0.33	2.34 ± 0.38	1.27 ± 0.13	<LOQ
o-Xylene	1.87 ± 0.11	0.92 ± 0.25	0.05 ± 0.02	0.04 ± 0.01	1.02 ± 0.14	<LOQ
α-Pinene	8.30 ± 0.24	6.84 ± 0.33	1.66 ± 0.27	0.80 ± 0.22	1.24 ± 0.17	0.87 ± 0.11
(*R*)-(+)-Limonene	8.69 ± 0.76	3.10 ± 0.15	<LOQ	0.05 ± 0.02	1.38 ± 0.16	0.79 ± 0.15
Myrcene	1.18 ± 0.22	1.76 ± 0.22	1.70 ± 0.16	2.16 ± 0.24	1.26 ± 0.33	<LOQ
Eucalyptol	3.45 ± 0.21	2.55 ± 0.48	4.70 ± 1.09	3.52 ± 0.48	7.49 ± 0.40	0.67 ± 0.06
Ocimene	1.69 ± 0.15	1.03 ± 0.07	1.44 ± 0.33	0.58 ± 0.19	1.57 ± 0.63	1.63 ± 0.51
Linalool	19.08 ± 0.14	12.92 ± 0.14	2.80 ± 0.64	2.40 ± 0.58	24.87 ± 0.78	4.44 ± 0.59
1,4-Dimethoxybenzene	1.31 ± 0.20	0.75 ± 0.53	60.18 ± 2.87	46.74 ± 2.45	0.07 ± 0.02	<LOQ
Indole	3.84 ± 0.13	2.97 ± 0.13	<LOQ	<LOQ	<LOQ	<LOQ
Eugenol	0.88 ± 0.09	0.56 ± 0.14	<LOQ	0.03 ± 0.01	0.53 ± 0.11	<LOQ
1,2,4-Trimethoxybenzene	<LOQ	<LOQ	59.16 ± 2.23	49.98 ± 2.72	<LOQ	<LOQ

[a]The data shown are the averages ± SD from three measures.
[b]The data shown are the averages ± SD from five measures.
LOQ: Limit of quantification.

compounds with a petals/nectar ratio close to 1 (myrcene, ethylbenzene, *p*, *m*-xylene, and eucalyptol), and with more weight in the positive axis of PC1 were 1,4-dimethoxybenzene and 1,2,4-trimethoxybenzene, which had a petals/nectar ratio of concentrations less than 1. These findings concur with those of Dobson et al. (1990) with petals being the main source of the aroma profile of the whole flower. However, in zucchini, this is influenced by the high concentration of 1,4-dimethoxybenzene and 1,2,4-trimethoxybenzene in nectar. Such findings were also reported by Bergstrom et al. (1995) in *Ranunculus acris* and agree with qualitative data obtained in the analysis of the aromas from whole living flowers. Figure 2 shows that the main difference between genders is the higher proportion of 1,4-dimethoxybenzene and 1,2,4-trimethoxybenzene in the air surrounding female flowers. This is in agreement with the sexual dimorphism that is typical in monoecious flowers such as those belonging to the Cucurbitaceae. The headspace air concentration of compounds from whole living flowers is a consequence of the volatilization from the nectary. Less nectar volatilization is likely to occur in male flowers than in females because the nectary of the male flower is an enclosed receptacle, opened only by three opercula, whereas female flowers have an open receptacle.

The second principal component (PC2) was related to the nectar aromas profile, which showed that factors with most weight in PC2 were 1,4-dimethoxybenzene and 1,2,4-trimethoxybenzene having a petals/nectar ratio less than 1, and myrcene, ethylbenzene, *p*,*m*-xylene, and eucalyptol with a petals/nectar ratio close to 1. Finally, the compounds with most weight in the third component (Figure 3), were eucalyptol and linalool, which were present in anther samples, with a petals/anther ratio less than 1, which appeared to be related to the reported attraction effect of linalool on pollinator insects (Laloi et al., 1999; Henning et al., 1999).

The effect of the cultivar on the aroma profile was not shown in this analysis because this relationship was very low compared to the other variables analyzed, i.e., flower gender and flower parts. There were some differences in certain compound concentrations between cultivar Chapin and the other two cultivars (Tosca and Consul) that were grouped together (data not shown) in the PCA analysis. However, this difference was not significant. As a result, the effect of variety on aroma profiles was not proven in this experiment.

In summary, quantitative determinations of floral volatiles in *C. pepo* showed that the main sources are petals and nectaries, with a lesser contribution to the total flower scent by anthers and stigmas. PCA indicated that compounds found in petal samples had the greatest influence on the aroma profile of the flower, although some of the compounds found in nectar had a higher concentration than in petals and thus contributed to the aroma profile of the whole flower. Nectar scent is dominated by 1,4-dimethoxybenzene and 1,2,4-trimethoxybenzene, whereas petal odor mainly contained linalool, limonene,

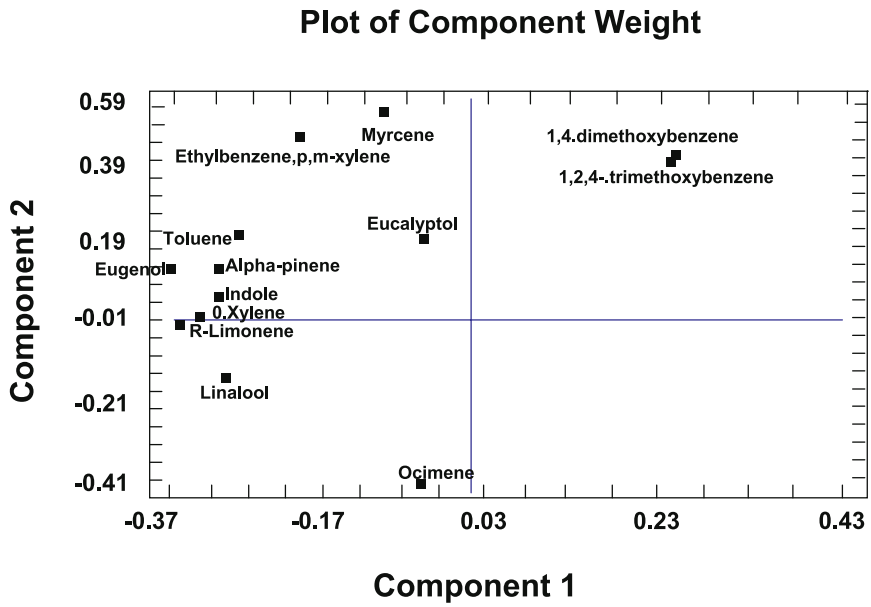

FIG. 1. Plot of samples of different flower parts, different cultivars, and genders on coordinate grid defined by principal components 1 and 2.

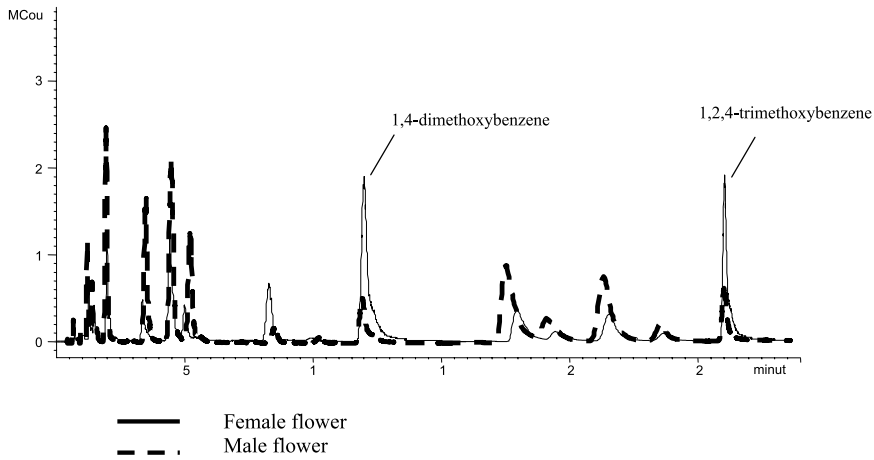

FIG. 2. MS/MS chromatograms of volatile compounds of whole living flower in both genders.

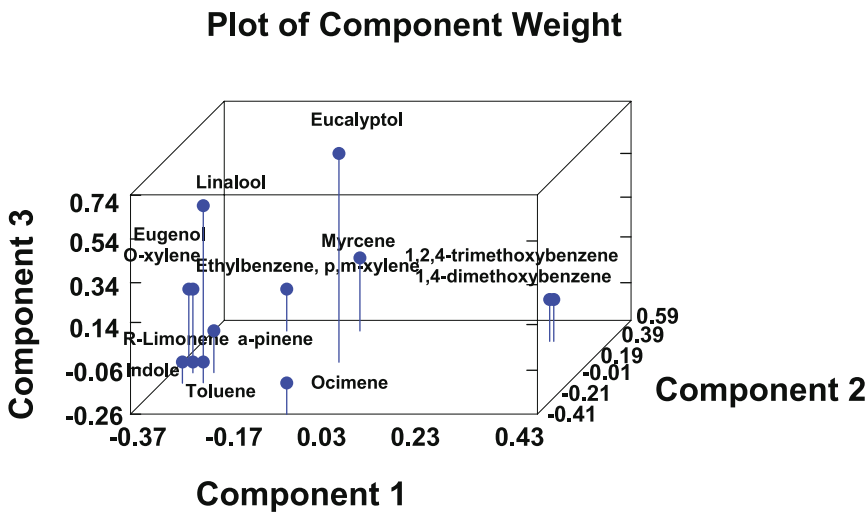

FIG. 3. Plot of samples of different flower parts, different cultivars, and genders on coordinate grid defined by principal components 1, 2, and 3.

and α-pinene. Indole was not present in nectar, but was in petals. Additionally, anther and stigmas had few compounds, with linalool and eucalyptol being the predominant compounds in these parts of the flower.

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SURFACE COMPOSITION OF MYRMECOPHILIC PLANTS: CUTICULAR WAX AND GLANDULAR TRICHOMES ON LEAVES OF *Macaranga tanarius*

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Abstract—Primary plant surfaces, covered with cuticles consisting of cutin and waxes, are important substrates for interaction with insects. The composition of leaf surfaces of the myrmecophilic plant *Macaranga tanarius* was studied. The prenylated flavanone nymphaeol-C was identified in surface extracts and was localized exclusively in glandular trichomes on the abaxial leaf side. The epidermal pavement cells surrounding these trichomes were covered with a smooth film of epicuticular wax from which few small wax crystals protruded. The epicuticular wax amounted to approximately 8 μg cm^{-2}, corresponding to 85% of the wax load on the adaxial as well as the abaxial leaf sides. The epicuticular wax mixtures from both leaf surfaces contained more than 70% primary alcohols, 14% fatty acids, 2% aldehydes, and traces of alkyl acetates, with chain lengths ranging from C_{20} to C_{38}. In contrast, the intracuticular wax layer was largely dominated by triterpenoid alcohols α-amyrin, β-amyrin, and lupeol. Consequently, these characteristic compounds are not available for direct contact with insects on the plant surface.

Key Words—Epicuticular wax, leaf surface, plant insect interactions, glandular trichomes, isoprenoids, flavanones, nymphaeol-C, *Macaranga tanarius*.

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INTRODUCTION

The surfaces of leaves, stems, flowers, and fruits are the stage for interactions between insects and their host plants. These aerial plant tissues, being in a primary state of development, are covered with a cuticle consisting of cutin and waxes (Walton, 1990). Within the latter, two layers of intracuticular and epicuticular waxes can be distinguished according to their location inside the cutin matrix and outside of it, respectively (Jeffree, 1986). The primary physiological function of the cuticle, to limit nonstomatal water loss, is likely associated with the intracuticular waxes (Baur, 1998), but the epicuticular waxes might also contribute to the transpiration barrier. Besides, the epicuticular waxes form the true surface of the plant organs and may serve important ecological functions in the interaction with insects and pathogens (Eigenbrode and Espelie, 1995).

The composition of plant cuticles has long been studied using superficial extraction of intact plant material with organic solvents (Walton, 1990). Solvent molecules enter rapidly into the deeper layers of the cuticle, thus releasing both epi- and intracuticular waxes (Jetter et al., 2000). Extracts reflect the total wax composition, averaging over the entire depth of the cuticular wax layers. The total wax mixtures of diverse plant species typically consist of homologous series of very long chain fatty acid derivatives, i.e., fatty acids, aldehydes, primary and secondary alcohols, ketones, and alkanes of chain lengths C_{20}–C_{36}, as well as alkyl esters with C_{38}–C_{70} (Tulloch, 1976). Besides, characteristic compounds such as triterpenoids and flavonoids can be present, in some species only in trace amounts and in others dominating the mixture (Wollenweber and Dietz, 1981; Baker, 1982).

In recent years, methods have been developed that allow independent and selective sampling from both the epi- and intracuticular waxes (Jetter et al., 2000; Jetter and Schäffer, 2001). Hence, it is now possible to describe the composition of plant surfaces more accurately. For example, it can be tested whether species-characteristic compounds are present at or near the plant surface and are hence available for direct contact with insects touching the plant cuticle. This is an important prerequisite for understanding the molecular mechanisms involved especially in early stages of interactions between insects and their host plants.

As one example of such an insect–plant interaction, the biology of ant–plant associations between the Asian pioneer tree genus *Macaranga* and its partner ants in the genera *Crematogaster* and *Camponotus* has been studied intensively (Fiala et al., 1994; Federle et al., 1997, 2000; Heil et al., 1998, 2001; Fiala et al., 1999). The partner ants nest inside the hollow shoots and feed predominantly on food bodies provided by the host plants. The ants actively search for and remove foreign objects touching the plant, thus providing indirect protection against epiphytes, herbivores, and pathogens. For various species of

the plant and ant genera involved, myrmecophilic or myrmecophytic associations are true mutualistic symbioses as they benefit both partners.

The stem waxes of various *Macaranga* species have been investigated, and in two instances their influence on the ant partners has been assessed. On one hand, host recognition by the colony-founding queen apparently involves surface cues of some *Macaranga* species (Inui et al., 2001). On the other hand, mechanical barriers located at the surface of various *Macaranga* species serve to protect partner ant species against generalist competitors (Federle et al., 1997). Thread-shaped epicuticular wax crystals that render the surfaces slippery for nonadapted ants generate these barriers. The surface crystals are formed by characteristic triterpenoids (Markstädter et al., 2000). In contrast, relatively little is currently known about the composition, properties, and functions of *Macaranga* leaf surfaces. Only in one species, *M. hypoleuca*, have adaxial and abaxial leaf waxes been studied in some detail (Markstädter et al., 2000). This investigation reports on the qualitative cuticular triterpenoids, aliphatic surface constituents, and large portions of the leaf waxes remain unidentified.

In the current study, we performed chemical analyses on *Macaranga tanarius* to address the questions (1) whether the cuticular waxes on adaxial and abaxial leaf surfaces differ, (2) whether gradients between the intra- and epicuticular wax layers on both sides of the leaves exist, (3) how much of the total cuticular wax is contributed by the intra- and epicuticular waxes, and (4) how the compositions of intra- and epicuticular wax layers on leaves differ from stems.

METHODS AND MATERIALS

Plant Material and Gland Preparation. Specimens of *M. tanarius* were taken from a continuous greenhouse culture in the Botanical Garden of the University of Würzburg, Germany. For the isolation of *M. tanarius* glandular trichomes, a protocol originally developed for preparing rose trichomes was adapted (Hashidoko and Urashima, 1995). Five mature leaves were harvested, cleaned carefully of food bodies using a paintbrush, and cut into pieces. Trichomes were sheared off the abaxial leaf surface by moving approximately 150 ml of glass beads with a diameter of 0.25–0.5 mm (Roth, Karlsruhe, Germany) in a highly viscous liquid, consisting of 650 ml of 0.4 M D-mannitol, 50 mM sodium L-(+)-ascorbate (both from Roth), and 5% glycerol (AppliChem, Darmstadt, Germany) at pH 6.8, in a 1-l round-bottom flask over the plant tissue. The flask was kept on a shaker for 90 min at 275 rpm. The resulting suspension was passed through a series of filtration steps by using nylon filters with different mesh sizes (125, 95, 74, 62, and 47 μm; Small Parts, Miami Lakes, FL, USA). Initially, big mesh sizes were employed to remove large fragments of leaflets and hair trichomes. At a mesh size of 62 μm, glandular

trichomes were collected nearly exclusively, whereas hair trichomes were only trapped on a mesh size of 47 μm. The filter residues were transferred into vials, and 3 ml of separation solution were added. The trichomes were ground into a paste, and 7 ml of chloroform and 10 μg of the internal standard of *n*-tetracosane were added. The mixture was vigorously agitated, and after phase separation the organic solution was removed, and the solvent was evaporated under a stream of N_2.

Sampling of Leaf Surface Compounds. Prior to use, gum arabic powder (Roth) was soxhlet-extracted in chloroform (Roth) for 3 hr. On freshly harvested leaves, several circles of 23 mm diam were marked with a felt pen, either on the adaxial or the abaxial side close to the center of the blade. The area inside the circles was cleaned carefully of food bodies with a dry paintbrush. Another paintbrush was used to apply an aqueous solution of gum arabic (ca. 0.8 mg ml^{-1}) onto the circular surface area. After 1 hr at 25°C, the hardened polymer film of gum arabic was lifted off the leaf together with adhering surface waxes. To separate wax constituents from gum arabic, the pieces of dry glue were transferred into vials containing a two-phase system of 7 ml each of chloroform and water. *n*-Tetracosane was added as internal standard, and the mixture was agitated vigorously. After phase separation, the organic phase was removed and concentrated under a gentle stream of N_2. The whole protocol could be repeated one more time on the adaxial side of the same leaf areas without damaging the cuticle or the underlying tissue, as was confirmed by scanning electron microscopy (SEM).

After the mechanical removal of epicuticular wax, the remaining cuticular wax was extracted with chloroform at room temperature. To this end, glass cylinders (13 mm diam) were gently pressed onto the leaf surface and filled twice with 1.5 ml of chloroform for 30 sec. Both chloroform solutions were combined, and *n*-tetracosane was added as internal standard. Alternatively, the total wax coverage was assessed by extraction of the cuticular wax from leaf surfaces without prior treatment with gum arabic.

Wax Analysis. Prior to GC analysis, chloroform was evaporated from all samples under a gentle stream of N_2 while heating the sample vials to 50°C. Next, all samples were treated with bis-*N*,*N*-(trimethylsilyl)trifluoroacetamide (BSTFA, Macherey-Nagel, Düren, Germany) in pyridine (30 min at 70°C) to transform all hydroxyl-containing compounds into the corresponding trimethylsilyl (TMSi) derivatives. The qualitative composition was studied with capillary GC (5890 II, Hewlett Packard, Avondale PA, USA; 30 m DB-1, 0.32 mm i.d., d_f = 1 μm; J & W Scientific, Folsom, CA, USA) with He carrier gas inlet pressure constant at 30 kPa and mass spectrometric detector (5971, Hewlett Packard). GC was performed with temperature-programmed injection at 50°C, oven 2 min at 50°C, raised by 40°C min^{-1} to 200°C, held for 2 min at 200°C, raised by 3°C min^{-1} to 320°C, held for 30 min at 320°C. The quantitative

composition of the mixtures was studied using capillary GC with flame ionization detection under the same gas chromatographic conditions described above, but H_2 carrier gas inlet pressure was programmed for 50 kPa at injection, held for 5 min, raised with 3 kPa min^{-1} to 150 kPa and held for 40 min at 150 kPa. Single compounds were quantified against the internal standard by manually integrating peak areas.

Identification of Nymphaeol-C. For identification of a novel surface component, intact leaves were extracted with chloroform. The resulting wax mixture was separated using thin layer chromatography (TLC) on silica gel with $CHCl_3$/EtOH (99:1) as mobile phase. Bands were stained with primuline, visualized under UV light, immediately removed from the plates, eluted with $CHCl_3$, and filtered. Finally, the solvent was removed in a stream of N_2, and samples were stored in the dark at 4°C. The target compound was detected in a fraction with R_f = 0.11. Hydroxyl groups were either transformed into TMSi ethers as described above, or acetylated by adding pyridine and Ac_2O to the dried fraction, heating the mixture to 70°C for 5 min, maintaining it at room temperature overnight, and then removing the solvent in a stream of N_2.

Circular dicroism (CD) spectra (25°C, MeOH, 0.02 cm cell) were recorded on a Jasco J-715 spectropolarimeter. ^{1}H NMR (400 and 600 MHz) and ^{13}C NMR (100 and 150 MHz) spectra were measured on Bruker Avance 400 and DMX 600 instruments, using $CDCl_3$ (δ 7.26 and 77.01) as a solvent and as the internal ^{1}H and ^{13}C standard. Proton-detected, heteronuclear correlations were measured using HMQC (optimized for $^1J_{HC}$ = 145 Hz) and HMBC (optimized for $^nJ_{HC}$ = 7 Hz) pulse sequences. EIMS of the underivatized compound was determined on a Fisons MD1000 instrument (70 eV) using the direct probe. Capillary GC analysis of the TMSi derivative was performed on a Hewlett-Packard 5890II gas chromatograph with FID and MS detection as described for wax analysis.

CD (CH_3OH) $\Delta\varepsilon_{207}$ −44.1, $\Delta\varepsilon_{222}$ +48.8, $\Delta\varepsilon_{293}$ −27.7, $\Delta\varepsilon_{333}$ +3.9; ^{1}H, ^{13}C NMR, and MS data were identical to those of the known (−)-nymphaeol-C isolated from *Hernandia nymphaefolia* (Yakushijin et al., 1980).

Scanning Electron Microscopy. Dried (overnight, 25°C) samples of untreated leaf disks and of leaf disks from the various treatments were mounted on aluminum holders, sputter coated with gold–palladium (Bal-Tec SCD005 Sputter Coater; 25 mA, 300 s; Balzers, Switzerland), and examined by SEM (Zeiss DSM 962, 15 kV; Zeiss, Oberkochen, Germany).

RESULTS AND DISCUSSION

Micromorphology of Leaf Surfaces. On *M. tanarius*, leaf hairs can be seen with the naked eye. Scanning electron microscopy revealed that hair trichomes

were unbranched and apparently unicellular, ranging from 10 to 15 μm in base diam and 100–150 μm in length, and ending in sharp points (Figure 1A). This type of trichomes was located mostly above vascular bundles on both sides of the leaf. A second type was restricted to the abaxial leaf surface, where they were located in shallow epidermal pits between principal veins (Figure 1A and B). These trichomes were characterized by segmental arrangement of eight cells in a circular head with a diameter of ca. 70 μm. Convex head segments were sharply delineated by sunken lines, likely above anticlinal cell walls.

Light microscopy showed that this cell shape and arrangement was present on fresh leaf material (data not shown) and could not be attributed to drying artifacts caused by sample preparation for SEM. This suggests that the trichome cells do not share a subcuticular cavity, as described for glandular trichomes on Lamiaceae leaves (Turner et al., 2000). Baker (1934) had reported similar trichomes for leaves of *Macaranga* species and called them bladder glands,

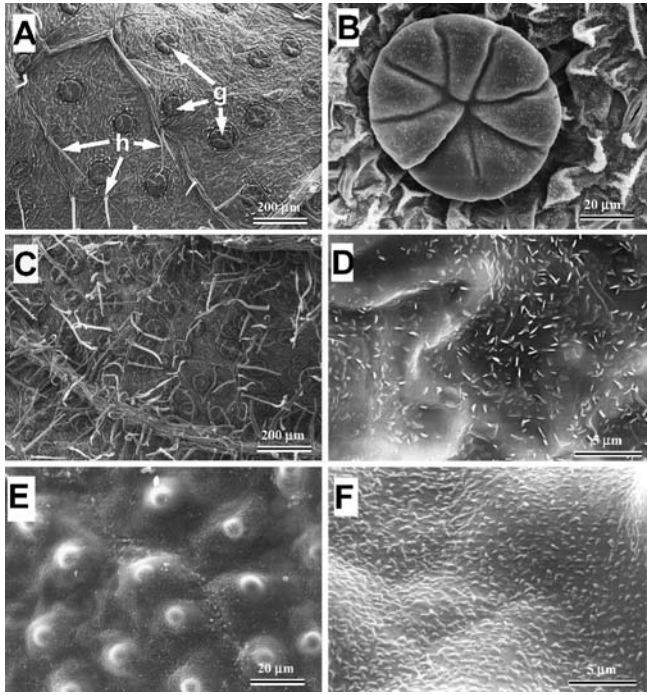

FIG. 1. Scanning electron micrographs of *M. tanarius* leaf surfaces. (A–B) Glandular trichomes (g) and hair trichomes (h) on the abaxial surface of mature leaves. (C) Abaxial surface of young leaf. (D) Detail of the wax structures on epidermal pavement cells of the mature abaxial surface. (E–F) Surface of pavement cells on the adaxial leaf.

whereas Elias (1983) designated comparable structures as scale-like nectaries. As neither the glandular character nor the nectary function has been verified for the structures on *M. tanarius* leaves, we will refer to them as scale trichomes.

On surfaces of young (<2 d) leaves of *M. tanarius*, both hair and scale trichomes were found in much higher density per surface area than on mature leaves (Figure 1C). In the course of leaf expansion, their numbers, sizes, and shapes stayed constant, whereas their densities gradually diminished. It can be concluded that both types of trichomes are formed early in leaf ontogenesis and reach maturity independently of epidermal pavement cells.

On epidermal pavement cells of both leaf sides, epicuticular wax structures were visible. The epicuticular waxes on the abaxial surface formed thin, upright platelets (length approximately 1 μm) with sharp edges (Figure 1D). In contrast, the adaxial surface was covered with a smooth wax film (Figure 1E), from which very small (approximately 500 nm), rounded granules protruded (Figure 1F). Compared to the surfaces of epidermal pavement cells, the scale trichomes were only sparsely covered with very small (approximately 500 nm) granules (Figure 1B), whereas the surface of the hair trichomes appeared smooth.

Identification of Nymphaeol-C. To prepare surface compounds for analysis, the adaxial and abaxial sides of intact *M. tanarius* leaves were first separately extracted with chloroform. While the two resulting solutions contained similar mixtures of typical cuticular wax constituents (see below), a single major compound was detected exclusively in the abaxial extract. As this compound had previously not been described in surface mixtures, its structure had to be assigned by a combination of spectroscopic techniques.

In a first experiment, the compound was purified from the crude surface extract by analytical TLC (R_f = 0.11 for chloroform/silica) and analyzed by GC-MS. The mass spectrum of the underivatized compound (Figure 2A) showed a prominent fragment *m*/*z* 69 that indicated the presence of one or more isoprenyl units. Other signals at *m*/*z* 368 ($[M-124]^+$), 221, 165, and 123 also matched with qualitative literature data for flavanones containing a dimethylallyl and a geranyl substituent (Yakushijin et al., 1980). In accordance with this tentative assignment, a molecular ion *m*/*z* 492 and a fragment *m*/*z* 477 $[M\text{-methyl}]^+$ were detected. The mass spectrum of the corresponding trimethylsilyl derivative (Figure 2B) further confirmed the prenylated flavanone structure by the presence of fragments *m*/*z* 73 and 147 (indicating multiple OTMSi groups), signals for *m*/*z* 780 $[M]^+$, 711 $[M\text{-dimethylallyl}]^+$, 643 $[M\text{-geranyl}]^+$, and for the corresponding daughter fragments *m*/*z* 765 $[M\text{-methyl}]^+$, 696 $[M\text{-dimethylallyl-methyl}]^+$, 628 $[M\text{-geranyl-methyl}]^+$.

Based on the spectral information given above, the novel surface component could be described as a prenylated flavanone with the overall formula $C_{30}H_{36}O_6$. Because reference data for possible isomeric representatives of this compound class had not been reported in the literature, the substitution pattern could not be

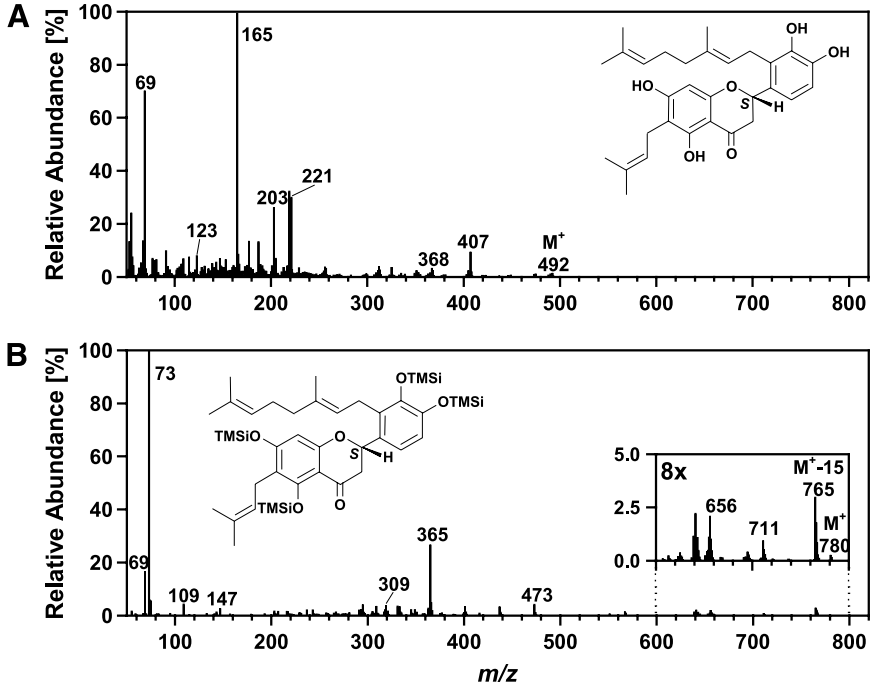

FIG. 2. Mass spectra of the prenylated flavanone nymphaeol-C. (A) Underivatized nymphaeol-C. (B) Trimethylsilyl derivate of nymphaeol-C.

assigned by using the fragmentation pattern alone. To allow the future identification of the compound employing GC-MS analysis, our mass spectral data had to be authenticated by other spectroscopic techniques. Therefore, in a second experiment, larger quantities of the compound were prepared by surface extraction of *M. tanarius* leaves, purified by TLC, and analyzed by NMR spectroscopy. The ^{1}H NMR spectrum showed the typical signals of a flavanone skeleton substituted with one geranyl, one γ,γ-dimethylallyl, and four hydroxyl groups. Both chemical shifts and coupling constants matched data previously reported for nymphaeol-C (Yakushijin et al., 1980). The constitution was corroborated by extended NOE, HMBC, HMQC, DEPT, and ^{13}C NMR measurements, proving that the novel surface constituent was indeed nymphaeol-C.

The absolute configuration at C-2 was confirmed to be *S* by comparison of the CD spectrum with that of (−)-nymphaeol-C (Yakushijin et al., 1980). Unfortunately, no CD data for the region <293 nm have as yet been published, apparently for instrumental reasons. Therefore, in this work we present these additional short wavelength CD data for the first time, thus making an unambiguous assignment of the compound easier in the future.

Localization of Nymphaeol-C in Scale Trichomes. The coincident occurrence of nymphaeol-C and scale trichomes, both restricted to the abaxial leaf surface of *M. tanarius*, suggested that the prenylated flavanone was produced and sequestered in these structures. This hypothesis was first tested by isolating the trichomes and subjecting them to chemical analyses. For this purpose, both types of trichomes were mechanically detached from the leaf surface and separated by differential filtration. As expected from the morphological data (see above), hair trichomes passed filter membranes with a mesh size of 62 µm, whereas scale trichomes were retained (Figures 3A and C). In the resulting scale fraction the amount of nymphaeol-C was 35 times higher than in the fraction of

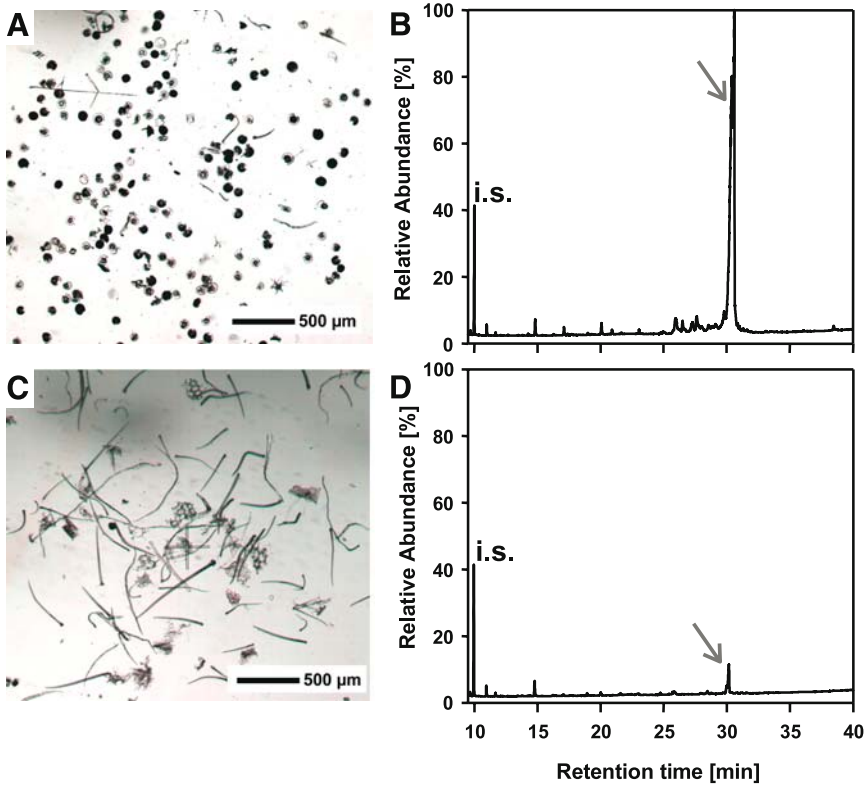

FIG. 3. Separation and extraction of scale and hair trichomes from *M. tanarius* leaves. (A) Light micrograph of the particle fraction from filtration through 62-µm mesh, containing mainly scale trichomes. (C) Light micrograph of the particle fraction from filtration through 47-µm mesh, containing mainly hair trichomes. (B and D) Normalized gas chromatograms showing quantities of nymphaeol-C (arrows) extracted from the fractions shown in (A) and (C), respectively (i.s.: internal standard *n*-tetracosane).

the hair trichomes (Figures 3B and D). Hence, the prenylflavanone is clearly associated with the scale trichomes, but not with the hair trichomes.

To further corroborate the localization of nymphaeol-C in these trichomes, we investigated the correlation between scale density and nymphaeol-C coverage per surface area. On individual leaves of various developmental stages, the amount of wax compounds per unit area was constant. In contrast, the amount of nymphaeol-C increased linearly with the number of scale trichomes ($r^2 = 0.93$ for linear regression, $P < 0.001$; Figure 4). It is, therefore, likely that this compound is accumulating exclusively in the scale trichomes, and not in the cuticular wax of the epidermal pavement cells. From the slope of the regression line, an average amount of 30 ng of nymphaeol-C per scale trichome can be inferred. Assuming a density of 1.4 g cm^{-3} for prenylated flavanones in a condensed phase (Kiehlmann et al., 1999), the neat compound should occupy 2.1×10^4 μm^{-3} per trichome, i.e., large portions of the head volume of approximately 3.5×10^4 μm^{-3} (calculated for 70 μm diam and 10 μm high). In accordance with this assessment, fluorescence microscopy of cross-sections through the scale trichomes showed green autofluorescence, similar to the appearance of flavonoid standards, throughout the volume of the head cells (data not shown). With all data

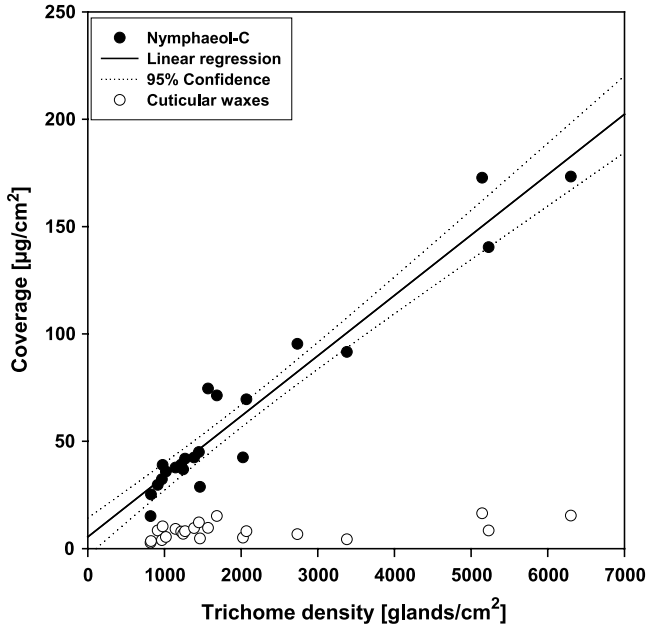

FIG. 4. Relationship between the coverage of nymphaeol-C or cuticular waxes and the trichome density.

taken together, the scale trichomes can be described as highly specialized glands, the head cells probably biosynthesizing and storing the prenylated flavanone.

Nymphaeol-C was first identified as a constituent of *Hernandia nymphaefolia* (Yakushijin et al., 1980). This compound had also been described as the major prenylated flavanone in fallen leaves of *M. tanarius* with a yield of 0.13 mg g^{-1} dry weight (Tseng et al., 2001). In the present study, an average of 60 μg cm^{-2} of nymphaeol-C was extracted from the abaxial leaf surface, corresponding to approximately 12 mg g^{-1} dry weight for mature leaves. As this value is more than 90 times higher than the result reported for fallen leaves, it is plausible that the entire amount of the prenylflavanone is located near the tissue surface. We conclude that nymphaeol-C is formed and stored exclusively in the glandular trichomes on the lower leaf surface of *M. tanarius*. Furthermore, the material is likely formed very early in leaf development and sequestered within the head cells without further turnover.

Although it has been reported that other prenylated flavonoids have cytotoxic properties inhibiting the growth of neighboring plants (Tseng et al., 2003), it is not clear whether this allelopathic effect represents the main ecological function of these constituents. The current results, localizing nymphaeol-C in scale trichomes restricted to the abaxial surface of *M. tanarius* leaves, show that this compound is exposed in high concentrations close to the plant surface. In analogy to the glandular trichomes of other plant families, the *Macaranga* scales might serve as defensive structures releasing allelochemicals upon mechanical damage (Werker, 1993). However, in sharp contrast to the essential oils produced by glandular trichomes of other plants, nymphaeol-C is not volatile and must exert its function(s) probably in direct physical contact between the plant surface and other organisms.

The present findings suggest a direct defensive function of nymphaeol-C against herbivores, in addition to the indirect defense exerted by partner ants on *M. tanarius*. This species is myrmecophilic, i.e., several relatively unspecific ant species can serve as partners that sometimes colonize the trees only late in development. This may leave *M. tanarius* plants without ant protection for long periods, additionally necessitating direct chemical defense mechanisms. This leads to the question whether other *Macaranga* species, either myrmecophytes (obligate ant–plants) or nonmyrmecophytes (including myrmecophilic species), have similar scale trichomes containing nymphaeol-C. Unfortunately, there are no published data that could be interpreted accordingly. In one report, only the total flavonoid amounts have been quantified for leaves of various *Macaranga* species including *M. tanarius* (Heil et al., 2002), but the flavonoid constituents and their localization have not been specified. In a preliminary survey, we found that scale trichomes, similar to those on *M. tanarius*, are present on the abaxial side of leaves of various *Macaranga* species. GC-MS analyses showed that in none of these cases—comprising the myrmecophytic

species *M. constricta*, *M. triloba*, and *M. winkleri*, the partially myrmecophytic species *M. hosei* and *M. pruinosa*, as well as the nonmyrmecophytic species *M. gigantea* and *M. heynei*—did the trichomes contain nymphaeol-C (data not shown).

Total Cuticular Wax Mixtures on M. tanarius *Leaves*. Cuticular waxes were extracted separately from the adaxial and the abaxial leaf surfaces, and analyzed using GC-FID and GC-MS. Preliminary tests had shown that this extraction was exhaustive for the soluble cuticular lipids, thus yielding total wax amounts. The adaxial leaf cuticle was found to contain 9.1 ± 2.5 μg cm^{-2} waxes, whereas the abaxial side (excluding the amount of nymphaeol-C) was covered with 10.9 ± 2.9 μg cm^{-2} of waxes (Figure 5).

On both leaf surfaces, characteristic patterns of diverse aliphatic compounds and small amounts of triterpenoids were detected, and overall 40 compounds were identified. In the total wax mixture on the adaxial leaf side, 13.6 ± 8.0% triterpenoid alcohols were found, mainly α-amyrin, β-amyrin, and lupeol, whereas 70.8 ± 7.0% were aliphatic compounds. The most abundant component class were the primary alcohols with 56.7 ± 11.5% (5.2 ± 1.8 μg cm^{-2}), followed by fatty acids with 11.0 ± 2.6% (1.0 ± 0.4 μg cm^{-2}) and aldehydes with 3.1 ± 2.2% (0.3 ± 0.2 μg cm^{-2}). Only 10.9 ± 5.2% of the adaxial wax mixture remained unidentified (Figure 6A). A similar pattern of compound classes was detected in total lipid extracts from lower leaf surfaces. In addition, the abaxial wax mixture contained small amounts of acetates with 3.5 ± 2.4% (0.4 ± 0.2 μg cm^{-2}) (Figure 6B).

The wax mixtures from both leaf sides contained homologous series of primary alcohols with chain lengths between C_{24} and C_{38} that had a strong predominance of even carbon numbers. The chain length distribution of

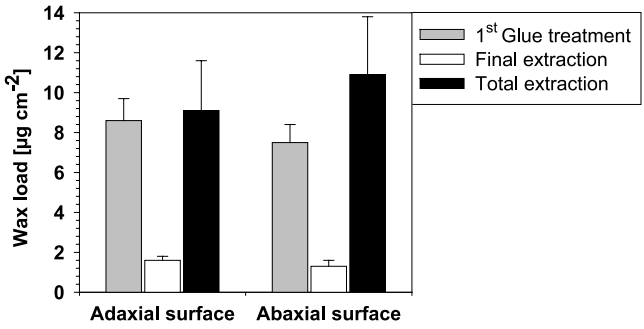

FIG. 5. Wax coverage on adaxial and abaxial leaf surfaces of *M. tanarius*. Yields from two independent experiments are given, one of them using selective mechanical removal with gum arabic and consecutive (final) extraction with chloroform, the other one using only extraction of bulk cuticular waxes (total extraction) with chloroform.

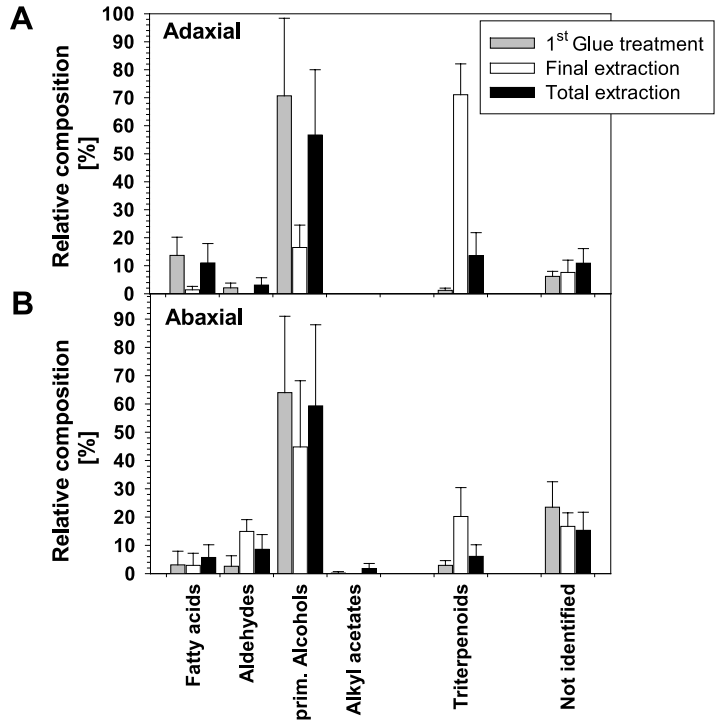

FIG. 6. Relative wax composition on leaf surfaces of *M. tanarius*. Amounts of the different component classes on (A) adaxial and (B) abaxial surfaces are given as percentages of respective wax mixtures.

alcohols from the upper leaf surface showed two maxima for C_{26} and C_{34}, whereas those from the lower leaf surface had only one maximum at C_{32}. The homologous series of fatty acids comprised chain lengths between C_{20} and C_{38}, while aldehydes with chain lengths between C_{26} and C_{34} were identified. Both compound classes were dominated by representatives with even carbon numbers and showed a maximum for C_{26} (Table 1).

Epi- and Intracuticular Wax Layers on M. tanarius *Leaves*. The contribution of epi- and intracuticular layers to the overall wax composition was assessed by successive mechanical and extractive probing. Hence, waxes were first removed from the surface using gum arabic as an adhesive. Two consecutive treatments of the adaxial surface yielded 8.6 ± 1.1 and 1.1 ± 1.0 μg cm^{-2} of waxes, respectively. A third gum arabic treatment caused the leaves to further dry and wilt, making a successive extraction for the investigation of intracuticular waxes impossible. Therefore, only a small number of samples were treated a third time

TABLE 1. RELATIVE COMPOSITION [%] OF INDIVIDUAL COMPOUNDS IN WAX MIXTURES SAMPLED SEPARATELY FROM BOTH SIDES OF *M. tanarius* LEAVES

Compound	Adaxial surface			Abaxial surface		
Chain length	First glue treatment	Final extraction	Total extraction	First glue treatment	Final extraction	Total extraction
Fatty acids						
20	0.2 ± 0.2	–	0.1 ± 0.2	–	–	–
22	0.3 ± 0.2	–	0.6 ± 1.0	–	–	–
24	1.2 ± 0.6	–	0.9 ± 0.4	0.4 ± 0.3	–	0.6 ± 0.4
26	3.2 ± 1.1	–	2.2 ± 0.9	1.0 ± 1.3	0.3 ± 0.6	1.7 ± 0.8
28	1.4 ± 0.6	1.4 ± 1.2	1.0 ± 0.4	0.6 ± 1.0	0.9 ± 1.6	1.2 ± 0.7
30	1.0 ± 0.4	–	0.7 ± 0.6	0.8 ± 1.7	0.6 ± 1.1	1.4 ± 1.3
32	1.8 ± 0.8	–	1.4 ± 0.4	–	1.1 ± 1.0	–
34	2.4 ± 0.4	–	3.1 ± 2.3	–	–	–
36	0.8 ± 0.3	–	0.4 ± 0.2	0.4 ± 0.5	–	0.7 ± 1.1
38	0.6 ± 0.1	–	0.3 ± 0.3	–	–	–
Aldehydes						
26	0.5 ± 0.3	–	0.6 ± 0.4	0.8 ± 0.9	14.9 ± 4.2	2.9 ± 1.9
28	0.4 ± 0.3	–	0.6 ± 0.3	0.7 ± 0.8	–	2.5 ± 0.9
30	0.2 ± 0.1	–	0.3 ± 0.2	0.9 ± 1.6	–	2.8 ± 1.8
32	0.7 ± 0.8	–	1.2 ± 1.1	0.2 ± 0.3	–	0.4 ± 0.5
34	0.3 ± 0.3	–	0.4 ± 0.5	–	–	–
Primary alcohols						
24	0.7 ± 0.9	–	0.4 ± 0.8	0.4 ± 0.3	0.4 ± 0.4	0.7 ± 0.4
26	20.8 ± 9.3	5.9 ± 3.7	17.3 ± 6.8	11.8 ± 3.3	–	15.1 ± 7.4
28	15.6 ± 3.9	4.5 ± 1.9	13.3 ± 3.6	13.5 ± 2.7	14.8 ± 3.9	15.4 ± 6.1
30	3.3 ± 1.4	–	2.8 ± 1.5	12.3 ± 5.7	12.5 ± 6.5	11.5 ± 4.9
32	9.7 ± 2.3	3.7 ± 1.7	7.8 ± 2.7	15.8 ± 9.7	11.3 ± 7.0	7.8 ± 3.9
34	14.2 ± 6.5	2.4 ± 0.7	10.5 ± 5.3	9.0 ± 3.5	4.0 ± 2.6	6.1 ± 4.2
36	2.4 ± 1.5	–	2.2 ± 0.9	0.8 ± 1.1	0.9 ± 1.6	1.5 ± 0.6
38	0.7 ± 0.3	–	0.7 ± 0.2	–	–	–
Alkyl acetates						
28 + 2	–	–	–	0.1 ± 0.2	–	0.9 ± 0.7
30 + 2	–	–	–	0.1 ± 0.3	–	0.9 ± 1.1
Triterpenoids						
β-Amyrin	0.7 ± 0.4	25.9 ± 4.1	5.4 ± 2.7	1.2 ± 0.7	5.8 ± 3.4	2.6 ± 1.6
α-Amyrin	0.4 ± 0.2	37.4 ± 4.5	7.0 ± 4.2	1.7 ± 1.0	12.1 ± 4.8	3.2 ± 1.9
Lupeol	tr	7.8 ± 2.3	1.1 ± 1.3	–	2.3 ± 2.0	0.3 ± 0.5

with glue, and found to yield no appreciable amounts of wax (data not shown). From this result, we conclude that the glue treatments had exhaustively removed a layer of waxes that was accessible to mechanical treatment. In contrast, a final extraction of the adaxial surface after two gum arabic treatments yielded 1.6 ± 0.2 μg cm^{-2} of waxes (Figure 5). This layer of wax must have been located inside a mechanical barrier that was likely imposed by the presence of the cutin

polymer matrix. Hence, in analogy to previous findings for other plant species (Jetter and Schäffer, 2001; Riedel et al., 2003; Vogg et al., 2004), the gum arabic samples can be interpreted as epicuticular waxes, while the final extraction yielded the remaining intracuticular waxes. This implies selective sampling of two distinct layers of waxes within the adaxial cuticle of *M. tanarius*, allowing a more accurate assessment of the wax composition at the surface of the leaf. In summary, on the adaxial leaf side 86% of the wax was located in the epicuticular layer.

For the abaxial side of the leaves, a first gum arabic treatment yielded 7.5 ± 0.9 μg cm^{-2} of waxes, while a consecutive extraction step released another 1.3 ± 0.3 μg cm^{-2} of wax (Figure 5). For this tissue, the mechanical treatment could not be repeated without disrupting the tissue. It can, therefore, not be directly assessed whether the mechanical treatment was exhaustive, and the selectivity of the sampling strategy cannot be evaluated. Nevertheless, the comparison of both experiments, i.e., the total wax extraction and the mechanical wax sampling, shows that the first gum arabic treatment likely released most of the epicuticular wax. The gum arabic samples give a fairly accurate representation of the abaxial surface composition. On the other hand, the samples from the successive extraction must largely reflect the intracuticular composition, even though they may have been contaminated with small amounts of epicuticular wax. The overall wax yields found in the second experiment for both adaxial and abaxial leaf surfaces were not significantly different from the corresponding results of the total wax extraction (Mann–Whitney *U*-test adaxial: $U = 4$, $P = 0.39$, abaxial $U = 7$, $P = 0.71$). Both experiments independently confirm each other.

The gum-arabic treated plant samples were inspected with SEM to visualize the mechanical removal of epicuticular waxes. After treatment with the glue, both adaxial and abaxial surfaces were smooth, whereas adjacent untreated areas showed the small crystal structures characteristic for native surfaces (see above). In transition regions between treated and untreated areas, a sharp edge could be seen (arrows in Figure 7A, B, and D). As a result of the gum arabic treatment, most of the gland heads were ripped off the abaxial leaf surface, exposing the end of short stalks that had apparently carried the gland scale. The stalks were located in shallow pits on the leaf surface, formed by the surrounding epidermal pavement cells (Figure 7C). The lower side of gum arabic represented a negative replica of the treated plant surface in which the outlines of pavement cells and stomata were clearly preserved. In addition, prominent structures with circular outlines comprising four or eight equal segments were visible. Both top and side views of these structures helped to identify them as glandular heads that had been broken off the abaxial leaf surface and were embedded in the gum arabic film (Figure 7E and F).

Especially on the adaxial leaf side, the chemical compositions of epi- and intracuticular waxes differed strikingly (Figure 6). The epicuticular layer

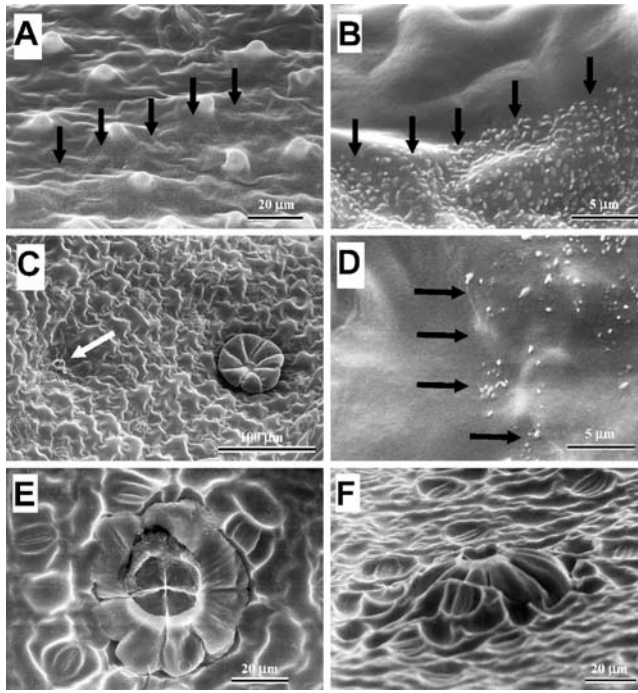

FIG. 7. Scanning electron micrographs documenting the treatment of *M. tanarius* leaf surfaces with gum arabic. (A–B) Adaxial leaf surface. (C–D) Abaxial leaf surface. (E–F) Lower surface of gum arabic films after treating the adaxial leaf surface.

contained large quantities of primary alcohols (70.1 ± 7.9%) and fatty acids (13.5 ± 4.4%), whereas only small amounts of triterpenoids were present (1.5 ± 0.9%). In contrast, the intracuticular wax was dominated by triterpenoids (71.1 ± 8.5%), whereas alcohols and fatty acids were found in relatively small percentages. Similar patterns could be observed for the epi- and intracuticular wax from the lower leaf surface, even though epi- and intracuticular waxes appeared to differ less than on the adaxial side (Figure 6). The relatively small differences between layers may have been in part due to contamination of final extraction products by epicuticular waxes remaining after the single gum arabic treatment (see above).

In summary, pronounced gradients existed between the epi- and intracuticular waxes on both sides of *M. tanarius* leaves. Both layers were not only mechanically, but also chemically distinct. On both sides of *M. tanarius* leaves, the cuticular triterpenoids were buried underneath a layer of aliphatic wax compounds, and are likely not available for direct contact with insects on the plant surface. This result leads us to question whether leaf cuticular triterpenoids serve

a direct function in moderating the behavior of insects living in contact with the plant surface, even though antifeeding properties have been reported for some triterpenoids (Schoonhoven et al., 1998).

The intracuticular localization of triterpenoids in *M. tanarius* leaf cuticles is of special importance for our understanding of the principles governing cuticle structure formation. On the one hand, it had previously been reported that other triterpenoids are also exclusively located in the intracuticular layer of *Prunus laurocerasus* leaves, which is a distantly related plant species (Jetter et al., 2000). This might reflect a general tendency of triterpenoids to accumulate in the intracuticular wax layer, possibly due to relatively high affinity to the cutin matrix. On the other hand, indirect evidence had shown that triterpenoids accumulate to high concentration in the epicuticular wax on stems of diverse *Macaranga* species (Markstädter et al., 2000). β-Amyrin is located at the surface of *M. tanarius* stems where it is involved in formation of epicuticular crystals that are important for the mechanical interaction with ants walking on the plant surface. Unfortunately, direct evidence is missing that would allow comparisons between the levels of triterpenoids in the intracuticular and epicuticular layers of the stems. It is consequently not clear whether gradients within the *Macaranga* stem wax exist, and currently it cannot be judged whether such triterpenoid gradients would be parallel or antiparallel to those described for *Prunus* and *Macaranga* leaves.

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EFFECTS OF ELEVATED CO_2 AND HERBIVORE DAMAGE ON LITTER QUALITY IN A SCRUB OAK ECOSYSTEM

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Abstract—Atmospheric CO_2 concentrations have increased dramatically over the last century and continuing increases are expected to have significant, though currently unpredictable, effects on ecosystems. One important process that may be affected by elevated CO_2 is leaf litter decomposition. We investigated the interactions among atmospheric CO_2, herbivory, and litter quality within a scrub oak community at the Kennedy Space Center, Florida. Leaf litter chemistry in 16 plots of open-top chambers was followed for 3 years; eight were exposed to ambient levels of CO_2, and eight were exposed to elevated levels of CO_2 (ambient + 350 ppmV). We focused on three dominant oak species, *Quercus geminata*, *Quercus myrtifolia*, and *Quercus chapmanii*. Condensed tannin concentrations in oak leaf litter were higher under elevated CO_2. Litter chemistry differed among all plant species except for condensed tannins. Phenolic concentrations were lower, whereas lignin concentrations and lignin/nitrogen ratios were higher in herbivore-damaged litter independent of CO_2 concentration. However, changes in litter chemistry from year to year were far larger than effects of CO_2 or insect damage, suggesting that these may have only minor effects on litter decomposition.

Key Words—Elevated CO_2, herbivory, litter quality, *Quercus myrtifolia*, *Quercus chapmanii*, *Quercus geminata*, Kennedy Space Center.

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INTRODUCTION

Increasing atmospheric carbon dioxide (CO_2) is likely to alter ecosystem processes, yet most research to date has focused on the direct effects of CO_2 on plant physiological processes, with less attention to indirect effects on ecosystem processes, including those mediated by plant herbivores. Elevated CO_2 can alter foliar chemistry directly, for example by reducing nitrogen (N) concentrations in green leaves (Lincoln et al., 1993; Agrell et al., 2000; Hall et al., 2005). Elevated CO_2 could also alter leaf chemistry indirectly by affecting the feeding behavior of herbivores, in turn eliciting plant synthesis of chemical defenses. For example, elevated CO_2 can increase foliar concentrations of polyphenols, possibly a response to a CO_2-induced increase in herbivore feeding (Agrell et al., 2000). Changes in plant chemistry affect the quality of herbivore diets and may result in behavioral and physiological changes that influence subsequent herbivory (Lindroth, 1996; Stiling et al., 1999). Whether such changes in foliar chemistry persist in senesced leaves is not clear (Curtis et al., 1989; Coûteaux et al., 1991; Kemp et al., 1994; Finzi et al., 2001) but is important to determine given the importance of litter chemistry for decomposition and nutrient cycling.

The quality of leaf litter is an important determinant of decomposition rates and nutrient dynamics in many systems (Swift et al., 1979). Litter nitrogen, lignin concentrations, and C/N ratios are often good predictors of decay rates within and among species (Cotrufo et al., 1994). Carbon-based compounds such as polyphenols also decrease litter quality and may exert some control on litter decomposition (Hättenschwiler and Vitousek, 2000).

Elevated CO_2 may affect litter quality through several mechanisms including chemical changes in green leaf tissue that persist beyond leaf abscission or changes in patterns of herbivory that influence subsequent chemistry. The quality of green leaf tissue can change as a result of elevated concentrations of atmospheric CO_2 (Lindroth et al., 1995; Hall et al., 2005). Generally, nitrogen concentrations in green leaves decline and C/N ratios increase under elevated CO_2 (Lincoln et al., 1993; Hall et al., 2005). There may also be increases in polyphenolic concentrations in green leaves under elevated CO_2 (Agrell et al., 2000). It is unclear, however, if these changes persist in senesced leaves (Curtis et al., 1989; Coûteaux et al., 1991; Kemp et al., 1994; Finzi et al., 2001). Given the significance of litter chemistry to decomposition processes and nutrient dynamics (Heal et al., 1997), it is important to study the links among elevated CO_2, foliar chemistry, and litter chemistry.

In addition to possible direct effects of elevated CO_2 on litter quality, another potential route by which elevated CO_2 might influence litter quality is through its impact on herbivores. Changes in plant chemistry affect the quality of herbivore diets and may result in behavioral and/or physiological changes that influence subsequent herbivory (Lindroth, 1996; Stiling et al., 1999). For

example, to compensate for lower nitrogen concentrations in leaves under elevated CO_2, insect herbivores often increase their consumption rates by 20–80% (Lincoln et al., 1993). However, lepidopteran larvae can exhibit slower growth rates when feeding on elevated CO_2 plants (Fajer et al., 1991) and become more susceptible to pathogens, parasitoids, and predators (Lindroth, 1996; Stiling et al., 1999). At our field site, which hosts the longest continuous study of the effects of elevated CO_2 on insects, herbivore populations decline markedly under elevated CO_2 (Stiling et al., 1999, 2002, 2003; Hall et al., 2005).

Changes in the feeding behavior of herbivores alter ecosystem processes. Consumption of plant tissue by insect herbivores can have direct affects on ecosystem productivity. Herbivores can also influence ecosystem function by changing organic matter added to the soil (Chapman et al., 2003; Frost and Hunter, 2004). These changes may come from herbivore by-products (Stadler et al., 2001; Frost and Hunter, 2004), alteration of the plant community via selective herbivory (Ritchie et al., 1998; de Mazancourt and Loreau, 2000), or by altering the chemical properties of plant litter (Chapman et al., 2003).

Plant foliage responds to herbivore activity in multiple ways including changes in nitrogen concentrations and induction of secondary compounds (Schultz and Baldwin, 1982). If these changes carry over to litter, subsequent decomposition rates and related nutrient transformations may be altered (Melillo et al., 1982; Scott and Binkley, 1997). Chemical alteration of litter quality may also occur when herbivory instigates premature leaf abscission, which effectively limits nutrient resorption and results in litter with higher nutrient concentrations (Kahn and Cornell, 1983).

Given that herbivore damage can influence subsequent foliar and litter quality, declines in herbivore density under elevated CO_2 have the potential to influence decomposition and nutrient dynamics. Therefore, elevated CO_2 has the potential to cause changes in litter quality through direct effects on foliar chemistry and indirect effects mediated by herbivores. In this study, we explore the impacts of CO_2, herbivory and their interactions on scrub oak litter chemistry.

METHODS AND MATERIALS

Study Site. Our study site lies within a two-hectare native scrub oak community located at Kennedy Space Center, Florida. The scrub oak forest is xenomorphic, largely consisting of evergreen or semi-evergreen trees with a mature canopy height of 3 to 5 m. This plant community is fire controlled and was last burned January 1996. Prior to site burning, the plant composition consisted primarily of oak species (76% *Quercus myrtifolia*, 15% *Quercus geminata*, and 7% *Quercus chapmanii*). The remaining 2% of the community included *Serenoa repens* (palmetto), *Myrica serifera* (wax myrtle), *Lyonia*

ferruginea (rusty lyonia), *Ceratiola ericoides* (Florida rosemary), and *Galactia elliottii* (milk pea). Continuous ground cover and longleaf pine (*Pinus palustris*), wiregrass (*Aristida beyrichiana*), and turkey oak (*Quercus laevis*) are absent. Fire in scrub communities is a stand-replacing disturbance that typically removes all aboveground vegetation. Plant regrowth is rapid and there is little change in species composition. When fire is suppressed, the scrub community transitions into a pioneer xeric hammock, which is defined by the retention of some scrub species and the lack of traditional hammock species. The last burn cycle was in 1996 prior to site set up. Sixteen 3.6-m-diam plots, each enclosed with a clear polyester film open-top chamber 3.4 m in height, were utilized to control CO_2 levels. Chambers were overlaid on an octagonal framework of PVC pipe with a removable access door and frustum to reduce dilution of air within the chamber by outside wind. After burning, all regrowth was cut to ground level in May 1996, and, since that time, the vegetation in eight of the chambers was kept at ambient levels of CO_2 while the other eight chambers were exposed to elevated CO_2 (ambient + 350 ppmV). Carbon dioxide is constantly supplied to the elevated CO_2 chambers. In ambient CO_2 chambers, the airflow is identical to that of the elevated CO_2 chambers. (See Dijkstra et al. (2002) for a detailed description of the site setup.) Three oak species dominate this community and are present in every chamber: *Q. myrtifolia* Willd, *Q. chapmanii* Sargent, and *Q. geminata* Small.

Litter Chemistry. Leaf litter was collected quarterly for 3 yr (2000, 2001, 2002) from litter trays placed inside each chamber. Litter was sorted by species and by herbivore damage type (undamaged, chewed, mined). The oak species are evergreen and abscise leaves throughout the year; samples within a year were combined. This resulted in 144 samples (16 chambers $\times$ 3 plant species $\times$ 3 damage categories) per year. Chambers (8 per treatment) acted as replicates. Litter samples collected in 2000 were assayed prior to samples collected in 2001 and 2002, which were assayed concurrently. Otherwise, all samples were processed identically. The air-dried litter was ground to a fine powder and stored at -80°C prior to analysis.

Percent dry weight nitrogen and carbon were estimated from leaf powder on a Carlo-Erba NA1500 model C/N analyzer (Milan, Italy). These data also provided estimates of litter C/N ratios. Subsamples of leaf powder were used to assess the effects of elevated CO_2 on litter concentrations of cellulose, hemicellulose, and lignin by sequential neutral detergent/acid detergent digestion on an Ankom fiber analyzer (Abrahamson et al., 2003).

Proanthocyanidins, an estimate of condensed tannin concentration, were assayed using n-butanol/HCL methods described in Rossiter et al. (1988). Total phenolics were estimated with the Folin–Denis assay (Swain, 1980), and gallotannins (hydrolyzable tannins) were estimated with a potassium iodate technique developed by Bate-Smith (1977) and modified by Schultz and

Baldwin (1982). Standards for tannin analysis were generated by multiple sequential washes of a bulk sample by acetone extraction. The small amount of available litter material for some species resulted in a bulk sample that was a mix of all species of litter for each year. All tannin assays produced colorimetric reactions, in proportion to tannin concentration, which were quantified using a BioRad microplate reader.

Statistical Procedures. Data were initially analyzed with the GLM procedure of SAS 8.2. However, the residuals of the ANOVA models failed the test for normality (Kery and Hatfield, 2003). Data were transformed and reanalyzed and again failed the test of normality. Data were finally analyzed with the repeated-measures GENMOD procedure of SAS 8.2 (SAS Institute, 1999) and the log likelihood ratio was maximized. With the development of generalized estimating equations (GEE), GENMOD is a nonparametric alternative for repeated-measures data (Littell et al., 2002), although *post hoc* analyses are not performed. The GENMOD procedure allows the explanatory variables to be selected and changes in the goodness-of-fit statistics are used to evaluate the contribution of each variable to the model. Thus, the importance of each additional variable and interaction can be assessed, allowing a sequence of models to be tested while taking into account main effects as well as interactions. The data presented contain only those effects that made significant contributions to the model.

RESULTS

Except for condensed tannins, all measures of litter chemistry varied among the species of oak (Table 1). The only consistent effect of elevated CO_2 on litter chemistry was higher condensed tannin concentrations under elevated CO_2 ($\chi^2 = 19.34$, $P < 0.001$) (Figure 1). All other effects of elevated CO_2 on

TABLE 1. LITTER CHEMISTRY OF THREE OAK SPECIES AT THE KENNEDY SPACE CENTER, FLORIDA

	Q. myrtifolia	*Q. chapmanii*	*Q. geminata*	*P* value
Condensed tannins	19.41 (0.68)	18.02 (0.81)	18.02 (0.71)	NS
Hydrolyzable tannins	20.46 (0.62)	23.38 (1.01)	19.68 (0.67)	0.001
Total phenolics	23.93 (0.58)	25.53 (1.05)	18.36 (0.57)	<0.001
Nitrogen	0.76 (0.01)	0.81 (0.02)	0.75 (0.02)	0.018
Carbon	48.71 (0.36)	46.97 (0.38)	46.59 (0.39)	<0.001
C/N ratio	66.44 (1.10)	60.58 (1.24)	65.23 (1.14)	0.002
Cellulose	22.62 (0.17)	19.05 (0.21)	25.09 (0.17)	<0.001
Hemicellulose	14.56 (0.19)	13.59 (0.22)	15.07 (0.35)	0.001
Lignin	14.27 (0.29)	10.54 (0.29)	12.47 (0.32)	<0.001
Lignin/nitrogen ratio	19.75 (0.56)	13.79 (0.40)	17.70 (0.55)	<0.001

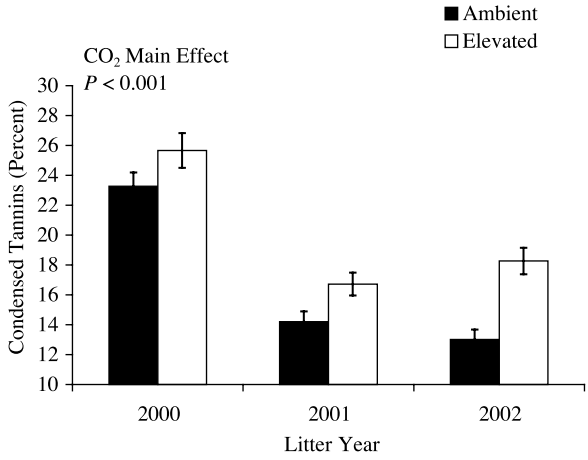

FIG. 1. Condensed tannin concentrations in litter across plant species. Data are the means of 72 samples and bars represent standard errors.

litter chemistry were either not significant (Table 2) or inconsistent among years (Figure 2a–c). The latter was one of our dominant findings—that interannual variation has a profound influence on the effects of elevated CO_2 on litter chemistry. A second result to emerge was that elevated CO_2 has no influence on litter nitrogen concentration or C/N ratio (Table 2). We have previously shown that in living green leaves of these oak species, nitrogen concentrations decrease whereas C/N ratios increase under elevated CO_2 (Hall et al., 2005). Apparently, these changes are lost by the time that leaves senesce.

Phenolic concentrations (condensed tannins, hydrolyzable tannins, total phenolics) were generally lower in chewed and mined litter than in undamaged

TABLE 2. RESULTS OF ANALYSES OF LITTER CHEMISTRY FOR CO_2 AND CO_2 × YEAR INTERACTIONS AT THE KENNEDY SPACE CENTER, FLORIDA

Litter chemistry	CO_2	CO_2 × year
Condensed tannins	$\chi^2 = 19.34$, $P < 0.001$	NS
Hydrolyzable tannins	NS	$\chi^2 = 20.39$, $P < 0.001$
Total phenolics	NS	NS
Nitrogen	NS	NS
Carbon	NS	NS
C/N ratio	NS	NS
Cellulose	NS	$\chi^2 = 9.54$, $P = 0.023$
Hemicellulose	NS	$\chi^2 = 11.86$, $P = 0.008$
Lignin	NS	NS
Lignin/nitrogen ratio	NS	NS

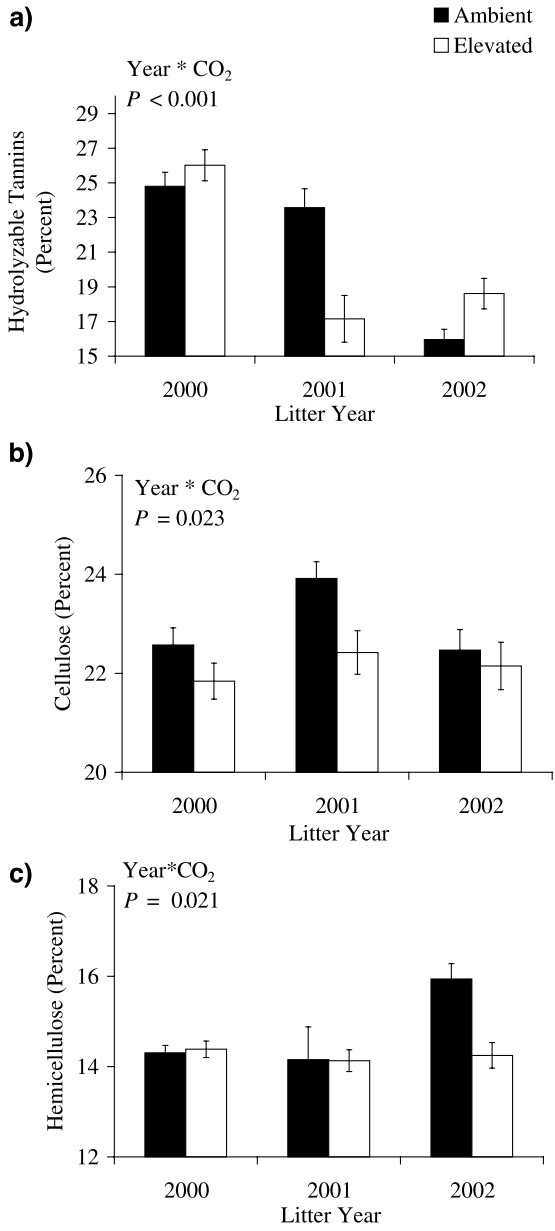

FIG. 2. Differences in litter chemistry between ambient and elevated CO_2 treatments across plant species by growing season. (a) Hydrolyzable tannins, (b) cellulose, (c) hemicellulose. Data are the means of 72 samples and bars represent standard errors.

litter (condensed tannins, $\chi^2 = 22.88$, $P < 0.001$; hydrolyzable tannins, $\chi^2 = 10.07$, $P = 0.006$; total phenolics, $\chi^2 = 15.55$, $P = 0.004$) (Figure 3a–c). However, mining only reduced hydrolyzable tannins and total phenolics in *Q. myrtifolia*

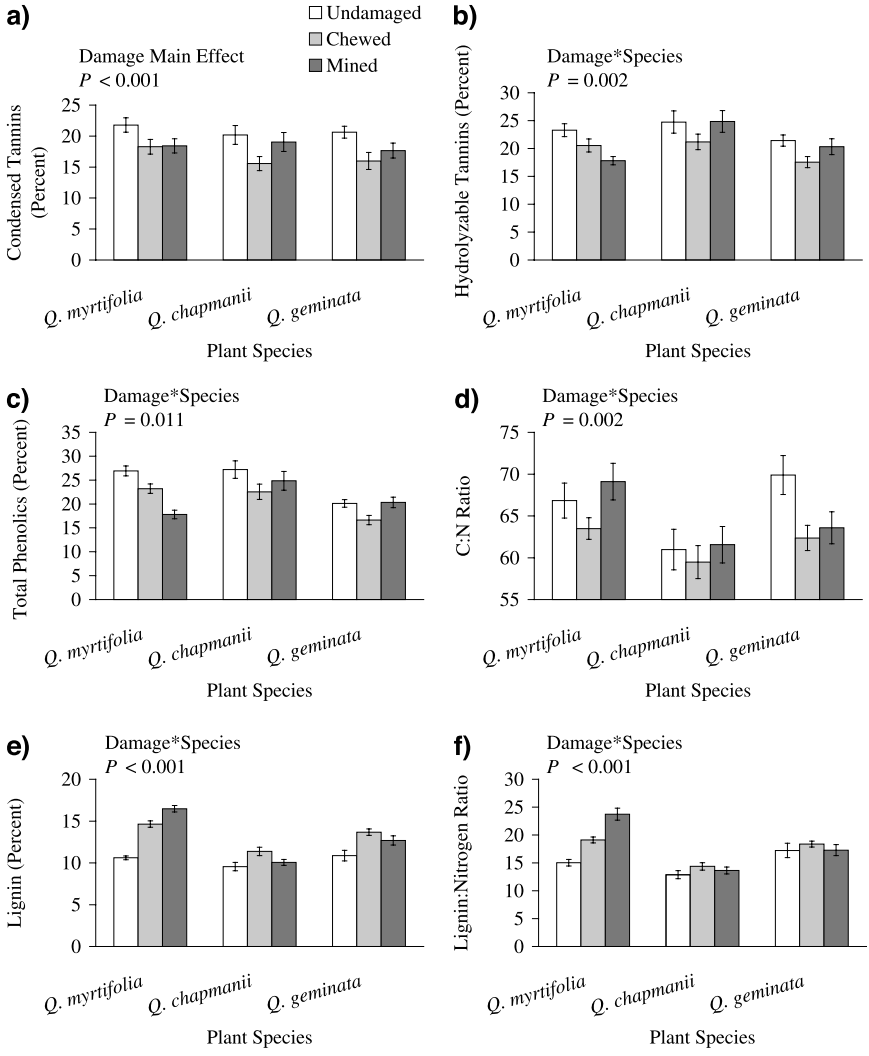

FIG. 3. Differences in litter chemistry among undamaged, chewed, and mined litter by plant species for (a) condensed tannins, (b) hydrolyzable tannins, (c) total phenolics, (d) C/N ratio, (e) lignin, (f) lignin/nitrogen ratio. Data are the means of 48 samples and bars represent standard errors.

(Figure 3b, c), and had no effect for the other species. Effects of damage on litter C/N ratios were inconsistent among the species ($\chi^2 = 20.73$, $P = 0.002$, Figure 3d) suggesting no dominant impact of damage on C/N ratios. Lignin concentrations and lignin/nitrogen ratios were higher in damaged litter from *Q. myrtifolia*, but

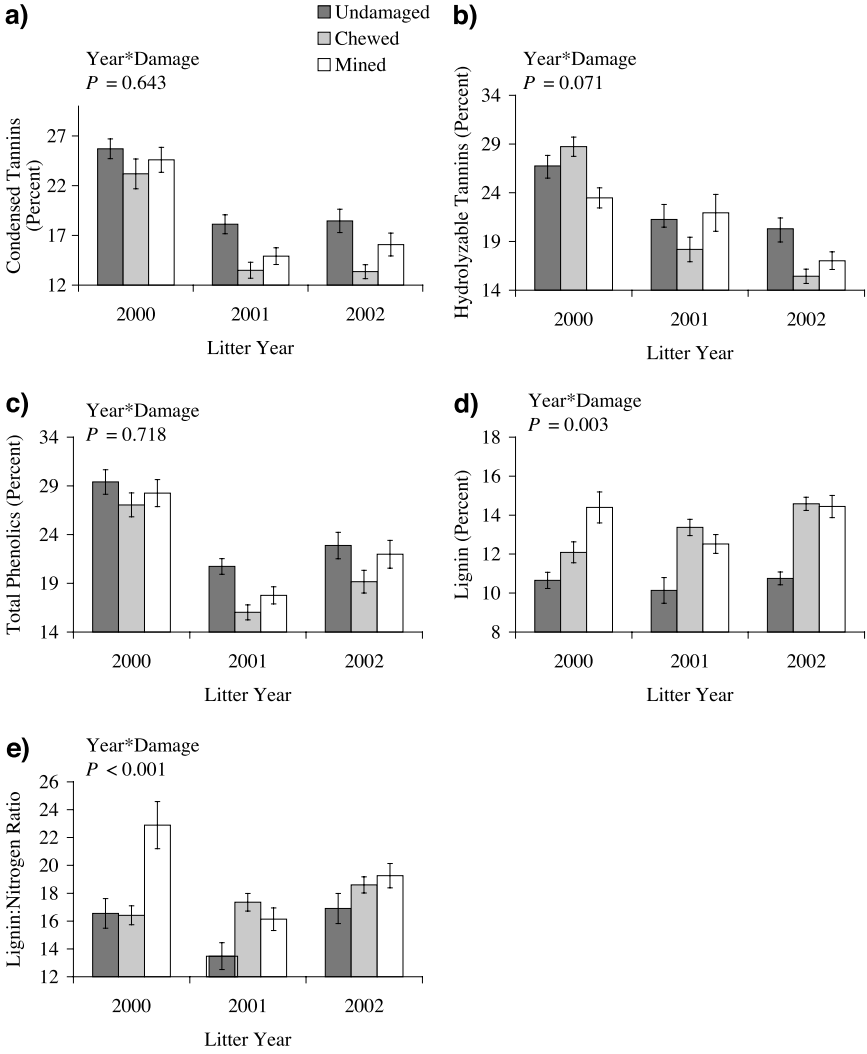

FIG. 4. Differences in litter chemistry of undamaged, chewed, and mined oak litter over time (a) condensed tannins, (b) hydrolyzable tannins, (c) total phenolics, (d) lignin, (e) lignin/nitrogen ratio. Data are the means of 48 samples and bars represent standard errors.

not from the other oak species (Damage × Species, $\chi^2 = 30.35$, $P < 0.001$, and $\chi^2 = 30.06$, $P < 0.001$ for lignin and lignin/nitrogen ratio, respectively, Figure 3e, f). As with the effects of CO_2, effects of damage on litter chemistry were relatively minor when compared to yearly variation from one growing season to the next (Figure 4a–e).

DISCUSSION

Climate and chemical composition of litter determine decomposition rates and nitrogen mineralization (Swift et al., 1979). Components influencing the quality of the litter include concentrations of nitrogen, lignin, and polyphenols. Typically, high concentrations of nitrogen are positively correlated with decomposition rates, whereas high lignin and polyphenolic concentrations are negatively correlated with decomposition rates (Swift et al., 1979). Low litter quality generally decreases decomposition, reducing nitrogen mineralization and soil nitrogen availability (Swift et al., 1979; Melillo et al., 1982). Previous studies of the effect of elevated CO_2 on litter quality have been equivocal. In some studies, increased levels of atmospheric CO_2 affected litter quality for several species (Coûteaux et al., 1991; Cotrufo et al., 1994; Parsons et al., 2004; Henry et al., 2005), whereas others have found no effect of elevated CO_2 on litter quality (Curtis et al., 1989; Finzi et al., 2001). Parsons et al. (2004) found that nitrogen concentrations declined and C/N ratios and condensed tannin concentrations increased under elevated CO_2 in paper birch (*Betula papyrifera*). Likewise, they found that mass loss was lower and decay rates were higher for birch litter from CO_2 environments compared to the control. Henry et al. (2005) found that lignin concentrations increased in grass and forb litter under elevated CO_2; however, they found no differences in total phenolic concentrations or percent nitrogen in the litter from elevated CO_2 environments. They found that the increase in lignin concentrations due to elevated CO_2 did not affect decomposition rates.

In our study, condensed tannin concentrations increased under elevated CO_2 regardless of species, herbivore damage, or growing season. Although the traditional measures of litter quality focus on lignin and nitrogen concentrations, there is growing consensus that polyphenols can have an effect on decomposition processes. Palm and Sanchez (1990) found that soluble polyphenolic concentrations were a better measure for predicting decomposition rates in leguminous litter in the tropics than were lignin or lignin/nitrogen ratios. Polyphenols may affect the rates of decomposition by influencing the composition and activity of the detritivore community (Hättenschwiler and Vitousek, 2000). In addition, polyphenols may alter the availability of nitrogen by binding with proteins and making the litter resistant to some detritivores, thus reducing the rates of nitrogen mineralization (Bernays et al., 1989). In our system, increased

condensed tannin concentrations under elevated CO_2 have the potential to affect ecosystem processes by slowing down litter decomposition and nutrient turnover. However, increases in condensed tannins were low (2–5%, Figure 1) and considerably smaller than interannual variation.

Prior work on green leaves in this system established that plant growth under elevated CO_2 reduced foliar nitrogen concentrations and increased C/N ratios by an average of 6 and 7%, respectively, across all three species (Hall et al., 2005). In contrast, secondary metabolites were unaffected in the green leaves (Hall et al., 2005). In this study of litter, however, there was no evidence of lower nitrogen concentrations or higher C/N ratios. Rather, the strongest CO_2 effects were seen in secondary metabolites, particularly condensed tannins (Figures 1 and 2a–c). Thus, differences in green leaf chemistry caused by growth under elevated CO_2 disappeared by the time leaves senesced. Few studies have directly compared foliar and litter chemistry under elevated CO_2. Curtis et al., (1989) found that nitrogen concentrations were lower and C/N ratios higher in green leaves of *Scripus olneyi* under elevated CO_2 but differences did not persist in senesced leaves. Finzi et al., (2001) found no effect of elevated CO_2 on total nonstructural carbohydrates or nitrogen in green leaves or in leaf litter of five tree species. Kemp et al., (1994), on the other hand, found significantly lower nitrogen concentrations in senesced foliage from *Poa pratensis* L. exposed to elevated levels of CO_2 compared to senesced foliage growing in ambient levels of CO_2. Similarly, Coûteaux et al., (1991) found that nitrogen concentrations were lower in leaf litter of chestnut trees grown in elevated CO_2 compared to ambient CO_2. The studies by Kemp et al., (1994) and Coûteaux et al., (1991) did not apply elevated CO_2 continuously to field plants, and this may account for the differences in their results from ours and other studies.

Phenolic concentrations were lower, whereas lignin concentrations and lignin/nitrogen ratios were higher, in litter following herbivore damage. Given that herbivores on oak generally induce increases in foliar phenolics (Schultz and Baldwin, 1982), it seems unlikely that the lower phenolic concentrations in damaged litter resulted directly from herbivore feeding. Rather, insect herbivores may be avoiding high phenolic leaves (Cooper-Driver et al., 1977; Bernays et al., 1989), leading to a preponderance of damage on low phenolic litter. This needs to be explored experimentally by manipulating herbivore abundance. Likewise, the apparent induction of lignification following damage (Figure 3e) requires experimental verification. Chapman et al., (2003) found that two insect herbivores from different feeding guilds (*Matsucoccus acalyptus* and *Dioryctria albovittella*) increased nitrogen concentrations and decreased C/N and lignin/nitrogen ratios in pinyon pine (*Pinus edulis*) litter. Moreover, these herbivore-induced changes resulted in increased litter decomposition rates. However, damaged litter in our study was lower in condensed tannins yet higher in

lignin and lignin/nitrogen ratios, changes that should have counteracting effects on decomposition rates. Herbivore-damaged litter may decompose more rapidly due to the decline in phenolic concentrations, particularly condensed tannins, compared to undamaged litter. Conversely, damaged litter may decompose more slowly due to higher concentrations of lignin and higher lignin/nitrogen ratios.

In our study, growing season had the dominant impact on litter chemistry so that yearly variation overwhelmed most effects of CO_2 or herbivore damage. Therefore, relative to the influence of seasonal variables, elevated CO_2 and herbivore activity may be less significant factors in leaf litter decomposition and ecosystem function. Although it is well known that climate and litter chemistry control the dynamics of decomposition (Swift et al., 1979), there has been little research on changes in litter quality based on yearly variation in climate. It is known, however, that the same plant species grown on different sites can vary in its litter chemistry (Vitousek et al., 1994; Scowcroft et al., 2000). Therefore, it is reasonable to expect within-site variation in litter chemistry from year to year due to climatic and other differences among the growing seasons. At our study site, climatic data available for precipitation and temperature did not offer insight to the yearly difference found in litter chemistry. Yearly precipitation, based on mean monthly data, varied only slightly with an average of 240 mm of precipitation recorded for 2000, 242 mm for 2001, and 263 mm for 2002. The average daytime temperature, based on mean monthly data, did not vary among years. The daytime mean temperature within CO_2 chambers was 27°C for all 3 years. The daytime mean temperature outside of chambers was 26°C for 2000 and 2002 and 25°C for 2001. Although precipitation and temperature did not vary among years, variation in monthly precipitation patterns based on timing of precipitation and litter fall may result in differential leaching of soluble compounds from year to year, thus leading to differences in litter chemistry. In addition to temperature and precipitation, differences in macro- and micronutrient supply as well as UV-B levels may affect litter quality (Heal et al., 1997; Horner et al., 1988). Given the magnitude of changes in litter chemistry from one growing season to the next, it is possible that decay rates and nitrogen mineralization will vary over time depending on the cohort of leaves that serve as substrate. Future work on the effects of climate change and herbivory on decomposition and nutrient cycling should occur over several years of study so that annual variation can be taken into account and key abiotic variables that drive variation in litter chemistry can be identified.

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ATTRACTION OF THE PARASITOID *Anagrus nilaparvatae* TO RICE VOLATILES INDUCED BY THE RICE BROWN PLANTHOPPER *Nilaparvata lugens*

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Abstract—*Anagrus nilaparvatae*, an egg parasitoid of the rice brown planthopper *Nilaparvata lugens*, was attracted to volatiles released from *N. lugens*-infested plants, whereas there was no attraction to volatiles from undamaged plants, artificially damaged plants, or volatiles from *N. lugens* nymphs, female adults, eggs, honeydew, and exuvia. There was no difference in attractiveness between plants infested by *N. lugens* nymphs or those infested by gravid females. Attraction was correlated with time after infestation and host density; attraction was only evident between 6 and 24 hr after infestation by 10 adult females per plant, but not before or after. Similarly, after 24 hr of infestation, wasps were attracted to plants with 10 to 20 female planthoppers, but not to plants with lower or higher numbers of female planthoppers. The attractive time periods and densities may be correlated with the survival chances of the wasps' offspring, which do not survive if the plants die before the wasps emerge. Wasps were also attracted to undamaged mature leaves of a rice plant when one of the other mature leaves had been infested by 10 *N. lugens* for 1 d, implying that the volatile cues involved in host location by the parasitoid are systemically released. Collection and analyses of volatiles revealed that 1 d of *N. lugens* infestation did not result in the emission of new compounds or an increase in the total amount of volatiles, but rather the proportions among the compounds in the blend were altered. The total amounts and proportions of the chemicals were also affected by infestation duration. These changes in volatile profiles might provide the wasps with specific information on host habitat quality and thus could explain the observed behavioral responses of the parasitoid.

Key Words—Host location, induced plant volatiles, rice, *Anagrus nilaparvatae*, *Nilaparvata lugens*, tritrophic interactions.

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INTRODUCTION

Herbivore-induced plant volatiles play a role in foraging behavior of predators and parasitoids of herbivores (Turlings et al., 1990; Takabayashi and Dicke, 1996; Sabelis et al., 1999; Dicke, 1999; Bertschy et al., 2001; Lou et al., 2002), and thus these volatiles are suggested to function as an indirect defense for plants (Vet and Dicke, 1992; Dicke, 1999; Sabelis et al., 1999; Turlings and Wäckers, 2004). So far, this phenomenon of indirect defense signaling has been reported in more than 23 plant species. It has been particularly well studied for corn, lima bean, cotton, and tobacco. In rice, a model monocot cereal species and one of the most important food crops in the world, however, few studies have examined the role of herbivore-induced volatiles in host or prey location by natural enemies of herbivores.

Nilaparvata lugens (Stål) is an important rice pest worldwide. It feeds on phloem sap, causing major physiological stress to the plant (Rubia-Sanchez et al., 1999; Watanabe and Kitagawa, 2000). Compared to chewing herbivores, sucking feeders that have been studied in the context of tritrophic interactions appear to cause only minor changes in volatile emissions, but these emissions are apparently used by natural enemies (Du et al., 1998; Turlings et al., 1998a; Birkett et al., 2003). Possible changes in volatiles profiles resulting from planthopper infestation have not yet been studied for any plant. In this study, we investigated the role of volatiles induced by the rice brown planthopper *N. lugens* in the host-searching behavior of *Anagrus nilaparvatae* Pang et Wang, an egg parasitoid of *N. lugens*. The parasitoid *A. nilaparvatae* is a major natural enemy of rice planthoppers, including *N. lugens* (Cheng and He, 1996). For host location, the wasp uses contact kairomones that are present in all developmental stages of the planthoppers as well as in their exuvia and honeydew (Lou and Cheng, 1994; Lou and Cheng, 2001), but volatiles released from hosts and by-products seem to have no attractiveness (Lou and Cheng, 1994). However, the volatiles emitted from planthopper-infested rice plants significantly attract the parasitoid (Lou and Cheng, 1996a). Nothing is known about the nature of these volatiles.

To elucidate the role of *N. lugens*-induced rice volatiles in the host-searching behavior of the parasitoid, we first measured the behavioral response of the parasitoid to the volatiles emitted from unmanipulated plants, artificially damaged plants, planthopper-infested plants, planthopper-damaged plants (planthoppers removed), as well as to volatiles from planthoppers alone or their by-products. Then, we checked the effect of infestation duration and planthopper density on attraction of the parasitoid. In an additional olfactometer experiment, it was shown that the volatiles are released systemically. Finally, we collected and identified the volatiles that were released from differently treated plants.

METHODS AND MATERIALS

Plants. The rice variety used was Zhe 852, which is susceptible to *N. lugens* (Lou and Cheng, 2003). Pregerminated seeds were sown in a greenhouse, and after 20 d, seedlings were transplanted into clay pots (16 cm diam × 10 cm height), each with 10 plants. Plants were watered daily, and each pot was supplied with 0.10 g of urea 10, 20, and 30 d after transplanting, respectively. All plants were placed in a controlled climate room that was maintained at 28 ± 2°C, 70–80% r.h., and 12 hr photophase. Thirty to 35 d after transplanting, plants were used for experiments. Plantings were continued at regular intervals so that enough plants of suitable age were available for experiments.

Insects. The *N. lugens* culture was originally obtained from the China National Rice Research Institute (CNRRI), Fuyang, Zhejiang, and maintained on Zhe 852 rice plants in a greenhouse. Late instar nymphs of *N. lugens* were captured from the greenhouse and reared on potted Zhe 852 rice plants, which were confined in plastic cages (11 cm diam × 40 cm high). Caged rice plants were maintained in a controlled climate room at 28 ± 2°C, 12 hr photophase, and 70–80% r.h. Newly emerged adults of *N. lugens* were collected daily and fed on potted fresh Zhe 852 rice plants. Using this procedure, we obtained *N. lugens* adults of uniform age.

A laboratory colony of the egg parasitoid *A. nilaparvatae* was started from individuals trapped in rice fields in Hangzhou, using Zhe 852 rice plants with *N. lugens* eggs as bait. The colony was propagated on *N. lugens* eggs in rice shoots enclosed in glass tubes (2.5 cm diam × 20 cm high), which were kept in a controlled climate room at 26 ± 2°C, 12 hr photophase, and 70–80% r.h. Each day the newly emerged wasps were collected into clean glass tubes with access to both water and honey solution and held for at least 2 hr to ensure mating. From the second generation onwards, female parasitoids were used in experiments less than 24 hr after emergence.

Bioassays. Responses of *A. nilaparvatae* females to rice volatiles were measured in a Y-tube olfactometer, which was described in detail by Lou et al. (2005). Briefly, the olfactometer consisted of a Y-shaped glass tube of 1 cm diam. The base and the two arms of the Y tube were all 10 cm in length. Each arm was connected to an odor source container (a glass box, 10 cm long, 10 cm wide, 30 cm high). An airstream was generated and divided in two, and each secondary airstream was led through a flowmeter, a tube with activated charcoal, a humidifier bottle, and one of the odor containers. Subsequently, the two airstreams were led through the two arms of the Y-tube olfactometer at 150 ml/min. The Y-tube olfactometer was placed in a box painted white with an artificial light source consisting of a single 25-W lamp placed above the arms of the Y tube. All bioassays were conducted between 0900 and 1700 hours, and the temperature in the room was maintained at 25–28°C.

Mated female parasitoids were introduced individually into the base tube of the olfactometer and given 10 min to walk toward the end of one of the arms. Choice for an odor source was defined as a female crossing a line 7 cm after the division of the base tube and remaining there for at least 1 min. If a parasitoid did not make a choice within 10 min, this was recorded as no response. For each odor source pair, at least 32 females were tested.

Response of A. nilaparvatae Toward Plant–N. lugens Complexes and Their Individual Components. In this experiment, the following odor sources were prepared:

1. Plant–*N. lugens* gravid female complex (PF). The potted plants were washed with running water and trimmed to leave 10 plants per pot. Plants were then individually infested with 10 gravid *N. lugens* females that were attached to the upper and lower position of the plant stems by using two parafilm bags, each with five females. One d later, the 10 plants (cut off at soil level and the cut end wrapped with a piece of moist cotton) with 100 females were used as an odor source for bioassays.
2. Plant–*N. lugens* nymph complex (PN). This treatment was nearly the same as in treatment (1). The only difference was that we used 4th–5th instar nymphs of *N. lugens* instead of gravid females.
3. *N. lugens* gravid female-damaged plants (FDP). This treatment was the same as in treatment (1), but 1 day after infestation, we removed the females and used the 10 plants, which now carried eggs, as an odor source for bioassays.
4. *N. lugens* nymph-damaged plants (NDP). The treatment was the same as in treatment (2), but 1 d after infestation, nymphs were removed and 10 plants were used as an odor source.
5. Mechanically damaged plants (MP). Ten plants were individually damaged with a needle at the lower and upper position of the stems by pricking each 150 times at the start of the experiment. Twenty-four hr later, the plants were damaged again using the same method and then were cut off at soil level and used as one odor source.
6. Unmanipulated plants (CP). Ten plants were cut off at soil level and served as one odor source.
7. *N. lugens* exuvia and honeydew. Exuvia and honeydew were collected from five glass tubes (2.5 cm diam $\times$ 20 cm) in which, for 24 hr, 20 4th–5th instar nymphs had been feeding on two rice plants that had been hung upside down, with their roots wrapped in wet cotton. Honeydew in the glass tubes was collected with a syringe, and the exuvia with forceps. Honeydew or exuvia was placed on a small piece of filter paper (3 $\times$ 3 cm) as an odor source.

8. *N. lugens* eggs. Ten plants were individually infested with 10 gravid N. lugens females. One day later, plants were cut at soil level and dissected under a microscope. All eggs were then carefully collected with a pin and placed on a small piece of filter paper (3 × 3 cm) as an odor source.
9. *N. lugens* nymphs and female adults. One hundred 4th–5th instar nymphs and female adults were individually collected and then used as odor sources.

The behavioral response of the parasitoid to the following pairs of odors were tested in the olfactometer: (1) blank control (BK) vs. CP, MP, PF, PN, NDP, or FDP, respectively; 2) PF vs. CP, MP, or PN, respectively; 3) PN vs. CP, or MP, respectively; and 4) BK vs. *N. lugens* exuvia, *N. lugens* eggs, *N. lugens* nymphs, *N. lugens* gravid females, or *N. lugens* honeydew, respectively.

Effect of Host Density and Infestation Duration on Attraction of A. nilaparvatae. In this experiment, we determined whether the duration of infestation or planthopper density had an effect on parasitoid attraction, using plant–female complexes infested with 1, 5, 10, 20, 40, or 80 gravid females per plant for 1 d. Plants without *N. lugens* served as controls. We used 10 plants per treatment.

To assess the effect of the duration of infestation, olfactometer tests were performed at 1, 2, 6, 12, 24, 48, and 72 hr after the plants had been infested by *N. lugens*. Each plant was infested with 10 gravid females and 10 plants were used per treatment. Plants without *N. lugens* served as controls.

Response of A. nilaparvatae to Undamaged Leaves of Infested Plants. In this experiment, we determined whether the *N. lugens*-induced rice volatiles were produced systemically and whether the infested leaf position had an effect on the attraction of the parasitoid to uninfested leaves. Plants with four fully expanded leaves, which were respectively assigned first, second, third, and fourth leaf position from the top to the base, were chosen. The uninfested leaves including leaf sheaths were individually collected and used for bioassay in a Y-tube olfactometer 24 hr after one of the fully expanded leaves (the first, second, third, or fourth leaf position) of plants was infested with 10 gravid *N. lugens* females who were fixed on the middle veins of the leaves by using two parafilm bags, each with five females. For example, if the first leaves of 10 plants had been infested by *N. lugens*, then we measured the response of the parasitoid to the volatiles released from either the second, third, or fourth leaves of the 10 plants, respectively. The corresponding leaves from uninfested plants (with two empty parafilm bags per plant at the same positions as the infested plants) served as controls.

Collection and Identification of Rice Volatiles. Rice volatiles were collected for 2 hr by using solid-phase microextraction (SPME, Supelco Co., Bellefonte, PA, USA) with a silica fiber coated with a 100-μm-thick film of

polydimethylsiloxane. For each treatment, 10 plants were used. The plant was cut off at soil level, and the cut part of the stem was wrapped in a piece of moist cotton to avoid desiccation. Ten plants were placed in a glass cylinder (5 cm diam × 30 cm), which was covered by a glass lid (5 cm diam) with a hole (1 mm diam) through which the SPME fiber was inserted for odor collection. The volatiles emitted from the following treatments were collected: plants that had been individually infested by 10 gravid *N. lugens* females for 1, 2, or 3 d, respectively; plants that had been individually infested by 10 *N. lugens* nymphs for 1 d; mechanically damaged plants (MP); and unmanipulated plants. We also collected volatiles from a control, (the glass cylinder without plants) to confirm that the system was clean. Each treatment was replicated three times. The experiment was conducted in a room at 26–28°C. After collecting each sample, the cylinder was rinsed with an acidic washing solution ($K_2Cr_2O_7$ 60 g/98% H_2SO_4 460 ml/H_2O 300 ml Caution! Highly corrosive!) followed by distilled water and then heated at 200°C for 1 hr. The SPME fiber was desorbed in a GC injector at 250°C for 15 min before use.

Analyses were done with a Hewlett-Packard (HP) 6890A gas chromatograph (GC) equipped with an HP-5 (30 m × 0.25 mm i.d., 0.25 μm film thickness) column. Volatile compounds collected on the SPME fiber were directly desorbed in the GC injector at 250°C for 1 min, in splitless mode. Following injection, the column was programmed from 40°C/2 min, 6°C/min to 250°C, hold for 2 min. Helium (1 ml/min) was used as the carrier gas. All compounds were analyzed by an HP 5970B mass spectrometer in the electron impact ionization mode. Compounds were identified by comparison of retention times and mass spectra with those of authentic standards. The authentic standard chemicals (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, limonene, cyclohexanol, linalool, β-cyclocitral, 1-undecene, *n*-tetradecane, *n*-hexadecane, dodecanal, *n*-heptadecane, *n*-octadecane, *n*-nonadecane, *n*-eicosane, and *n*-henicosane were purchased from Sigma Chemical (St. Louis, MO, USA); tetradecanal was purchased from ChemExper, Belgium. The relative proportions of the compounds in the blend were calculated according to their peak areas.

Statistical Analysis. Differences in behavioral responses of the parasitoid to pairs of odors were determined by χ^2 tests. Volatiles data were analyzed by ANOVA. If the ANOVA analysis was significant ($P < 0.05$), Fisher LSD *post hoc* tests to detect significant differences between groups were conducted. Data were analyzed with Statistica (SAS Institute Inc., Cary, NC, USA).

RESULTS

Response of A. nilaparvatae Toward Plant–N. lugens Complexes and Their Individual Components. Volatiles released from unmanipulated plants and

mechanically damaged plants were no more attractive to the parasitoid than blank controls, whereas the volatiles from plant–*N. lugens* complexes (PF, PN), and *N. lugens*-damaged plants (FDP, NDP) were attractive (Figure 1). The plant–*N. lugens* complexes were also more attractive than unmanipulated plants or mechanically damaged plants. No difference was observed in attraction of the parasitoid to volatiles emitted from plant–nymph complex and those from plant–gravid female complex (Figure 1).

The parasitoid was not attracted to the volatiles released from *N. lugens* materials, including *N. lugens* nymphs, female adults, eggs, honeydew, or exuvia (Figure 2).

Effect of Host Density and Infestation Duration on Attraction of A. nilaparvatae. N. lugens density had a significant effect on attraction of the parasitoid (Figure 3B). Plants that had been infested with 10 or 20 gravid females per plant for 1 d were more attractive than unmanipulated plants, whereas plants that were infested with 1, 5, 40, or 80 gravid females per plant were no different from uninfested plants (Figure 3B).

There was a similar effect of duration of infestation on the attraction of the parasitoid (Figure 3A). Plants that had been infested with 10 gravid *N. lugens* females for 6–24 hr were more attractive to the parasitoid than unmanipulated

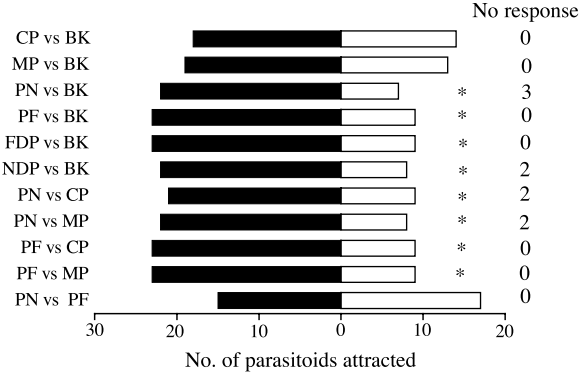

FIG. 1. Numbers of *A. nilaparvatae* adult females attracted by volatiles released from pairs of odors: Unmanipulated plants (CP) vs. blank control (BK); mechanically damaged plants (MP) vs. BK; *N. lugens* nymph–plant complex (PN) (infested for 1 d) vs. BK; *N. lugens* gravid female–plant complex (PF) (infested for 1 d) vs. BK; *N. lugens* gravid female-damaged plants (FDP) (females removed 1 d after infestation) vs. BK; *N. lugens* nymph-damaged plants (NDP) (nymphs removed 1 d after infestation) vs. BK; PN vs. CP; PN vs. MP; PF vs. CP; PF vs. MP; and PN vs. PF. For explanation of treatments and methodology see "Methods and Materials." Asterisks indicate significant differences between members of a pair ($P < 0.05$, χ^2 test).

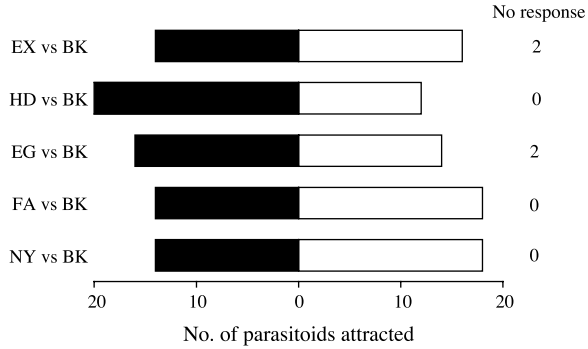

FIG. 2. Number of *A. nilaparvatae* adult females attracted by volatiles released from pairs of odors: *N. lugens* exuvia (EX) vs. blank control (BK); *N. lugens* honeydew (HD) vs. BK; *N. lugens* eggs (EG) vs. BK; *N. lugens* female adults (FA) vs. BK; and *N. lugens* nymphs (NY) vs. BK.

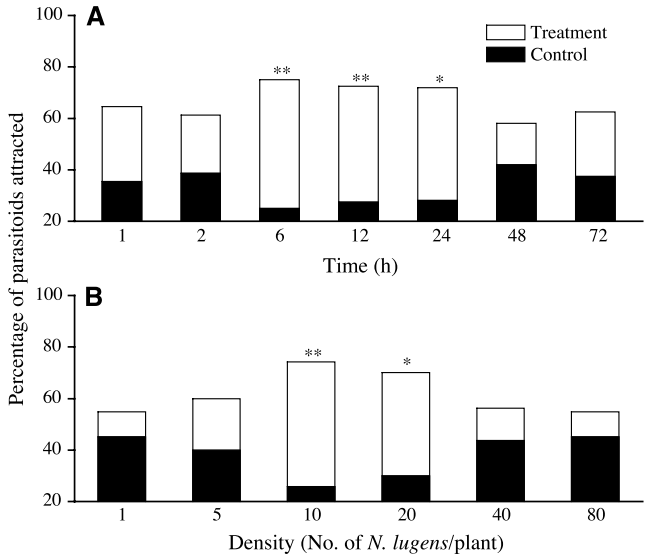

FIG. 3. Percentage of *A. nilaparvatae* adult females attracted by volatiles released from pairs of odors: unmanipulated plants (Control) vs. (A) plants that were individually infested by 10 *N. lugens* gravid females for 1, 2, 6, 12, 24, 48, or 78 hr (Treatment); (B) plants that were individually infested with 1, 5, 10, 20, 40, or 80 *N. lugens* gravid females for 1 d (Treatment). Asterisks indicate significant differences between members of a pair (*$P < 0.05$, **$P < 0.01$, χ^2 test).

plants, but plants that had been infested with 10 gravid *N. lugens* gravid females for a shorter (1–2 hr) or longer time (48–72 hr) were no different than uninfested plants (Figure 3A).

Response of A. nilaparvatae to Undamaged Leaves of Infested Plants. Infestation of one of the fully expanded leaves by *N. lugens* resulted in an increase in attractiveness of the other three undamaged leaves, and the leaf position that was infested by *N. lugens* had no effect on this attractiveness (Figure 4).

Volatiles Profile Comparisons. More than 20 compounds were collected from the headspace of rice plants by SPME, 16 of which were identified, including 8 aliphatic hydrocarbons, 3 aldehydes, 3 terpenoids, and 2 alcohols (Table 1). Of these compounds, aliphatic hydrocarbons, specifically 7 aliphatic hydrocarbons, dominated the blends, comprising more than 48% of the total peak areas of compounds emitted in every assay (Table 1). In addition, two green leafy volatiles, (*E*)-2-hexenal and (*E*)-2-hexen-1-ol, as well as tetradecanal and unknowns 1, 3, and 4 were also relatively abundant in the headspace of the rice plants (Table 1).

Compared to unmanipulated plants, *N. lugens* infestation did not induce rice plants to release new compounds, nor did the total amount of volatiles

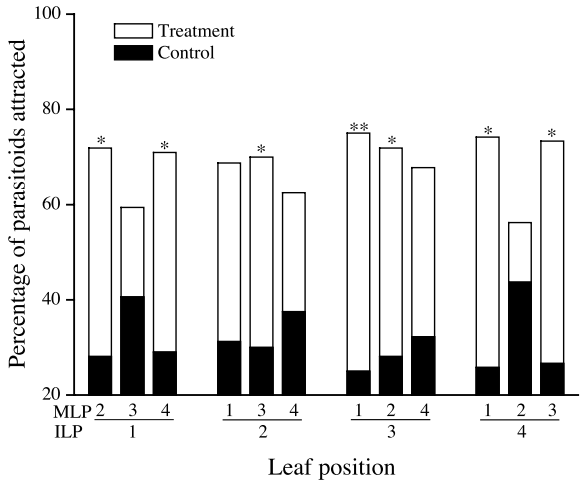

FIG. 4. Percentage of *A. nilaparvatae* female adults attracted by volatiles released from pairs of leaves: undamaged individual leaves, growing at positions 1–4, of 10 damaged plants on which a single leaf, at position 1–4, was infested with 10 *N. lugens* gravid females for 24 hr (Treatment) vs. the same leaves of unmanipulated plants (Control). MLP, measured leaf position; ILP, infested leaf positions. Asterisks indicate significant differences between members of a pair (*$P < 0.05$, **$P < 0.01$, χ^2 test).

TABLE 1. PROPORTIONS (% OF TOTAL PEAK AREA) OF VOLATILE COMPOUNDS EMITTED FROM UNMANIPULATED PLANTS (CP), MECHANICALLY DAMAGED PLANTS (MP), AND *Nilaparvata lugens* GRAVID FEMALE INFESTED PLANTS FOR 1 (PF1), 2 (PF2), OR 3 (PF3) DAYS, AND *N. lugens*-NYMPH INFESTED PLANTS FOR 1 DAY (PN1)[a]

Chemical	CP	MP	PF1	PF2	PF3	PN1
1. (*E*)-2-Hexenal	5.69 ± 1.26 bc	6.73 ± 0.40 ab	5.26 ± 0.75 bc	4.93 ± 0.18 bc	4.04 ± 0.59 c	8.32 ± 1.07 a
2. Unknown 1	7.63 ± 0.20 1ab	7.92 ± 1.32 a	5.55 ± 0.90 bc	5.29 ± 0.40 c	3.67 ± 0.54 c	5.25 ± 0.41 c
3. (*E*)-2-Hexen-1-ol	6.67 ± 0.89 ab	8.73 ± 1.19 a	3.75 ± 0.21 cd	2.95 ± 0.72 d	2.03 ± 0.05 d	5.76 ± 1.36 bc
4. Unknown 2	0.23 ± 0.03 a	0.24 ± 0.03 a	0.28 ± 0.06 a	0.18 ± 0.02 a	0.32 ± 0.04 a	0.23 ± 0.03 a
5. Limonene	0.21 ± 0.06 a	0.24 ± 0.05 a	0.25 ± 0.08 a	0.20 ± 0.03 a	0.31 ± 0.08 a	0.23 ± 0.03 a
6. Cyclohexanol	1.09 ± 0.19 a	1.19 ± 0.15 a	0.39 ± 0.08 bc	0.22 ± 0.03 bc	0.21 ± 0.03 c	0.57 ± 0.11 b
7. Linalool	0.17 ± 0.03 bc	0.19 ± 0.05 bc	0.24 ± 0.03 bc	0.34 ± 0.07 b	1.25 ± 0.11 a	0.13 ± 0.03 c
8. β-Cyclocitral	0.59 ± 0.06 a	0.90 ± 0.15 a	0.94 ± 0.18 a	0.84 ± 0.19 a	1.10 ± 0.14 a	0.90 ± 0.25 a
9. 1-Undecene	0.98 ± 0.07 bc	1.48 ± 0.28 ab	1.26 ± 0.31 abc	1.77 ± 0.31 a	1.97 ± 0.07 a	0.74 ± 0.19 c
10. Unknown 3	3.76 ± 0.20 c	4.09 ± 0.67 bc	4.31 ± 0.69 bc	4.04 ± 0.63 bc	5.75 ± 0.81 ab	6.28 ± 0.59 a
11. *n*-Tetradecane	4.24 ± 0.56 bc	3.95 ± 0.21 bc	5.24 ± 0.52 b	7.21 ± 0.74 a	4.18 ± 0.82 bc	3.00 ± 0.52 c
12. *n*-Hexadecane	2.40 ± 0.35 a	2.71 ± 0.51 a	2.48 ± 0.24 a	2.73 ± 0.55 a	1.75 ± 0.46 a	1.85 ± 0.61 a
13. Dodecanal	0.23 ± 0.03 b	0.50 ± 0.19 b	0.35 ± 0.04 b	0.27 ± 0.11 b	1.16 ± 0.11 a	0.41 ± 0.06 b
14. *n*-Heptadecane	29.37 ± 0.60 e	30.30 ± 2.16 de	41.72 ± 3.12 ab	44.64 ± 1.33 a	38.31 ± 0.47 bc	34.84 ± 0.77 cd
15. Tetradecanal	2.39 ± 0.18 a	3.90 ± 2.54 a	3.08 ± 0.37 a	2.98 ± 0.48 a	5.86 ± 0.93 a	3.88 ± 0.44 a
16. *n*-Octadecane	3.65 ± 0.19 a	2.85 ± 0.44 b	1.45 ± 0.23 c	0.86 ± 0.20 c	0.69 ± 0.17 c	1.44 ± 0.23 c
17. Unknown 4	3.36 ± 0.04 c	2.94 ± 0.27 c	3.52 ± 0.70 c	10.20 ± 1.10 b	14.18 ± 0.97 a	4.03 ± 0.42 c
18. *n*-Nonadecane	8.48 ± 0.27 a	5.97 ± 0.96 b	5.36 ± 0.42 b	3.24 ± 0.08 c	3.16 ± 0.15 c	5.64 ± 0.48 b
19. *n*-Eicosane	6.52 ± 0.18 a	3.96 ± 0.53 b	3.49 ± 0.34 b	1.00 ± 0.17 c	0.38 ± 0.09 c	2.92 ± 0.47 b
20. *n*-Henicosane	2.98 ± 0.27 a	1.76 ± 0.27 b	2.30 ± 0.36 ab	0.79 ± 0.14 c	0.49 ± 0.15 c	2.05 ± 0.50 ab
Total peak area ($\times 10^8$)	9.79 ± 1.10 ab	11.43 ± 1.28 a	8.52 ± 0.98 ab	7.78 ± 0.83 b	7.02 ± 1.20 b	9.15 ± 1.30 ab

[a]For explanation of treatments and methodology see "Methods and Materials." Data represent the mean of three replications. Different letters in the same row indicate significant differences among treatments ($P < 0.05$, Fisher LSD *post hoc* tests).

change with duration of infestation (Table 1). However, *N. lugens* infestation altered the proportions of chemicals in the blend (Table 1). Compared to unmanipulated plants, *N. lugens*-infested plants (infested for 1 d) had a higher proportion of *n*-heptadecane and lower proportions of (*E*)-2-hexen-1-ol, cyclohexanol, *n*-octadecane, *n*-nonadecane, and *n*-eicosane. Moreover, 1 d of nymph infestation increased the proportions of (*E*)-2-hexenal and unknown 3 (Table 1). With duration of *N. lugens* infestation, the proportions of dodecanal, linalool, and unknown 4 increased, whereas the proportions of *n*-nonadecane, *n*-eicosane, and *n*-henicosane decreased (Table 1). Between the volatiles emitted from *N. lugens* nymph-infested plants and those from gravid female-infested plants, there was no significant difference except for the chemicals (*E*)-2-hexenal and unknown 3, both of which were released in higher proportions by nymph-infested plants (Table 1).

Mechanical damage resulted in no increase in the total volatiles compared to unmanipulated plants. However, the total volatiles released from mechanically damaged plants were higher than those from plants infested by *N. lugens* for 2–3 d (Table 1). The proportions of chemicals that decreased after *N. lugens* infestation did not decrease after artificial damage, except for the chemicals *n*-octadecane, *n*-nonadecane, *n*-eicosane, and *n*-henicosane (Table 1).

DISCUSSION

A. nilaparvatae was attracted to volatiles released from *N. lugens* plant complexes (damaged for 1 d), but not to those from unmanipulated plants or artificially damaged plants. Wasps also were not attracted to volatiles from *N. lugens* nymphs, female adults, eggs, honeydew, or exuvia. Moreover, no obvious difference was observed in attraction between *N. lugens* nymph–plant complex and gravid female–plant complex. These results were similar to those observed in a tritrophic system consisting of the phloem feeder *Phenacoccus herreni*, cassava, and an encyrtid parasitoid (Bertschy et al., 2001). This suggests that volatiles induced by *N. lugens* feeding on rice are used in host location by *A. nilaparvatae*, as has been reported for many parasitoids of insect larvae (Dicke and Vet, 1999; Turlings and Wäckers, 2004). *Nilaparvata lugens* is an insect pest with completely overlapping generations; all developmental stages can co-occur in the same plant and field. Therefore, the indirect association of *N. lugens* feeding with egg presence is reliable, suggesting that the response of the parasitoid to *N. lugens* feeding-induced volatiles is adaptive.

Anagrus nilaparvatae females were not attracted to volatiles from undamaged rice plants. It is commonly reported that healthy plants are unattractive to parasitoids (Turlings et al., 1990; Guerrieri et al., 1993; Potting

et al., 1995; Finidori-Logli et al., 1996; Du et al., 1996; Steidle and Schöller, 1997), which may be attributed to the minor amounts of volatiles released by healthy plants or the low reliability of such volatiles in informing the parasitoid of host presence (Vet et al., 1991; Vet and Dicke, 1992). Volatiles released by artificially damaged plants, such as green leaf volatiles, are often attractive to parasitoids (Turlings et al., 1990; Steinberg et al., 1993; Geervliet et al., 1994; Mattiacci et al., 1994; Potting et al., 1999; Hoballah and Turlings, 2005). Here, however, artificially damaged rice plants were not attractive to *A. nilaparvatae* females possibly because the limited amount of damage resulted in only minor changes to the green leafy volatiles (*E*)-2-hexen-1-ol and (*E*)-2-hexenal.

Nilaparvata lugens infestation for 1 d did not result in detectable production of new compounds, nor was there an increase in the total amount of volatiles compared to controls. However, infestation did alter the relative proportions among the volatiles compared to undamaged and artificially damaged plants. Thus, *N. lugens* infestation appears to alter the plant volatiles profile only slightly, which may be common for phloem feeders and is in contrast to the substantial induction caused by tissue feeders. For instance, Turlings et al. (1998a) found that the aphid *Rhopalosiphum maidis* induced no measurable emissions of volatiles in maize, even after heavy infestation, whereas feeding by caterpillars caused major increases in the volatile emissions. Similarly, infestation by the silverleaf whitefly *Bemisia tabaci* did not appear to induce volatiles emissions in cotton (Rodriguez-Saona et al., 2003). Phloem-sucking insects, such as rice planthoppers and aphids, inflict only minor physical damage to host plants, which is likely to reduce the chances of triggering a physiological reaction in the plant (Turlings et al., 1998a). Quantitative (amount) (e.g., apple fruit, Hern and Dorn, 2001) and/or qualitative changes (production of new compounds or changes in proportions) (e.g., maize, Turlings et al., 1990) in volatiles from plants in response to herbivory have been reported in many plant–herbivore systems, providing exploitable cues for parasitoids (Dicke, 1999; Gouinguené et al., 2001). The attraction of *A. nilaparvatae* females to *N. lugens*-infested plants is best explained by a change in the proportions among the volatiles, a qualitative change.

Systemic release of herbivore-induced volatiles in plants has been reported in many plant species (Turlings and Tumlinson, 1992; Dicke et al., 1993; Cortesero et al., 1997; Guerrieri et al., 1999; Halitschke et al., 2000; Neveu et al., 2002; Röse and Tumlinson, 2004). We too found an attraction of the parasitoid to the undamaged leaves of plants in which one of the mature leaves had been infested by *N. lugens* for 1 d. Wasps were similarly attracted to undamaged, mature leaves of plants that had been damaged by *N. lugens* on another leaf. Although the mature leaves of plants mainly export their photoassimilates to roots and younger leaves (sinks) via the phloem, a solute

exchange does occur between the phloem and the xylem (Fisher, 2000). Thus, the wound signal from infested mature rice leaves may reach other undamaged mature leaves via the xylem, eliciting the undamaged leaves to release volatiles.

We also studied the effect of host density and infestation duration on attraction of *A. nilaparvatae*, and both had a significant effect. The degree of attraction of the parasitoid and the *N. lugens* density or the infestation duration did not fit a dose related positive relationship as reported in other studies (Turlings et al., 1990; Gols et al., 2003), but rather reached a maximum at intermediate densities, with no attraction at low or very high infestations. A similar effect was found for the duration of infestation; wasps were not attracted early on, but attraction was strong after 6–24 hr of infestation, and then declined after 48 hr. The time lag for the induction of volatiles attractive to parasitoids has also been observed in other studies (Turlings et al., 1998b; Guerrieri et al., 1999), suggesting the involvement of active physiological and biochemical processes that result in changes in volatile profiles. A threshold for damage level has also been reported for the attraction of an aphid parasitoid to broad bean plants infested with varying aphid densities (Powell et al., 1998; Guerrieri et al., 1999), as well as for induced direct resistance in plants (Karban and Baldwin, 1997; Lou and Cheng, 1997). These results suggest that an adequately strong elicitation signal is needed for the induction of direct and indirect resistance.

The decline in attraction of the parasitoid to plants that had been infested by either 10 *N. lugens* females for 2–3 d or 30–40 females for 1 d may be related to the survival chances of the parasitoid offspring. *Nilaparvata lugens* feeds and oviposits on the same plant even if plants are heavily damaged. Heavy damage in the field by 50–90 female adults per hill (about 20 plants) causes susceptible plants to wilt and eventually die after ~10 d (Li et al., 1996). The parasitoid spends 9–10 d in the host egg before it emerges as an adult at appropriate temperatures of 25–28°C (Lou and Cheng, 1996b), and developing wasps may die due to desiccation if the plant dies before parasitoid emergence. This may explain the declining response with increasing levels of plant damage. Discrimination between heavily and lightly damaged plants may be possible due to the differences in volatile profiles. For example, volatiles released from plants that were infested by 10 females for 1 d showed lower proportions of unknown 4 and higher proportions of *n*-nonadecane, *n*-eicosane, and *n*-henicosane compared to plants that were infested by 10 females for 2–3 d. These differences could provide important cues on the profitability status of the plants for the parasitoid. To our knowledge, this is the first study to report a decrease in attractiveness in tritrophic interactions after heavy herbivore infestation. The possible adaptive value of the lack of response to heavily infested plants may warrant further investigation.

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ROQUEFORTINE/OXALINE BIOSYNTHESIS PATHWAY METABOLITES IN *Penicillium* SER. *Corymbifera*: *IN PLANTA* PRODUCTION AND IMPLICATIONS FOR COMPETITIVE FITNESS

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Abstract—Three strains of each of the seven taxa comprising the *Penicillium* series *Corymbifera* were surveyed by direct injection mass spectrometry (MS) and liquid chromatography–MS for the production of terrestric acid and roquefortine/oxaline biosynthesis pathway metabolites when cultured upon macerated tissue agars prepared from *Allium cepa*, *Zingiber officinale*, and *Tulipa gesneriana*, and on the defined medium Czapek yeast autolysate agar (CYA). A novel solid-phase extraction methodology was applied for the rapid purification of roquefortine metabolites from a complex matrix. *Penicillium hordei* and *P. venetum* produced roquefortine D and C, whereas *P. hirsutum* produced roquefortine D and C and glandicolines A and B. *P. albocoremium*, *P. allii*, and *P. radicicola* carried the pathway through to meleagrin, producing roquefortine D and C, glandicolines A and B, and meleagrin. *P. tulipae* produced all previously mentioned metabolites yet carried the pathway through to an end product recognized as *epi*-neoxaline, prompting the proposal of a roquefortine/*epi*-neoxaline biogenesis pathway. Terrestric acid production was stimulated by all *Corymbifera* strains on plant-derived media compared to CYA controls. *In planta*, production of terrestric acid, roquefortine C, glandicolines A and B, meleagrin, *epi*-neoxaline, and several other species-related secondary metabolites were confirmed from *A. cepa* bulbs infected with *Corymbifera* strains. The deposition of roquefortine/oxaline pathway metabolites as an extracellular nitrogen reserve for uptake and metabolism into growing mycelia and the synergistic role of terrestric

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acid and other *Corymbifera* secondary metabolites in enhancing the competitive fitness of *Corymbifera* species *in planta* are proposed.

Key Words—*Penicillium* ser. *Corymbifera*, roquefortine C, glandicoline A, glandicoline B, meleagrin, *epi*-neoxaline, terrestric acid, *Allium cepa.*

INTRODUCTION

Taxa of the *Penicillium* ser. *Corymbifera* are pathogenic agents causing blue mold storage rot upon a variety of flower and vegetable bulbs (Saaltink, 1971; Prince et al., 1988; Vincent and Pitt, 1989; Bertolini and Tian, 1996; Filtenborg et al., 1996), especially *Allium cepa* (onion) (Overy et al., 2005a). Seven species are recognized within the ser. *Corymbifera*: *P. albocoremium* (Frisvad) Frisvad, *P. allii* Vincent and Pitt, *P. hirsutum* Dierckx, *P. hordei* Stolk, *P. radicicola* Overy and Frisvad, *P. tulipae* Overy and Frisvad, and *P. venetum* (Frisvad) Frisvad. Extracellular enzyme profiling of the series demonstrated that all of the *Corymbifera* (with the exception of *P. hordei*) produce high levels of cellulase and hemicellulases compared to other *Penicillia*, but do not excrete extracellular proteases (Overy et al., 2005b). The *Corymbifera* extracellular enzymatic profile corresponds to the composition of *A. cepa* cells (high levels of cellulose, hemicellulose, and pectin with only a 1% protein content per cell dry weight; Mankarios et al., 1980; Séné et al., 1994), indicating that *A. cepa* bulbs are an ideal ecological niche for these specialized fungal invaders. An exception to this is *P. hordei*, which produces moderate levels of proteases (Overy et al., 2005b), reflecting this species' known association with cereal grains.

The *Corymbifera* species are reported to produce the alkaloids roquefortine C and sometimes meleagrin (Frisvad and Filtenborg, 1989; Overy and Frisvad, 2003); however, the extent to which each of the taxa produce intermediates of the roquefortine/oxaline (r/o) biosynthesis pathway (Figure 1) has not been evaluated for the series. Roquefortine C is a diketopiperazine, synthesized from tryptophan, histidine, and dimethylallyl pyrophosphate (Ohmomo et al., 1979). Both dehydrohistidyl–tryptophanyl–diketopiperazine and 3,12-dihydroroquefortine (roquefortine D) are known precursors to roquefortine C (Kozlovsky et al., 1996). C^{14} labeling experiments by Reshetilova et al. (1995) have demonstrated that roquefortine C is a precursor to glandicolines A and B (Kozlovsky et al., 1994), meleagrin (Kawai et al., 1984), and oxaline (Nagel et al., 1976), a series of biosynthetically related and structurally similar metabolites comprising the r/o biosynthesis pathway. A few reports of antibiotic activity (Kopp and Rehm, 1979) and neurotropic properties (Scott et al., 1976) of roquefortine C have prompted multiple studies into roquefortine production (Boichenko et al., 2002), uptake (Reshetilova et al., 1994; Kulakovskaya et al., 1997), and metabolism (Reshetilova and Kozlovsky, 1990).

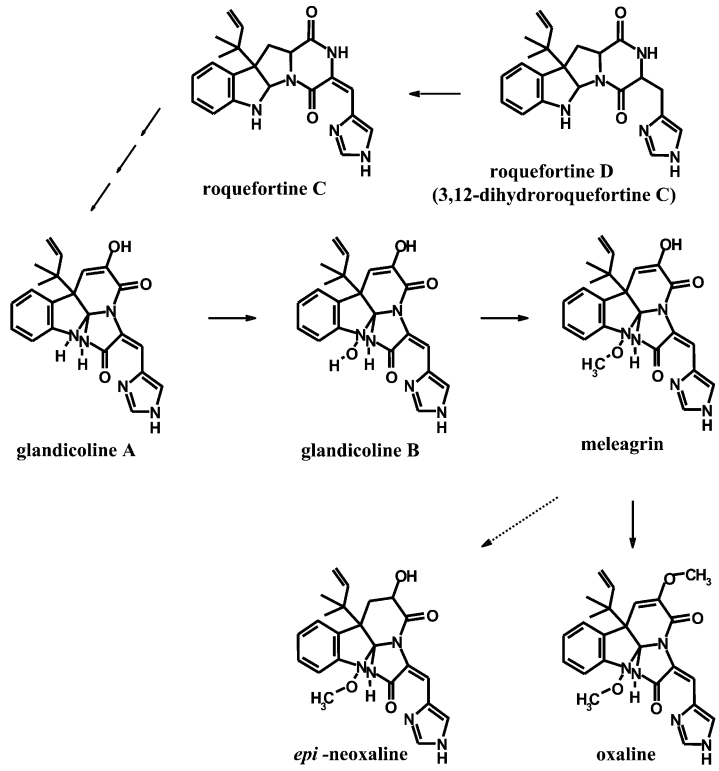

FIG. 1. Roquefortine/oxaline biosynthesis pathway as proposed by Reshetilova et al. (1995) and the proposed roquefortine/*epi*-neoxaline biogenesis pathway proposed for *Penicillium tulipae*.

The use of alkaloids as an exclusive, transportable nitrogen source has been previously proposed in plants (Wink, 2003), and we hypothesize that this strategy is also employed by fungi. Roquefortine C biosynthesis and excretion from fungal mycelia has been determined to be dependent upon the quantity of exogenous roquefortine C present in the surrounding media (Kulakovskaya et al., 1997). Additionally, ^{14}C-roquefortine C experiments demonstrated that roquefortine C was not only taken up by growing mycelia, but was also incorporated into protein and mycelial residues (Reshetilova et al., 1986, 1994; Reshetilova and Kozlovsky, 1990; Kulakovskaya et al., 1997). From *in vitro* experimentation on strains of *P. crustosum*, it was determined that transport of roquefortine C across the cellular membrane occurs via both energy-dependent and energy-independent processes (Reshetilova et al., 1994; Kulakovskaya

et al., 1997); thus, Kulakovskaya et al. (1997) proposed that roquefortine C may function as an extracellular nitrogen source for the producing organism.

Recent research efforts culturing ser. *Corymbifera* species upon host tissue-derived media have led to the stimulation and structure elucidation of a series of novel naphthalene lactones (Overy and Blunt, 2004). Preliminary investigation into the r/o biosynthesis pathway metabolites indicated that discrepancies in production might exist with the reported literature (Frisvad and Filtenborg, 1989). Media-dependent production of terrestric acid, a low molecular weight metabolite produced by five out of seven of the *Corymbifera* species, has also been observed (unpublished results). We conducted experiments testing the degree to which stimulation of the r/o biosynthesis pathway metabolites and terrestric acid occurs when *Corymbifera* strains are grown on plant-based media. To facilitate analysis of r/o metabolites from complex matrices, a preferential solid-phase extraction (SPE) methodology was applied. Subsequent *in planta* studies using the common host *A. cepa* (yellow onion) were carried out (1) to see if production of terrestric acid, the r/o pathway metabolites, and additional secondary metabolites, associated with the *Corymbifera* strains occurred, and (2) to suggest an ecological, synergistic function for these metabolites *in planta*.

METHODS AND MATERIALS

Fungal Isolates. The following isolates were revived from IBT culture collection (BioCentrum-DTU, Kgs. Lyngby, Denmark) and streak plated on Czapek yeast autolysate agar (CYA) to ensure purity: *P. albocoremium* (Frisvad) Frisvad (IBT 21502, IBT 22521, IBT 21071), *P. allii* Vincent and Pitt (IBT 3772, IBT 4112, IBT 21503), *P. hirsutum* Dierckx (IBT 12398, IBT 13033, 19340), *P. hordei* Stolk (IBT 4154, IBT 21532, IBT 23024), *P. radicicola* Overy and Frisvad (IBT 10693, IBT 10696, IBT 22526), *P. tulipae* Overy and Frisvad (IBT 3458, IBT 10676, IBT 10681), and *P. venetum* (Frisvad) Frisvad (IBT 22111, IBT 23039, IBT 23040). Spore suspensions of each isolate were then prepared by removing a loopful of conidia from axenic cultures into a semisolid medium (for media recipes, see Frisvad and Samson, 2004) and vortexed.

Preparation of Macerated Plant Tissue Agar. Plant tissue-based agars were prepared according to Overy and Blunt (2004). Bulbs of *A. cepa* (red and yellow onion) and *Tulipa gesneriana* (tulip) and tubers of *Zingiber officinale* (ginger) were inspected for symptoms of infection, and healthy tissue was selected and individually macerated in a blender. The macerate was transferred into weighed 2-l screw cap bottles, and deionized water was added to yield a

40% slurry (w/v) to which agar was added (2%; w/v). The mixture was autoclaved at 121°C for 20 min and then poured into 9-mm Petri dishes (15 ml/dish).

Strain Cultivation and Extraction. The selected ser. *Corymbifera* strains were three-point inoculated onto CYA and the various plant extract agar plates and incubated in darkness at 25°C for 14 d. Following this, colony extraction was divided into two parts. For metabolomic profiling by direct infusion electrospray mass spectrometry (Di-ESI-MS), three plugs were removed from each culture and individually extracted according to Smedsgaard (1997) [using a one-step extraction with ethyl acetate (EtOAc)]. For solid-phase extraction (SPE), an entire colony from one selected strain from each species (see Table 1) was removed in strips from the plate and inserted into 14-ml glass vials to which 8 ml of EtOAc were added. The vials were sonicated for 1 hr and the extraction solvent was subsequently decanted into clean 14-ml glass vials and evaporated *in vacuo* using a Christ rotational vacuum concentrator (RVC). The dry residues were dissolved in 2 ml of EtOAc, filtered through 0.45-μm PTFE filters to remove conidia and particulates, and evaporated to dryness under a stream of nitrogen in weighed glass vials. Selected extracts were subsequently dissolved in a volume of EtOAc to give the same concentration of raw extract, and a 1-ml subsample of the extract was dried down to be used for solid-phase extraction (equivalent to approx. 1 g of extract).

In Planta *Infection Trials.* Infection trials of *A. cepa* (yellow onion) bulbs and *Z. officinale* tubers were carried out using the selected ser. *Corymbifera* strains according to Overy et al. (2005a,b). Tubers and bulbs were purchased in

TABLE 1. SUMMARY OF SER. *corymbifera* SECONDARY METABOLITES PRODUCED *In Planta* IN INFECTED *A. cepa* BULBS[a]

Species	*In planta* secondary metabolite production
Penicillium albocoremium	Andrastin A, b. acid A, cyclopenin, gland A&B, meleagrin, roq C, viridicatin
Penicillium allii	Andrastin A, cyclopenin, fulvic acid, gland A&B, meleagrin, roq C, viridicatin
Penicillium hirsutum	Compactin, cyclopenin, gland A&B, roq C, terrestric acid, viridicatin
Penicillium hordei	Roq C (1/3), terrestric acid (1/3)
Penicillium radicicola	Andrastin A, cyclopenin, gland A&B, meleagrin, roq C, terrestric acid, viridicatin
Penicillium tulipae	Andrastin A, *epi*-neoxaline, gland A&B, meleagrin, roq C, terrestric acid
Penicillium venetum	Andrastin A, cyclopenin, roq C, terrestric acid, viridicatin

[a]Secondary metabolites were confirmed by LC-DAD-HR-MS analysis from EtOAc tissue extracts of three different species strains individually inoculated into separate *A. cepa* bulbs.
b. acid A = Barceloneic acid A; gland A&B = glandicolines A and B; roq C = roquefortine C.

late spring and inspected for signs of damage or infection (bulbs had their outer skin removed). Tubers or bulbs exhibiting signs of damage or infection (this included physical damage, sunken lesions, brown spots, or visible rot) were discarded. Surface sterilization was done in three steps in a laminar flow bench: submerging in 96% ethanol for 1 min, then in a 3% hypochlorite solution for 1 min, and resubmerging in 96% ethanol for 1 min. Once dry, bulbs were inoculated with a spore suspension in the center of the basal root plate and tubers through the epidermis with a flame sterilized needle, and placed into perforated plastic bags, and incubated in the dark at 20°C. After 3 wk, explants approximately 2 cm^3, consisting of infected tissue displaying visible sporulation (including exposed conidiophores and hyphae), were removed to 14-ml screw cap vials to which 8 ml of EtOAc were added. Control samples were similarly prepared using visually uninfected tubers and bulbs. The vials were shaken on a rotary shaker for 2 hr, the extraction solvent subsequently decanted into clean 14-ml screw cap vials, and evaporated to dryness *in vacuo* prior to SPE.

To confirm the identity of the fungal cultures excised and extracted for analysis, tissue explants were removed from the infected tissues where sporulation was visible and placed onto Petri dishes containing CYA media (+chloramphenicol, 100 ppm). Petri dishes were then incubated in the dark at 25°C and monitored for 3–7 d. Colonies emerging from the tissue explants were transferred to streak plates from which purified cultures were three-point inoculated onto CYA and incubated in the dark at 25°C for 7 d for identification based upon colony characteristics and micromorphology.

Solid-Phase Extraction. Solid-phase extraction columns were packed with approximately 300 mg of Silica 60 (0.015–0.040 mm, Merck, Darmstadt, Germany) pressed between two disks of 3-mm Vyon sheet (porous high-density polyethylene, FilterServe, West Midlands, UK) in 2-ml disposable syringes. The columns were placed onto a vacuum manifold, wetted with 2 ml of MeOH, and conditioned with 2 ml toluene/EtOAc/formic acid (TEF, 5:4:1, v/v/v). Sample extracts were redissolved in 200 µl TEF and loaded onto the column. The loaded column was then washed with 3.5 ml of TEF (fraction 1) and then with 1.5 ml EtOAc/MeOH (1:1, v/v; fraction 2). The fractions were dried *in vacuo* and resuspended in MeOH for liquid chromatography–ultraviolet–mass spectrometry (LC-UV-MS) evaluation.

Time Scale Study. P. tulipae IBT 3458 was three-point inoculated onto 18 CYA plates and incubated in the dark at 25°C. Three plates were grown to 5, 6, 7, 8, 9, and 10 d. One colony was removed from each dish with a scalpel, further cut into 2- to 3-mm rectangles, transferred to a 16-ml vial, and shaken twice with 10 ml EtOAc for 1 hr. The EtOAc phases from the double extraction were combined, evaporated *in vacuo*, and redissolved in 1000 µl MeOH for LC-UV-MS analysis. From the culture plates, ten plugs (6 mm ID) were taken from

the agar outside the colonies, transferred into a 4-ml vial, and shaken twice with 3 ml EtOAc for 1 hr. The combined EtOAc phases were evaporated *in vacuo* and redissolved in 500 μl MeOH for LC-UV-MS analysis.

LC-UV-MS Analysis. Extracts fractionated by solid-phase extraction were filtered through a 0.45-μm PFTE syringe filter and analyzed by reversed-phase chromatography combined with photodiode array detection (DAD) and high-resolution mass spectrometry on an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a LCT (Waters-Micromass, Manchester, UK) orthogonal *Time of Flight* mass spectrometer operated in the positive electrospray ionization (ESI^+) mode as described in detail by Nielsen and Smedsgaard (2003). Samples were separated on a Phenomenex Luna C_{18} (II) column (2×50 mm, with 3-μm particles) using a flow of 0.3 ml/min. A linear acetonitrile–water (AcN:H_2O) gradient system (starting at 15% AcN and increasing to 100% AcN over 20 min and holding for 5 min) was used to elute compounds from the column. The water (MilliQ) contained 10 mM ammonium formate and 20 mM formic acid (both analytical grade) and AcN (gradient grade) containing 20 mM formic acid.

Peaks, from UV and/or ESI^+ MS, excluding those found in negative controls, were matched against the Mycology reference standard database (~550 compounds; Nielsen and Smedsgaard, 2003). Metabolites not available as reference standards were dereplicated (tentatively identified) by UV spectra combined with high-resolution ESI^+ spectra as illustrated in Nielsen and Smedsgaard (2003), and matched against literature data of *Corymbifera* species and other *Penicillia* as well as Antibase 2003 (ca. 30,000 microbial secondary metabolites, Wiley & Sons, Hoboken, NJ, USA). ESI^+ spectra confirmed by deconvolution, v.s., by plotting ion traces of ions found in the background subtracted spectra of the peak of interest. Target analyses of selected metabolites, andrastin A, barceloneic acid A, cyclopenin, compactin, *epi*-neoxaline, fulvic acid, glandicolines A and B, meleagrin, roquefortine C, terrestric acid, viridicatin, and viridicatol, were performed by plotting their $[M+H]^+$ ions ± 0.01 Da.

Di-ESI-MS Fingerprinting Analysis. Plug extracts developed from each of the three selected strains of the ser. *Corymbifera* species grown on the various experimental media were additionally fingerprinted by using direct infusion positive ES-MS on a QTOF (Waters-Micromass) orthogonal quadrupolequad-rupole *Time of Flight* mass spectrometer. A constant flow of 15 μl methanol/min was delivered from an Agilent 1100 (Agilent Technologies), and 1 μl of sample was injected into the carrier stream. Just prior to the source, 5 μl/min of water with 2% formic acid were added, giving a combined flow of 20 μl/min entering the ESI source. Spectra were collected from 100 to 1000 Da/e, at a rate of 1 spectrum/sec. The scans collected during the elution time (about 15 scans) were summarized into one scan for further processing. As roquefortine C is produced

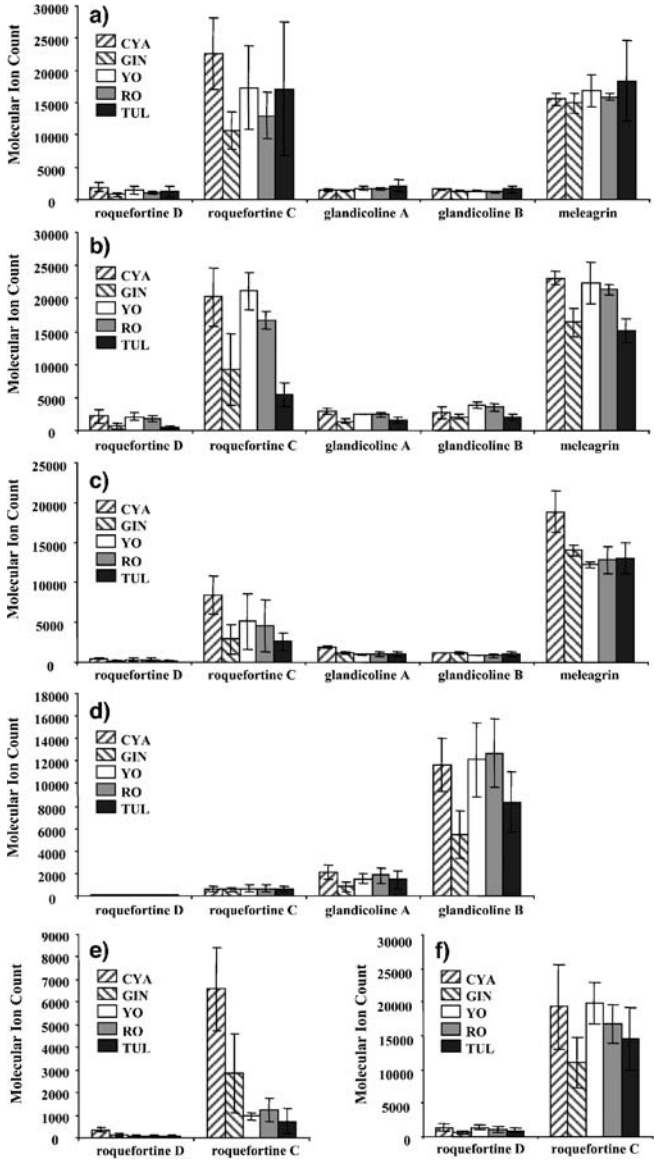

FIG. 2. Average molecular ion count calculated for roquefortine/oxaline biosynthesis pathway metabolites as produced by *Corymbifera* strains grown on Czapek yeast autolysate (CYA), ginger (GIN), yellow onion (YO), red onion (RO), and tulip (TUL) agars. (a) *Penicillium albocoremium*, (b) *Penicillium allii*, (c) *Penicillium radicicola*, (d) *Penicillium hirsutum*, (e) *Penicillium hordei*, (f) *Penicillium venetum*.

by all species and under all conditions, protonated roquefortine C (390.1930 Da/e) was used for internal mass calibration. In case of high roquefortine C intensity, the 13-C isotope was used instead. The ions from protonated terrestric acid and protonated metabolites from the roquefortine/oxaline biosynthesis pathway from each strain were averaged to give a representative species measure.

RESULTS

P. albocoremium, *P. allii*, and *P. radicicola* proceeded through the r/o pathway to meleagrin as an end product, producing roquefortine D and C, glandicolines A and B, and meleagrin. *P. hirsutum* produced roquefortine D and C and glandicolines A and B with glandicoline B accumulating as the end product. Both *P. hordei* and *P. venetum* produced roquefortine D and C, with roquefortine C accumulating as an end product. Although *P. tulipae* produced roquefortine D and C, glandicolines A and B, and meleagrin, it differed from other species in the production of *epi*-neoxaline. The time scale study of *P. tulipae* strain IBT 3485 (Figure 3b) showed that the internal mycelial concentration of meleagrin was stable after 7 d, whereas *epi*-neoxaline accumulation continued to increase gradually as colony diameter increased. Only *epi*-neoxaline could be detected as diffusing into the agar adjacent to the colonies after 8 d (3–4 ng/cm^2 detected), corresponding to approx. 20 times less than the internal mycelial concentration. Interestingly, meleagrin did not diffuse into the media.

Regardless of the media type applied for growth, r/o pathway metabolite expression for all of the *Corymbifera* species was carried through to the respective end product (Figures 2a–f, 3a). Some variation in the production of the various pathway metabolites was present between the three strains surveyed for each species as demonstrated by the error bar length for several of the media. It is important to note that the colony fitness of the species strains upon the various media were approximately equivalent as judged by the amount of sporulation as well as colony diameter (although colonies developing on TUL, YO, and RO agars did tend to produce more biomass). Colonies developing on ginger agar were an exception as they had a smaller diameter compared to the others, yet were otherwise phenotypically comparable to the CYA controls.

In general, when the margin of error was taken into consideration, the average r/o pathway metabolite expression observed for the *Corymbifera* strains grown on the various media remained relatively constant as compared to expression on CYA. For *P. albocoremium*, a decreased average production of the intermediate roquefortine C was observed on GIN and RO media; however, production of the remaining intermediates and the pathway end product meleagrin was comparable to production on CYA (Figure 2a). For *P. allii*, a

decreased production of all r/o pathway intermediates and the end product meleagrin was observed from GIN and TUL agar extracts compared to CYA, RO, and YO agars (Figure 2b). An average production of all r/o pathway metabolites was observed for *P. radicicola* strains grown on the various plant-derived media compared to production on CYA (Figure 2c). Of all the plant media used for *P. hirsutum* strains, GIN agar resulted in a decreased production of glandicoline B compared to CYA, RO, and YO agars (Figure 2d). Strains of *P. hordei* showed a definite decrease in roquefortine D, even more so in roquefortine C, on all of plant-based media (Figure 2e). However, *P. venetum* strains had relatively consistent production of these compounds on all plant-based media (although slightly lower on ginger agar; Figure 2f). For *P. tulipae* strains, an overall reduced average production of *epi*-neoxaline was observed when strains were cultured on GIN, RO, and YO media compared to CYA; in contrast, an increased expression of meleagrin production was observed for TUL compared to CYA, GIN, and YO media (Figure 3a).

Stimulation of terrestric acid production was evident when molecular ion counts were compared between culture extracts from plant-derived media and CYA (Figure 4). Terrestric acid stimulation was most pronounced for *P. venetum* grown on YO, RO, and TUL agars. For *P. hirsutum*, terrestric acid production was also stimulated on YO, RO, and TUL agars. In the case of *P. radicicola* and *P. tulipae* strains, terrestric acid stimulation was pronounced on TUL agar; however, stimulation did occur on YO and RO agars. Terrestric acid stimulation for *P. hordei* strains was noted from YO and RO agars. For all of the species, only minor terrestric acid stimulation occurred on GIN agar compared to the other plant-derived media. The greatest production stimulation occurred from *P. venetum* strains. Terrestric acid production for *P. albocoremium* and *P. allii* strains did not occur on CYA or the other plant extract agars.

With the exception of *P. hordei*, all of the ser. *Corymbifera* series species produced extensive rot (root disk covered in sporulating mycelium) in yellow onion bulbs after 3-wk incubation. Internal and external tissue necrosis was most extensive in *P. albocoremium*, *P. allii*, *P. hirsutum*, and *P. venetum* samples (with *P. hirsutum* and *P. venetum* being most extensive) and characterized by total necrosis of the root disk and meristematic region, tissue discoloration and rot of the outerleaf, first leaves, and leaf initials accompanied by pronounced sporulation of the bulb cavity (Figure 5a, d). Although the external symptoms of the *P. tulipae* and *P. radicicola* infection were similar in appearance, the internal decay caused by these strains was limited to necrosis and sporulation of the root disk, and meristematic tissue with the discoloration of first leaf and leaf initials (Figure 5b, e). For the *P. hordei* isolates, only minor sporulation was apparent, extending to cover one third of the root disk surface and internal discoloration limited to the root disk region (Figure 5c, f). In addition to the aforementioned compounds, the *Corymbifera* species we tested

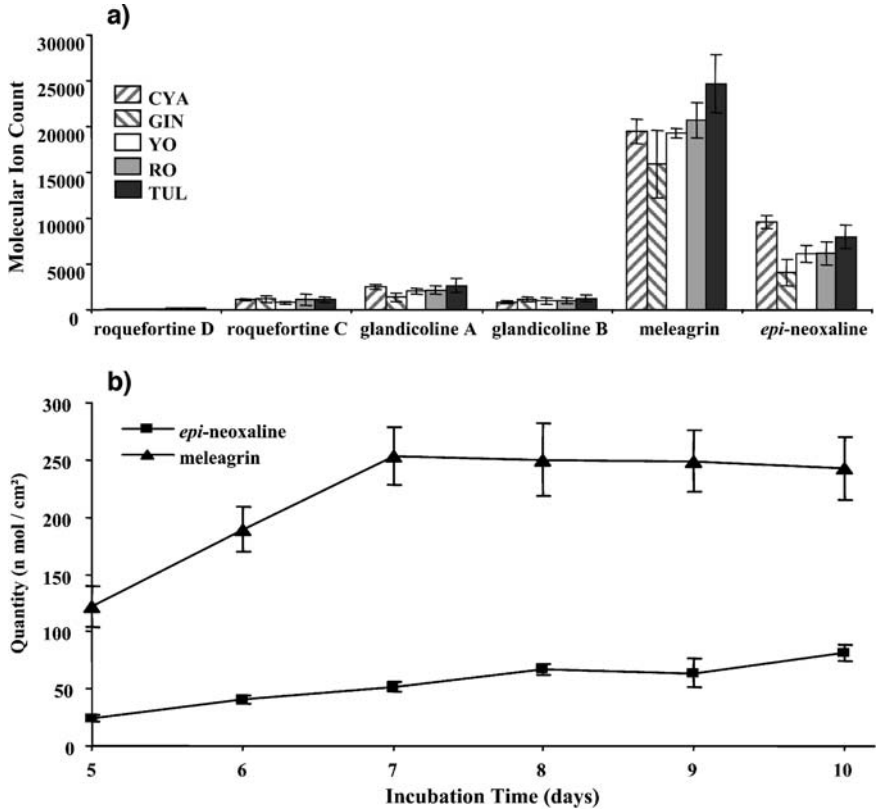

FIG. 3. Summary of roquefortine/*epi*-neoxaline biogenesis pathway metabolites produced by strains of *P. tulipae*. (a) Average molecular ion count calculated for *P. tulipae* strains grown on Czapek yeast autolysate (CYA), ginger (GIN), yellow onion (YO), red onion (RO), and tulip (TUL) agars. (b) Production of meleagrin and *epi*-neoxaline by *P. tulipae* strain IBT 3458 grown on CYA from days 5 to 10.

produced a variety of secondary metabolites (Table 1). None of the *Corymbifera* species we tested produced a rot on *Z. officinale* tubers.

DISCUSSION

Most fungi can absorb and utilize inorganic nitrogen (nitrates and ammonium) as well as organic nitrogen-containing compounds (proteins and amino acids) as a source of nitrogen. This is essential for the biosynthesis of complex molecules (i.e., amino acids, proteins, and nucleic acids); however, larger

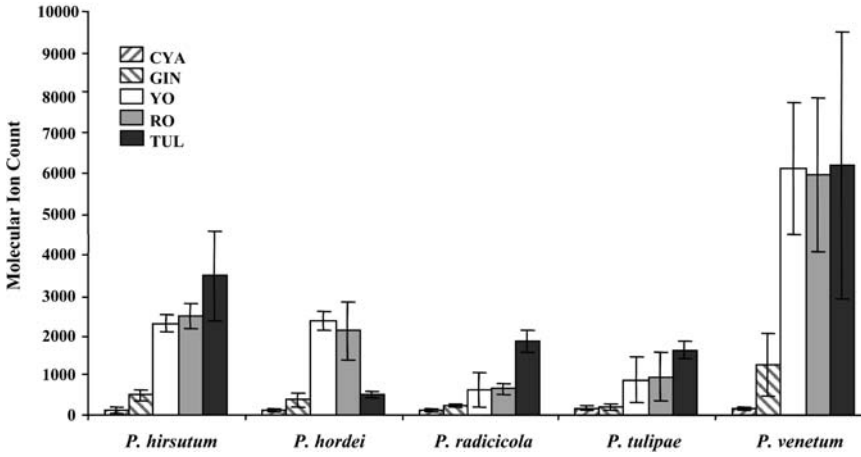

FIG. 4. Average molecular ion count of terrestric acid produced by *Corymbifera* strains grown on Czapek yeast autolysate (CYA), ginger (GIN), yellow onion (YO), red onion (RO), and tulip (TUL) agars.

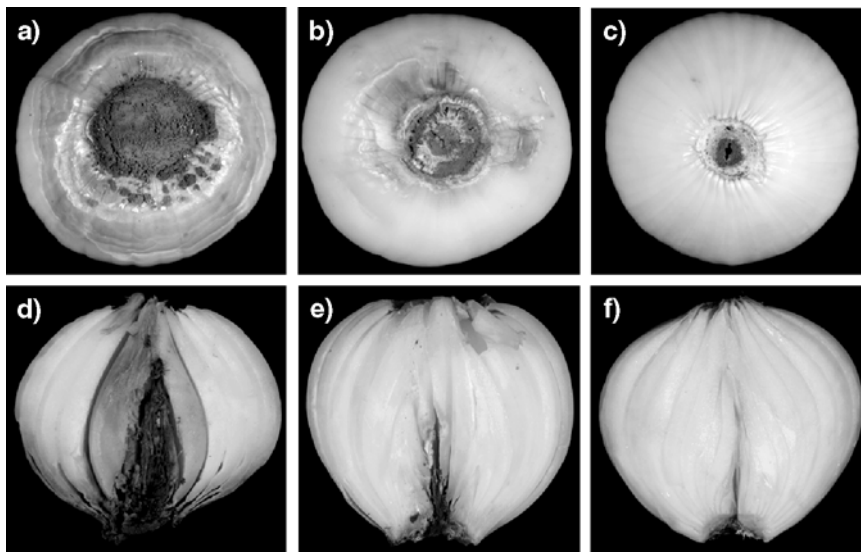

FIG. 5. Pathological symptoms displayed by *Allium cepa* bulbs infected with *Corymbifera* strains following 3-wk incubation in the dark at 20°C. (a–c) Root disk region of *A. cepa* bulbs inoculated with (a) *P. hirsutum*, (b) *P. radicicola*, and (c) *P. hordei*. (d–f) Bisection of *A. cepa* bulbs inoculated with (d) *P. hirsutum*, (e) *P. radicicola*, and (f) *P. hordei*.

extracellular polymers such as proteins must first be broken down by proteases into smaller units prior to transport. With the exception of *P. hordei*, all of the ser. *Corymbifera* species do not produce exogenous proteases and are, therefore, unable to breakdown extracellular proteins into transportable amino acids (Overy et al., 2005b). Perhaps in compensation, roquefortine C (and subsequent products of the r/o pathway) may serve as exogenous nitrogen sources. The fact that these compounds can enter and exit mycelia via both energy-independent and succinate energy-dependent mechanisms (Kulakovskaya et al., 1997) may make them particularly advantageous. In this case, nitrogen would not have to be actively pumped into mycelia. Following colonization and growth, r/o biosynthesis pathway intermediates and products accumulated in mycelia and bulb tissue infected by *P. albocoremium*, *P. allii*, *P. hirsutum*, *P. radicicola*, and *P. venetum* strains. Once accumulated in colonized tissue, these alkaloids could subsequently be taken up into growing hyphae and/or germinating conidia, metabolized into primary metabolites, and incorporated into proteins (Reshetilova et al., 1986; Reshetilova and Kozlovsky, 1990) to facilitate colonial expansion. Moreover, the antimicrobial properties reported for roquefortine C may help prevent the utilization of accumulated reserves of this amino acid resource by bacterial secondary infectors.

All of the ser. *Corymbifera* species produce and excrete organic acids: *P. hirsutum*, *P. hordei*, *P. radicicola*, *P. tulipae*, and *P. venetum* produce terrestric acid, whereas *P. albocoremium* produces barceloneic acids A and B, and *P. allii* produces fulvic acid (Overy and Frisvad, 2003, Frisvad and Samson, 2004); all of which are confirmed to be produced *in planta* in infected *A. cepa* bulbs. While r/o metabolites are deposited during colony development, accumulation of the *Corymbifera* organic acids *in planta* would lower the pH of the plant cellular environment. The pH optimum for roquefortine C excretion is 6–7 and uptake is 4.5 for *P. crustosum* strains (Kulakovskaya et al., 1997). Acidification of the infected region during colony development would therefore facilitate the uptake and subsequent metabolism of deposited r/o pathway alkaloids into germinating conidia and developing hyphae. Regional acidification would have an additional bactericidal effect, preventing the growth and competition of host-associated bacteria.

Several additional fungal secondary metabolites were produced *in planta* by *Corymbifera* strains. These compounds may act synergistically with terrestric acid and the r/o metabolites to improve the pathogenic ability and competitive fitness of the infecting strains. In addition to terrestric acid and roquefortine C, both cyclopenin and viridicatin have antibacterial properties (Bracken et al., 1954; Taniguchi and Satomura, 1970). Viridicatin also possesses phytotoxic properties, as is reported to inhibit root and shoot development in rice seedlings (Taniguchi and Satomura, 1970). Viridicatin may also act synergistically with the protein farnesyl transferase (PFTase)

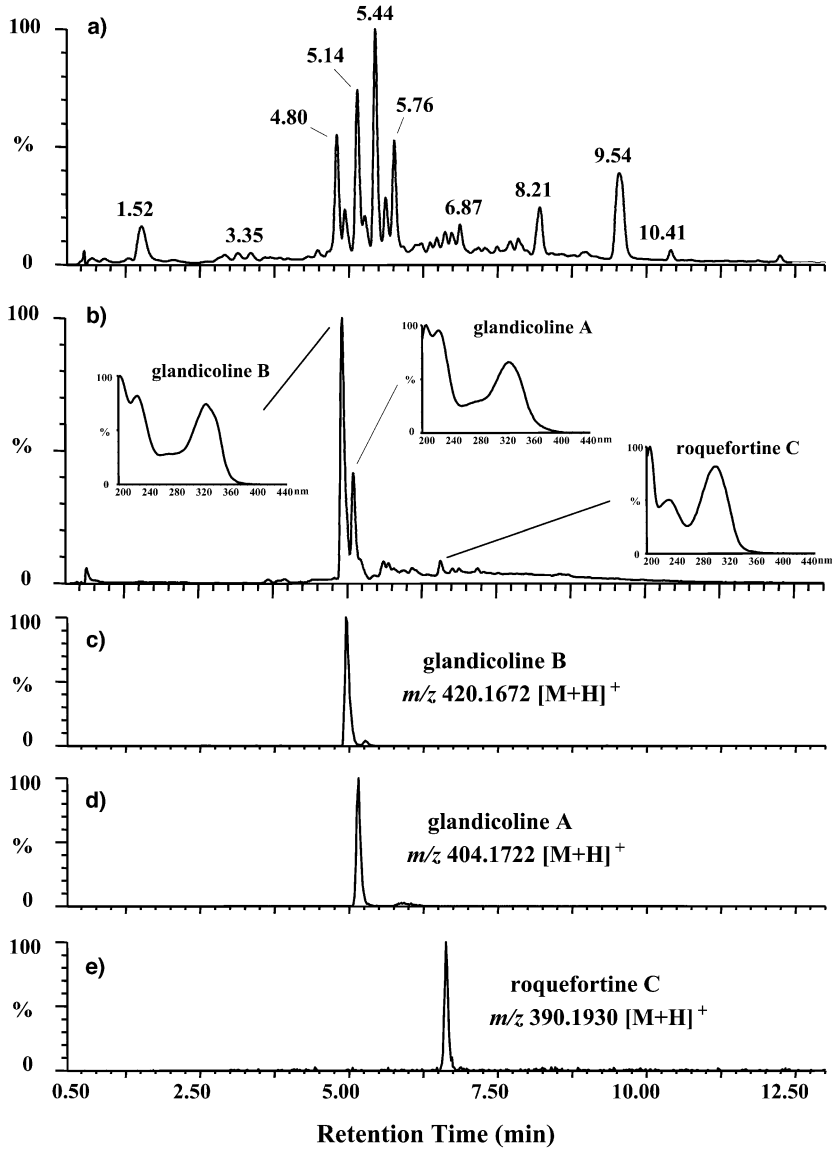

FIG. 6. LC-UV-MS traces demonstrating the preferential separation of r/o pathway metabolites from a *P. hirsutum*-infected *A. cepa* tissue extract: (a) crude extract, UV trace 300 nm (*y*-axis = relative absorbance); (b) following SPE, UV trace 300 nm (*y*-axis = relative absorbance); (c–e) accurate mass confirmation of $[M + H]^+$ ion using 100 ppm search window (*y*-axis = relative ion count).

inhibitors barceloneic acid A (Jayasuriya et al., 1995) and andrastin A (Omura et al., 1996) to weaken plant cell response to fungal infection (Overy et al., 2005c). PFTase inhibitors disrupt cellular signaling and division in developing plant cells (Morehead et al., 1995; Qian et al., 1996) resulting in decreased growth rates and stunted plants (Running et al., 2004). Compactin, produced *in planta* by *P. hirsutum* strains, is a well-known hydroxymethylglutaryl (HMG)–CoA reductase inhibitor, an enzyme responsible for the production of mevalonic acid, the biochemical building block involved in terpene biosynthesis. Although compactin has not been examined for phytotoxicity, lovastatin (a structurally similar compound) does inhibit cellular development in cultured carrot cells (Chen et al., 1987). Both compactin and lovastatin have reported antifungal properties (Brown et al., 1976; Huang et al., 1999), attributed to the inhibition of ergosterol biosynthesis in other fungi (Huang et al., 1999).

Terrestric acid production is stimulated by plant-based media. This compound is formed from the condensation of a C_4-TCA cycle intermediate with the methylene group of a fatty acid (Turner, 1971). Production of this fungal metabolite is, therefore, directly dependent upon the catabolism of glucose or other simple sugars to pyruvate during glycolysis. Ser. *Corymbifera* species produce an increased quantity of extracellular cellulases and hemicellulases as compared to other *Penicillia* (Overy et al., 2005b). This characteristic extracellular enzyme expression is ideal for the liberation and mycelial uptake of simple sugars through the enzymatic degradation of plant cell wall structural polymers (that are present in high concentrations in *A. cepa* cells; Mankarios et al., 1980). We conclude, therefore, that increased catabolic rates in the *Corymbifera* strains we tested were responsible for stimulating the production of TCA intermediates and subsequently terrestric acid production following an increased rate of respiration.

The SPE technique employed in this survey was successful in the preferential separation of the targeted r/o pathway metabolites from the various complex matrices (Figure 6), allowing the profiling of the r/o biosynthesis pathway in each of the ser. *Corymbifera* species. The r/o pathway profiles produced for five out of the seven species were in accordance with literature reports, with *P. hirsutum* and *P. tulipae* as exceptions (Frisvad and Filtenborg, 1989; Overy and Frisvad, 2003). *P. hirsutum* has been reported in the past to produce meleagrin based on TLC and HPLC-DAD analysis (Frisvad and Filtenborg, 1989); however, the results here based on preferential purification by SPE followed by LC-UV-MS analysis clearly demonstrates that glandicoline B is the r/o pathway end product for this species rather than meleagrin. Results from SPE profiling for *P. tulipae* allowed for the structural confirmation of *epi*-neoxaline (unpublished data), a metabolite previously reported as being similar to neoxaline and produced by *P. tulipae* (Overy and Frisvad, 2003). As *epi*-neoxaline rather than meleagrin was excreted by the *P. tulipae* strain IBT 3458 into the surrounding media, and because of the

progression of structural differences between glandicoline B, meleagrin, and *epi*-neoxaline, we propose that *epi*-neoxaline is the end product in the roquefortine/*epi*-neoxaline biogenesis pathway for *P. tulipae* (Figure 1).

In general, when cultured *in vitro*, terrestric acid production was stimulated, whereas the overall production of r/o metabolites was comparable to the CYA controls for most of the *Corymbifera* grown on most of the plant tissue agars. However, all of the ser. *Corymbifera* species demonstrated only minor stimulation of terrestric acid and a decreased overall production of r/o metabolites when cultivated on GIN media. Colony size on GIN media was reduced in comparison to CYA controls; however, sporulation and general colony morphology were comparable. None of these species have been reported to produce a rot on ginger and failed to grow when inoculated. An inability to grow on this substrate *in planta* may have resulted in decreased overall production of r/o metabolites when cultured *in vitro* on GIN media. This trend was also apparent for *P. hordei* when cultured *in vitro* and *in planta* on *A. cepa* (yellow onion), and for *P. allii* cultured on TUL agar. *P. hordei* did not cause rot in infected *A. cepa* bulbs, and *P. allii* is nonpathogenic on tulip bulbs (Overy et al., 2005a). With the exception of *P. hordei*, all of the ser. *Corymbifera* species are capable of producing rot on *A. cepa* (yellow onion), accompanied by the *in planta* production of terrestric acid and associated r/o pathway metabolites. The rot produced by *P. radicicola* and *P. tulipae* strains was less severe compared to the remainder of the infectious *Corymbifera*, which corresponds to a reduced production of r/o pathway end products when strains were cultured *in vitro* on YO agar. We propose that *in vitro* activities of fungi on plant-based media are predictive of *in planta* metabolite expression and pathogenic activity.

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EGGSHELL COMPOSITION OF SQUAMATE REPTILES: RELATIONSHIP BETWEEN EGGSHELL PERMEABILITY AND AMINO ACID DISTRIBUTION

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Abstract—Most snakes and lizards produce eggs with flexible shells that interact with the environment to maintain water balance. Geckos produce rigid eggshells that are independent of an external source of water and can be oviposited in more open, dryer locations. In this study, we analyzed and compared the amino acid composition of 24 lizard species, six snake species, and four outgroups (including avian and reptilian elastin and chicken eggshell). Rigid Gecko eggshells had significantly lower levels of seven of the 17 amino acids evaluated. Multivariate analysis showed that proline was the most important amino acid in distinguishing between these two groups of eggshells, occurring at significantly higher levels in flexible eggshells. High levels of proline have also been observed in the eggshells of other species. Proline and other amino acids are associated with the alleviation of water and salt stress in plants.

Key Words—Amino acid analysis, eggshells, squamate reptiles, proline, water relations, water–soil relations.

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INTRODUCTION

Most species of squamate reptiles (lizards and snakes) are oviparous (Pianka and Vitt, 2003). Packard et al. (1982) have demonstrated that there are two types of squamate eggshells: flexible and rigid. Females of oviparous species with flexible eggshells deposit the eggs in nests in the soil or in organic debris, which serves as a source of the water necessary for successful development (Tracy, 1980; Packard et al., 1982). The passage of water from the surrounding medium through the eggshell and into the interior of the egg can be bidirectional, depending upon hydric dynamics (Sexton et al., 1979; Andrews and Sexton, 1981). In contrast, the eggs of those species with rigid eggshells are independent of an external source of water and can be oviposited in relatively open, dry locations (Sexton and Turner, 1970; Pianka and Vitt, 2003).

Krampitz et al. (1972) determined the amino acid composition of eggshells of some reptiles and birds and proposed that analyses of the amino acid composition of those eggshells represented "a basis for heterogeneously structured proteins which might be class-dependent if not even species-specific." Later, Kriesten (1975) determined that the flexible eggshells of the lizard *Iguana iguana* contained high levels of the amino acid proline. His finding was confirmed by Cox et al. (1982, 1984).

In this work, we will expand the scope of these earlier studies by surveying the relative abundance of the amino acids in the eggshells of a variety of oviparous squamate reptiles to determine what role they, and in particular proline, might play in the dynamics of water transfer between soil and egg from oviposition to hatching. We will demonstrate that there is a distinct difference between the amino acid composition of flexible vs. rigid eggshells. We will also briefly review the literature about the relationship between amino acid content and the hydric environment of other disparate taxa.

METHODS AND MATERIALS

Sample Collections. We examined the eggshells of 24 species of lizards of seven families and of six species of snakes in three families (Table 1). We obtained eggs collected in nature or from eggs oviposited by captive females. The geographic range of the species are: the United States of America, the Republic of Panama, Australia, northern Africa, southern Asia, Pacific atolls, the Galapagos Islands, and Caribbean Islands.

Procedure. Eggshell samples for amino acid samples were obtained by cutting 5-mm sections from each whole, air-dried shell. Samples were weighed, washed in distilled water, and hydrolyzed in constant boiling HCL at 105°C for 24 hr. We then dried the samples using a Speed Vac concentrator. We

determined the amino acid composition of each sample using a Beckman 119C analyzer with a program optimized for resolving elastin crosslinking amino acids (Mecham and Lange, 1982). We performed triplicate analyses on each sample. We selected the extracellular, fibrous proline-rich proteins, reptilian elastin and keratin (Sage and Gray, 1979), and avian elastin (Foster et al., 1975) as controls as well as chicken eggshell (Leach et al., 1981), because of the proteinacious, fibrous nature of reptile eggshells (Sexton et al., 1979; Cox et al., 1982; Packard et al., 1982; De–Salle et al., 1984; Heulin, 1990).

Statistical Analysis. We used Nei's index of genetic similarity (Nei, 1972) to compare pairs of samples. We employed *t*-tests to evaluate individual amino acids and multivariate tests to examine sets of amino acids. Discriminant function analysis was used to identify which set of amino acids best separated the various squamate taxa. Factor analysis was used to examine the relationships among amino acids to identify a smaller set of composite variables (factors), which were then used to examine differences among the squamate groups. In all cases, overall test alphas were maintained at $P = 0.05$; all tests were two-tailed. When several individual analyses (e.g., *t*-tests on each amino acid) were performed, the individual test alphas were divided by the number of comparisons (the Bonferroni adjustment) to conserve an overall alpha of 0.05. The statistical tests chosen assume that the character traits of taxa are independent. Because of phylogenetic relationships, this is likely not to be true, and so type 1 errors are likely to be inflated (Garland et al., 1993). The impact of phylogenetic relationship is addressed in the discussion.

RESULTS

Proline was the most abundant amino acid of the 17 amino acids examined for 29 of the 30 species of squamate eggshells although there was considerable variation among taxa (Table 1). Such variation could result from genetic or environmental factors. To determine if differences in eggshell composition have a genetic component, we compared the overall similarity of pairs of congeneric species with pairs of more distantly related species with the expectation that more closely related species would be more similar than more distantly related ones. In addition, if eggshell composition is largely genetically determined, then sets of species with similar diets should not be different from sets of comparisons of species with different diets. For this analysis, we used all species within the same family that differed at a relatively gross level of diet similarity, comparing within and between herbivorous and carnivorous species.

From the available data, there were five possible comparisons we could make between pairs of species within the same genus (three species of

TABLE 1. RELATIVE AMOUNT OF 17 AMINO ACIDS (X/1000 RESIDUES) IN THE EGGSHELLS OF 24 LIZARD SPECIES, 6 SNAKE SPECIES AND 4 OUTGROUPS

Family	Species	PRO	ASP	GLU	ALA	THR	SER	GLY	CYS	VAL	MET	ILE	LEU	TYR	PHE	LYS	HIS	ARG
Iguanidae[A1]	*Iguana iguana*	435	46	41	65	27	37	73	11	52	2	21	30	47	15	15	51	32
	Basiliscus vittatus	402	52	46	78	22	38	75	67	38	2	11	35	37	17	13	49	21
	Amblyrhynchus cristatus	377	47	69	65	23	49	71	17	49	1	26	34	33	25	17	50	45
	Conolophus pallidus	364	45	62	75	26	50	71	16	55	4	27	38	34	26	22	42	40
	Sceloporus undulatus	351	55	40	75	21	65	99	4	69	2	14	31	68	19	17	43	26
	Anolis stratulus	342	50	39	84	27	44	113	37	31	6	15	30	67	8	19	69	18
	Crotaphytus collaris	341	54	49	74	22	55	80	18	49	1	20	44	36	18	16	90	34
	Anolis auratus	338	56	38	86	22	50	106	20	37	6	18	35	71	12	10	72	23
	Anolis limifrons	336	56	37	86	21	50	105	22	37	6	18	34	70	12	13	72	23
Helodermatidae[A1]	*Heloderma suspectum*	426	42	47	46	42	36	75	0	30	6	22	42	43	22	20	58	41
Scincidae[A1]	*Saproscincus mustelinus*	420	39	47	81	28	33	65	2	25	3	24	44	43	16	29	54	50
	Eumeces anthracinus	343	37	63	48	50	57	53	9	35	5	36	54	45	9	19	66	63
	Eumeces fasciatus	333	27	69	65	50	56	59	33	44	5	41	59	33	13	8	46	59
Agamidae[A1]	*Pogona barbata*	370	46	55	66	42	39	84	28	40	14	18	35	85	14	29	0	36
Eublepharidae[A1]	*Hemitheconyx sp.*	347	45	37	61	40	49	78	87	44	0	11	36	75	7	15	48	20
	Eublepharis macularius	341	43	61	58	34	39	63	9	51	11	27	42	49	16	29	70	58

Teiidae[A1]	*Cnemidophorus sexlineatus*	341	38	66	57	48	51	61	7	33	4	36	55	52	10	17	61	61
	Cnemidophorus uniparens	340	42	69	75	38	36	76	12	51	10	21	31	55	12	21	53	33
Gekkoniidae[B]	*Gehyra oceanica*	178	73	99	92	84	80	81	44	40	3	22	56	27	11	18	47	45
	Hemidactylus sp.	138	76	102	86	83	122	102	39	38	4	26	53	14	19	24	46	31
	Gonatodes albogularis	138	136	58	32	87	90	69	27	31	5	31	94	28	18	46	82	26
	Lepidodactylus lugubris	134	67	94	87	71	81	94	40	45	2	28	66	35	18	26	36	75
	Sphaerodactylus sp.	134	74	99	89	76	106	112	35	41	5	27	48	14	17	24	65	34
	Hemidactylus mabouia	122	80	110	81	89	125	107	38	42	5	20	48	13	17	20	62	18
Colubridae[A2]	*Liochlorophis vernalis*	384	35	53	47	38	46	66	47	62	7	15	31	45	13	6	66	38
	Opheodrys aestivas	337	58	56	61	37	47	66	54	60	8	18	36	43	16	8	55	38
	Pituophis catenifer	328	56	61	54	36	57	83	77	35	6	18	39	48	12	10	57	26
	Lampropeltis triangulum	290	63	66	56	35	60	106	90	27	6	18	39	51	10	11	58	20
Viperidae[A2]	*Cerastes cerastes*	369	62	55	49	34	48	85	45	31	6	24	40	53	11	10	53	22
Boidae[A2]	*Python molurus*	338	36	50	71	38	42	120	0	71	5	15	42	42	21	15	51	43
Outgroups	Reptile elastin	130	3	24	184	18	11	319	1	151	2	17	58	34	13	7	4	8
	Avian elastin	128	2	12	176	3	5	352	0	175	0	19	47	12	24	4	0	5
	Chicken eggshell	119	75	97	40	57	60	97	109	79	30	36	48	14	15	34	33	55
	Reptile keratin	101	23	40	30	15	57	303	55	67	1	18	81	127	23	3	13	45

Superscript categories A1 and A2 are lizard and snake species with flexible eggshells, respectively; B represents gecko species with rigid eggshells (shaded in gray). Within each family, species are ordered by declining levels of proline.

Anolis providing three comparisons, and two species each of *Eumeces* and *Cnemidophorus*, each providing a single paired comparison). We then randomly chose five pairwise comparisons of species from different genera within the same family. Pairs of congeneric species were significantly more similar to each other than were pairs of other confamilial species (Nei's $I = 0.995$ vs. 0.976; $t = 3.06$, $df = 8$, $P = 0.016$), providing evidence that variation in eggshell composition has a significant genetic component.

Within the iguanid lizards, three species, *I. iguana*, *Amblyrhynchus cristatus*, and *Conolophus pallidus*, are primarily vegetarian, providing three pairwise comparisons. The remaining six species of iguanids largely eat an animal diet. To maintain the same level of taxonomic relationship, we used only one species of *Anolis* (*A. stratulus*), which provided a total of four iguanid species for six pairwise comparisons. Vegetarian species had eggshell amino

TABLE 2. MEANS ± SE FOR EACH AMINO ACID AND THE FIRST FIVE PRINCIPLE COMPONENTS FOR LIZARDS WITH FLEXIBLE SHELLS AND GEKKONID LIZARDS WITH HARD SHELLS

Shell Variable	Lizards with flexible shells $N = 18$	Lizards with hard shells $N = 6$	*t*-Test for equality of means		
			t	*df*	*P* (two-tailed)
PRO	**363.8** ± 8.01	**140.8** ± 7.82	**15.13**	**22**	**<0.001**
TYR	**52.3** ± 3.89	**21.7** ± 3.83	**4.27**	**22**	**<0.001**
ASP	**45.6** ± 1.82	**84.3** ± 10.51	**−5.87**	**22**	**<0.001**
THR	**32.4** ± 2.50	**81.6** ± 2.85	**−10.54**	**22**	**<0.001**
SER	**46.3** ± 2.11	**100.5** ± 8.21	**−9.26**	**22**	**<0.001**
GLU	**51.9** ± 2.88	**93.7** ± 7.38	**−6.43**	**22**	**<0.001**
LEU	**39.3** ± 2.08	**60.9** ± 7.25	**−3.99**	**22**	**<0.001**
LYS	18.3 ± 1.43	26.1 ± 4.08	−2.32	22	0.030
GLY	78.1 ± 4.05	94.1 ± 6.77	−1.99	22	0.059
ALA	69.1 ± 2.87	77.7 ± 9.24	−1.20	22	0.242
CYS	22.1 ± 5.33	37.1 ± 2.37	−1.59	22	0.127
VAL	42.7 ± 2.57	39.5 ± 2.06	0.69	22	0.496
MET	4.9 ± 0.90	3.9 ± 0.54	0.65	22	0.520
ILE	22.4 ± 2.01	25.8 ± 1.65	−0.92	22	0.370
PHE	15.0 ± 1.28	16.7 ± 1.23	−0.72	22	0.479
HIS	55.2 ± 4.41	56.4 ± 6.73	−0.14	22	0.888
ARG	37.9 ± 3.56	38.3 ± 8.17	−0.05	22	0.962
Factor 1	**0.12** ± 0.092	**−1.32** ± 0.162	**−7.83**	**22**	**<0.001**
Factor 2	**0.58** ± 0.079	**−1.22** ± 0.081	**−12.29**	**22**	**<0.001**
Factor 3	−0.37 ± 0.254	0.50 ± 0.326	1.81	22	0.084
Factor 4	0.14 ± 0.171	0.73 ± .228	1.81	22	0.085
Factor 5	0.04 ± 0.092	−0.23 ± 0.320	−1.14	22	0.265

Values in bold significantly differ between the two groups of lizards.

acid compositions more similar to each other than did carnivorous species. The nine paired comparisons of species with similar diets were not significantly different from the 12 paired comparisons of species with different diets (t = 0.835; df = 19; P = 0.41), suggesting that diet is not a significant determinant of differences in eggshell composition.

The key question is whether eggshell composition differs between squamates with flexible vs. hard-shelled eggs. Relative concentrations of seven of the 17 amino acids differed significantly between geckos and the other squamates when individually compared (Table 2). Factor analysis reduced the17 amino acids to five principal components (Factors), which accounted for 80.8% of the original variation. Both of the first two factors, which together accounted for 53.2% of the variation, differed significantly between geckos and the other lizard taxa (Table 2). Graphing Factor 1 against Factor 2 shows how each separates the two groups of lizards (Figure 1). Snakes are interspersed with the

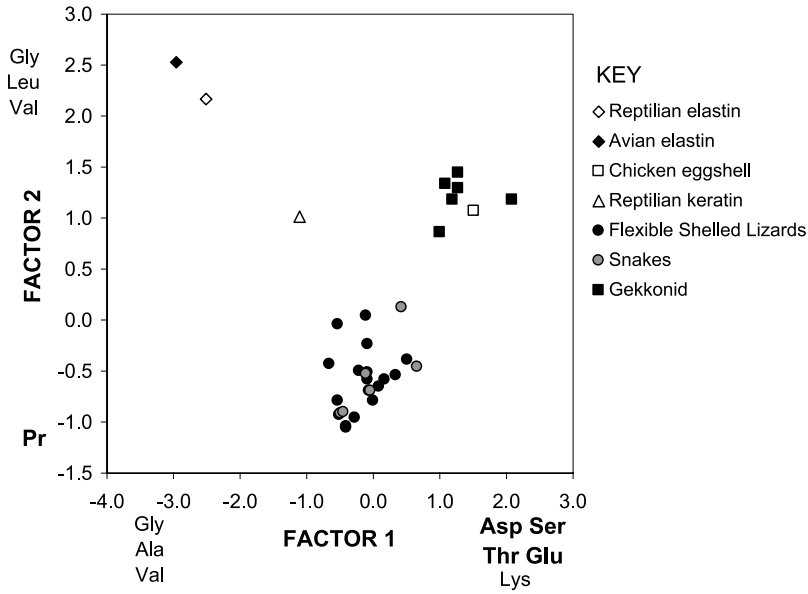

FIG. 1. Clustering of samples by factor analysis. The variables that load highest in Factor 1 are aspartic acid, serine, threonine, and glutamic acid, all of which are positively associated with Factor 1. The variable that loads highest in Factor 2 is proline and it is negatively associated with Factor 2. Variables that are more weakly correlated are included (but not in bold), and whether they are positively or negatively associated is indicated by their placement on the axis. Both factors clearly separate the flexible shelled lizards and snakes from the Geckos, although Factor 2 shows the better separation.

flexible-shelled lizards and, interestingly, the chicken eggshell nests with the hard-shelled geckos. The variables that load highest in Factor 1 are threonine, glutamic acid, aspartic acid, and serine (loading values: 0.85, 0.83, 0.81, and 0.78, respectively). Rigid eggshells are associated with high levels, flexible eggshells with lower values. The variable that loads highest in Factor 2 is proline (loading value, −0.91). High values of factor 2 are associated with flexible eggshells, lower values with rigid eggshells.

Stepwise discriminant function was used to determine which set of amino acids can best be used to separate the geckos from the flexible egg-shelled lizards. Proline, by itself, accounted for 90.8% of the differences between these two groups. Threonine, isoleucine, and phenylalanine significantly contributed to discrimination between the two groups, but together added less than 5% to the model. The best discriminant function (−4.193 + 0.028*Pro + 0.092*Ile − 0.098 Thr − 0.143*Phe) effectively separates members of these two groups. When the discriminant function is applied to the snakes, it places them all in the flexible-shelled lizards. When the equation is applied to the outgroups, it places the two elastin samples (reptile and avian) with the lizards and the chicken eggshell and reptilian keratin with the hard-shelled geckos.

DISCUSSION

We have clearly demonstrated that the amino acid composition of the eggshells of 24 species of flexible shelled squamate reptiles from nine different families differs from that of the six species of rigid-shelled geckos (Figure 1). Eggshell composition was shown to have a significant genetic composition, and, thus, it is possible that the differences observed are due to genetic relatedness and not to environmental adaptation. Recent phylogenetic analysis of the squamate reptiles places the Gekkoniidae and the Eublepharidae in a clade distinct from other squamates (Townsend et al., 2004). The eggshells of the two species of Eublepharidae in this study cluster with the other flexible shelled squamates and not with the more closely related Gekkoniidae. In addition, the clustering of the chicken eggshell with the rigid-shelled Geckos argues further that the differences observed in eggshell composition is an adaptation and not an artifact of common ancestry. The placement of snakes, all with flexible eggshells, within the flexible shelled lizards is consistent with both an adaptive explanation (all have flexible eggshells) and a phylogenetic one (see Townsend et al., 2004).

The eggs of some squamate species with flexible eggshells (e.g., *Sceloporus scalaris* and *S. virgatus*) can gain two to four times their mass through the influx of water from the surrounding medium (Andrews, 1997).

Proline, the predominant amino acid of pliable eggshells, is hydrophobic, and proline-rich sequences generally form extended structures and flexible regions that are hard to crystallize (Williams, 1994). During early external development of the egg, the inner surface of the eggshell first becomes covered by the yolk sac and later by the chorio-allantoic membrane (Andrews, 2004; Ackerman and Lott, 2004). Andrews (1997) has demonstrated that in *S. scalaris* and *S. virgatus* (members of the flexible eggshell group of squamates) much of the water influx during the incubation period occurs through the chorio-allantoic membrane. Hence, the eggshell serves as a porous, flexible barrier between the environment and the embryo with its associated embryonic membranes.

The presence of high levels of proline is not restricted to squamate eggshells. High levels have been reported from egg capsules of the skate, *Raja erinacea* (Cox et al., 1987), and from the eggshell of *Drosophila melanogaster* (Gigliotti et al., 1989; Waring et al., 1990). Proline also serves a conspicuous role in the alleviation of water and salt stress in plants (Larcher, 2003; di Toppi and Pawlik-Skowronska, 2003). Many species of plants, faced with drought or an excess of salt in the external environment, induce the synthesis of proline in the root system, and this serves as a protectant against negative hydric changes in the external environment (Kuznetsov and Shevyakova, 1999; de Ronde et al., 2000; Ramanjulu and Sudhakar, 2000).

Although the rigid eggshells of geckos contain lesser amounts of proline than flexible eggshells, they do contain higher levels of four other amino acids (Table 2). Of these four, aspartic acid/asparagine (ASX) and glutamic acid/glutamine (GLX) have been observed to accumulate in some plant species as nonspecific reactions to salt stress (Larcher, 2003). The high levels of these two amino acids in gecko eggshells may play an analogous role and may have indirectly played a role in accounting for the wide distribution of some species of geckos in the islands of the South Seas (Pianka and Vitt, 2003). The thesis is that gecko eggs, deposited on trees that were later blown into the sea, could have been transported by wind and wave to islands lacking that particular species, thus establishing a new colony.

These findings have further implications for: (1) the study of eggshell morphology of a variety of animals, (2) biochemical dynamics involved in hydric relationships between a wide variety of organisms and their environment, and (3) evolutionary relationships of various taxa.

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SCHELORIBATID MITES AS THE SOURCE OF PUMILIOTOXINS IN DENDROBATID FROGS†

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Abstract—The strawberry poison frog *Dendrobates pumilio* (Anura: Dendrobatidae) and related poison frogs contain a variety of dendrobatid alkaloids that are considered to be sequestered through the consumption of alkaloid-containing arthropods microsympatrically distributed in the habitat. In addition to ants, beetles, and millipedes, we found that adults of two species of oribatid mites belonging to the cohort Brachypylina, trophically a lower level of animal than ants and beetles, contain dendrobatid alkaloids. Gas chromatography/mass spectrometry (GC/MS) of hexane extracts of adult *Scheloribates azumaensis* (Oribatida: Acari) revealed the presence of not only pumiliotoxin 251D (8-hydroxy-8-methyl-6-(2′-methylhexylidene)-1-azabicyclo[4.3.0]nonane), but also precoccinelline 193C and another coccinelline-type alkaloid. From the corresponding extracts of an unidentified *Scheloribates* sp., pumiliotoxin 237A (8-hydroxy-8-methyl-6-(2′-methylpentylidene)-1-azabicyclo[4.3.0]nonane) was detected as a minor component, and identified by synthesis. The presence of related alkaloids, namely deoxypumiliotoxin 193H, a 6,8-diethyl-5-propenylindolizidine, and tentatively, a 1-ethyl-4-pentenynylquinolizidine, were indicated by the GC/MS fragmentation patterns, along with at least another six unidentified alkaloid components. Thus, one possible origin of pumiliotoxins,

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coccinellid alkaloids, and certain izidines found in poison frogs may be mites of the genus *Scheloribates* and perhaps related genera in the suborder Oribatida.

Key Words—Dendrobatid alkaloid, pumiliotoxins, Dendrobatidae, *Scheloribates azumaensis*, Oribatida, *Dendrobates pumilio*, oribatid mites.

INTRODUCTION

The mystery as to why batracotoxin alkaloids are shared in poison dart frogs and toxic passerine birds was solved recently by the finding that melyrid beetles (genus *Choresine*) are a prey item common to both these species (Dumbacher et al., 2004). The origins of the other frog alkaloids—groups of pumiliotoxins, allopumiliotoxins, decahydroquinolines, indolizidines, quinolizidines, coccinellines, pyrrolizidine oximes, pyrrolidines, and piperidines that are found in three genera of dendrobatid frogs (*Dendrobates*, *Phyllobates*, and *Epipedobates*) and in at least three other genera of different anuran families (*Mantella*, *Pseudophryne*, and *Melanophryniscus*) (Daly et al., 1984, 1987, 1999, 2003; Vences et al., 1998; Mortari et al., 2004)—have not yet been fully identified. These alkaloids have been detected in skin extracts from dendrobatid and mantellid frogs in nature, but are not present in those of captive-raised frogs. Furthermore, the fact that alkaloids fed to frogs are readily accumulated in the skin and remain there for months suggests that their origins are dietary (Daly et al., 1994).

Pyrrolidines, piperidines, decahydroquinolines, indolizidines, and quinolizidines are known to exist in myrmicine ants, coccinellines in ladybirds, and pyrrolizidine oximes in millipedes (as summarized by Daly et al., 2000). Pumiliotoxins, suggested as being of arthropod origin (Daly et al., 2002), have been reported recently in formicine ants of the genera *Brachymyrmex* and *Paratrechina* (Saporito et al., 2004), and enzymatic oxidation of pumiliotoxins to more potent allopumiliotoxins has been reported in dendrobatid frogs (Daly et al., 2003). Ants are, therefore, one of the possible origins for the indicated groups of alkaloids. No evidence of biosynthesis, however, has been obtained among ants.

On the other hand, it is known in Dendrobatidae that toxicity is linked to aposematic coloration and to dietary specialization as microphagus foragers, which mainly dependent on ants and mites (Vences et al., 1998, 2003; Vences and Kniel, 1998; Summers and Clough, 2001; Santos et al., 2003; Summers, 2003). In the strawberry poison frog *Dendrobates pumilio*, regarded as an ant–mite specialist (Lieberman, 1986), Donnelly (1991) has reported that mites represent 49% of the prey items consumed by *D. pumilio* males, 38% by females, and 56% by juveniles. Certain species of ants are well-known predators of Oribatida (Masuko, 1994), and it is likely that alkaloids are accumulated via the food chain from soil mites. Therefore, other species of poison frogs may be ant–mite specialists, and

these frogs may similarly accumulate those groups of alkaloids from mites or mite-eating ants microsympatrically distributed in the habitat.

As preyed mite candidates in litter, there are three suborders of Acari: Mesostigmata, Astigmata, and Oribatida—and each suborder contains species emitting non-alkaloids compounds. Hydroxymethylnaphthoquinones are known to come from *Uroactinia hirschmanni* Hiramatsu (Mesostigmata, Uropodidae) (Sakata et al., 1997), but most species of Mesostigmata seem to be non-secreting. A total of 88 compounds have been isolated from 61 species of Astigmata belonging to 10 families as opisthonotal gland secretions with species-specific blends. Some of the compounds function either as alarm, aggregation, or sex pheromones, or as defense allomones (Kuwahara, 2004). These "Astigmata compounds" (Sakata and Norton, 2001) consist of 26 monoterpenes, two sesquiterpenes, eight aromatics, four aliphatic aldehydes, a ketone, two novel fatty acids, a novel alkyl formate, and 14 fatty acid esters together with 31 hydrocarbons (Kuwahara, 2004).

The same excretory gland is also distributed in the following four glandulate cohorts of Oribatida: Parhyposomata, Mixonomata, Desmonomata, and Brachypylina, but not in the non-glandulate cohort of Palaeosomata and Enarthronota (Sakata and Norton, 2001). At present, compounds from a total of 11 species of three cohorts have been studied: six species belonging to Desmonomata, *Trhypochthoniella crassus* (Sakata et al., 1995), *Nothrus palustris* (Shimano et al., 2002), *T. japonicus* (Sakata et al., 2003), *Trhypochthoniellus* sp. (Sakata et al., 2003), *Hermannia convexa* (Raspotnig et al., 2005a), and *Platynothrus peltifer* (Raspotnig et al., 2005b); three species of Mixonomata, *Collohmannia gigantea* (Raspotnig et al., 2001), *Nehypochthonius porosus* (Sakata and Norton, 2001); *Perlohmannia* sp. (Sakata and Norton, 2001), and two species of Parhyposomata (the most basal group with the gland), *Parhypochthonius aphidinus* and *Gehypochthonius urticinus* (Sakata and Norton, 2001). Major components are identified as "Astigmata compounds" together with aromatics not yet found in Astigmata, and no evidence of alkaloids has been reported.

In this study, we analyzed the secretion of two species (*Scheloribates azumaensis* and unidentified *Scheloribates* sp.) belonging to Brachypylina (one of the four glandulate cohorts of Oribatida) and demonstrated the presence of pumiliotoxins, coccinellid alkaloids, and other related alkaloids. We believe that oribatid mites belonging to Brachypylina are one important origin of pumiliotoxins and other alkaloids in poison frogs.

METHODS AND MATERIALS

Mites. Scheloribates azumaensis (Acari: Oribatida, belonging to the cohort Brachypylina), was isolated from a crop field in Fukushima, Japan, and main-

tained successively in a laboratory at the Upland Farming Division of the Tohoku National Agricultural Experimental Station, and raised in a culture dish (90 mm i.d. × 20 mm in height) with a wet floor made of calcium sulfate, and fed with dried chlorella (Nihon Chlorella Co. Ltd., Yokohama, Japan) at room temperature. The species (holotype, female, dark reddish brown) is 424 μm in body length and 288 μm in width (Enami et al., 1996), and its tritonymph (opaque or transparent in color) has an average body length of 436.8 μm. The other species, with a similar body size, is an unidentified *Scheloribates* sp. It was collected from organic soils around an athletic field at Kyoto University, and was raised on the same diet and under the same conditions. The rearing culture dish of each species was placed in a zip-lock plastic bag (240 × 170 mm, 0.04 mm in film thickness) to retain moisture, and maintained at room temperature.

GC/MS and GC/FT-IR Analyses. Gas chromatography/mass spectrometry (GC/MS) was carried out with a Hewlett Packard HP-5989B GC/MS operated at 70 eV in the split-less mode, using an HP-5 capillary column (0.25 mm i.d. × 30 m, 0.25 μm in film thickness, Hewlett Packard) with He as the carrier gas at a flow rate of 1.23 ml/min at a temperature programmed to change from 60°C (2 min) to 290°C at a rate of 10°C/min and then held for 5 min. A Bio-Rad FT-IRD instrument coupled with a GC system was used under the same conditions to obtain vapor phase IR spectra.

Preparation of Mite Hexane Extracts for GC/MS and GC/FT-IR Analyses. A group of mites (5–20) was transferred to a conical-bottomed glass tube (8 mm i.d. × 30 mm in height, handmade) with a needle, and soaked for three min in 3 μl of hexane with a microsyringe (10 μl, Hamilton Co.). The extract collected by the microsyringe was subjected to GC/MS and GC/FT-IR analyses without being concentrated.

Preparation of Authentic Pumiliotoxin 237A. Pumiliotoxin 237A (8-hydroxy-8-methyl-6-(2′-methylpentylidene)-1-azabicyclo[4.3.0]nonane) was prepared from 8-hydroxy-8-methyloctahydro-5-indolizidinone (Barrett and Damiani, 1999) as a key compound, by aldol condensation, dehydration, and reduction, following previously reported methods as summarized in Figure 1 (Fox et al., 1991; Santos and Pilli, 2001). GC t_R = 14.29 min, GC/MS *m*/*z* (%): 237(M^+, 16), 222(3), 194(26), 176(6), 166(71), 148(10), 123(16), 112(14), 95(7), 85(21), 70(100), 55(13) and 43(46).

RESULTS

The GC profile of *S. azumaensis* adults was composed of at least four major peaks (A, 1′, 2′, and 3′) [Figure 2(1)], among which the third (peak A,

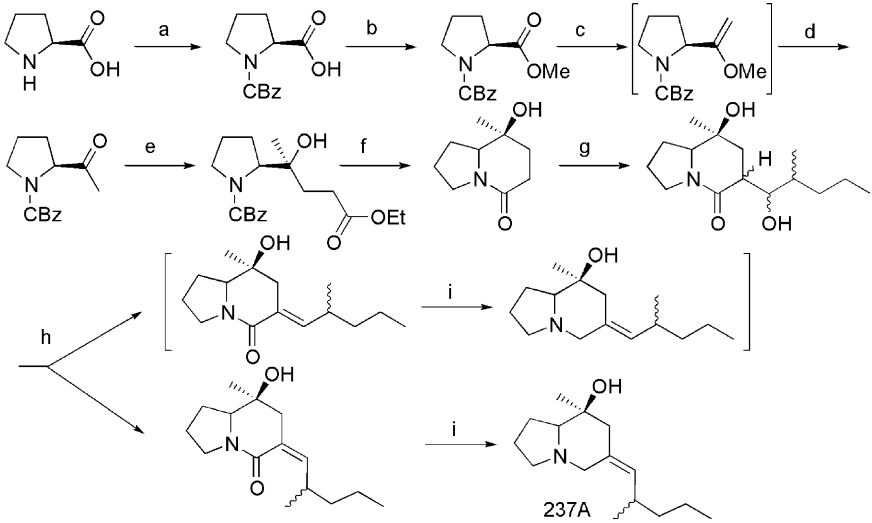

FIG. 1. Synthesis of pumiliotoxin 237A and its isomers, based on reported methods (Fox et al., 1991; Barrett and Damiani, 1999; Santos and Pilli, 2001). (a) $ClC(O)OCH_2Ph$, NaOH, THF; (b) H^+/MeOH; (c) Tebbe reagent, NaOH; (d) HCl/acetone, (e) ethyl ω-trichlorotitanylpropionate/CH_2Cl_2, (f) H_2, Pd/C, (g) LDA, THF, −78°C, 2-methylheptanal; (h) DCC, PhMe, CuICl, 110°C, or MsCl, pyridine, then KOH, or $(CF_3CO)_2O$, DBU, CH_2Cl_2, DMAP, −40°C; (i) $LiAlH_4/AlCl_3$ (3:1) in Et_2O, rt.

t_R = 15.72 min) gave an M^+ ion at *m/z* (%): 251(15) and base ion at *m/z* 166(100), together with characteristic fragment ions at *m/z* 236(3), 218(2), 208(15), 194(15), 176(7), 148(8), 137(9), 123(13), 112(12), 96(9), 84(17), 70(79), 55(7), and 43(19) (Figure 3). The mass spectrum was identical (quality index: 96) to that of pumiliotoxin 251D listed in the mass spectral libraries (Wiley Library, 6th edn., in HP Analytical CD-Rom MS ChemStation Libraries, version A.00.00) and to data previously reported (Daly et al., 1999). The IR spectrum (vapor phase, ν cm^{-1}) indicated the following absorption bands: 3543.1 (–OH, w), 2965.92 (s), 2936.14 (sh), 2884.92 (sh), 2797.13 (m), 2749.97 (sh), 1460.86 (w), 1382.48 (w), 1310.16 (w), 1140.23 (w), 1094.33 (w), and 962.62 (w). Although intensities and positions around the C–H stretching band region look a little different, appearances of all the other parts were identical to those of pumiliotoxin 251D (Daly et al., 1999). The compound was, therefore, concluded to be pumiliotoxin 251D.

The compound represented by peak 1′ [t_R = 7.00 min, Figure 2(1)] gave an M^+ ion at *m/z* (%): 150(39) and base ion at 121(100), together with ions at 135(53), 107(71), 93(27), 79(14), 67(16), 55(7), and 41(9). The mass spectrum indicates none of the alkaloid characteristics, and is similar to that for 2-(2-

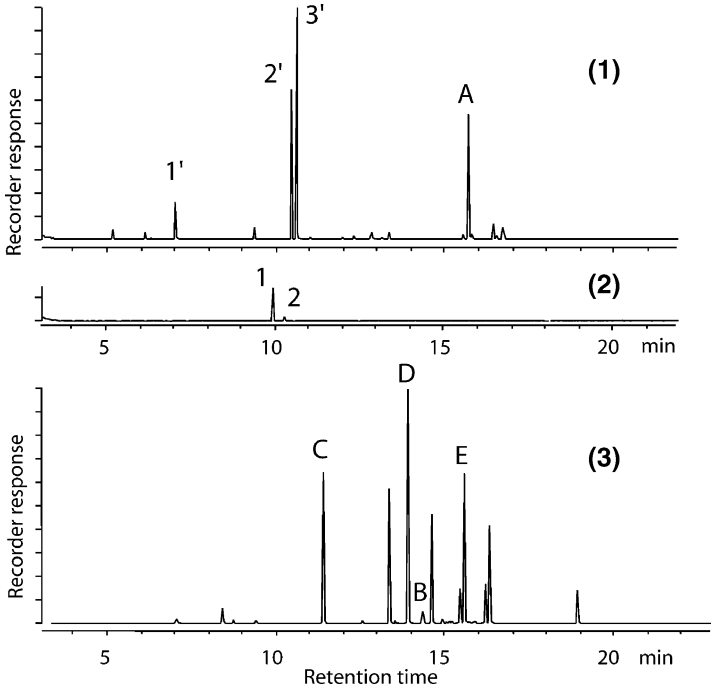

FIG. 2. Typical gas liquid chromatogram of *Scheloribates azumaensis* [(1) and (2)] and of *Scheloribates* sp. [(3)]: (1) 10 adults of *S. azumaensis*; (2) nymphal stages, 20 mites of *S. azumaensis*; (3) 5 adults of *Scheloribates* sp. Peak A: pumiliotoxin 251D. Peak B: pumiliotoxin 237A. Peak 1: geranial. Peak 2: 3-hydroxybenzene-1,2-dicarbaldehyde. Peak C: 8-deoxypumiliotoxin 193H. Peak D: a 6,8-diethyl-5-propenylindolizidine. Peak E: tentatively, a 1-ethyl-4-pentenynylquinolizidine. Peak 1′: 2-(2-pentenyl)-2-cyclopenten-1-one. Peak 2′: precoccinelline 193C. Peak 3′: a coccinelline-type alkaloid.

pentenyl)-2-cyclopenten-1-one in the mass spectral library. The compound represented by peak 2′ (t_R = 10.45 min) had an M^+ ion at *m*/*z* (%): 193(71) and base ion at 122(100), together with ions at 178(63), 164 (54), 150(99), 136(83), 108(28), 95(16), 82(30), 70(55), 55(21), and 41(22). The mass spectrum is very similar to that of the beetle alkaloid precoccinelline 193C (Daly et al., 1999). The compound represented by 3′ (t_R = 10.63 min) had an M^+ ion at *m*/*z* (%): 193(18) and base ion at 152(100), together with ions at 178(16), 164(12), 150(24), 136(25), 122(27), 110(11), 96(5), 82(6), 70(15), 55(6), and 41(6). The mass spectrum also suggests that it is another coccinelline-type alkaloid.

The hexane extracts from nymphs consisted of two components [Figure 2(2)]; one (peak 1) at t_R = 9.90 min with an M^+ ion at *m*/*z* (%): 152(8) and base

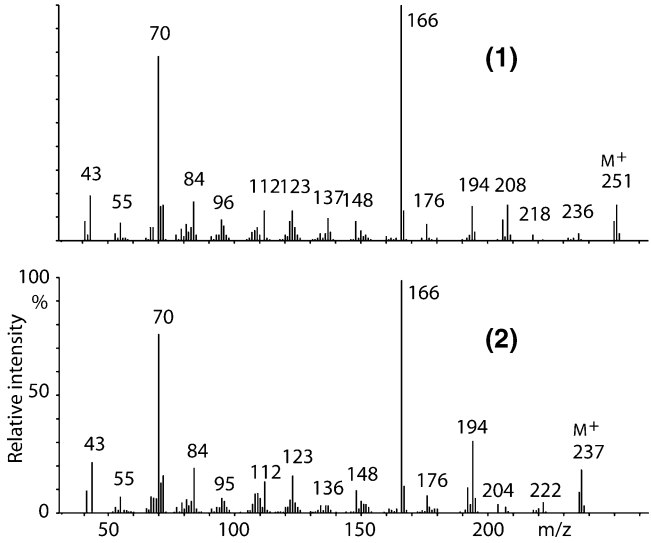

FIG. 3. Comparison of mass spectra between (1) compound A (pumiliotoxin 251D, t_R = 15.71 min) in *Scheloribates azumaensis* and (2) compound B (pumiliotoxin 237A, t_R = 14.31 min) in *Scheloribates* sp. Both compounds are homologs.

ion at 69(100), together with ions at 137(12), 123(9), 109(9), 94(23), 84(30) and 41(78), and the other (peak 2) at t_R = 10.21 min with an M^+ ion at *m*/*z* (%): 150(66) and base ion at 121(100), together with ions at 122(62), 93(43), and 65(33). From a comparison of GC t_Rs and mass spectra with authentic compounds, the compound at t_R = 9.90 min was identified as geranial [2(*E*)-3, 7-dimethyl-2,6-octadienal] (Kuwahara et al., 1980), and that at t_R = 10.21 min as 3-hydroxybenzene-1,2-dicarbaldehyde (Sakata and Kuwahara, 2001). Recorder responses of both peaks were also small and almost one-tenth of the intensity for peak A in adults.

GC/MS of the extract from *Scheloribates* sp. revealed that a minor component, peak B (t_R = 14.31 min) [Figure 2(3)], had a similar mass spectrum to pumiliotoxin 251D; an M^+ ion at *m*/*z* (%): 237(18) and base ion at *m*/*z* 166(100), together with the following diagnostic ions; 222(5), 194(31), 176(8), 148(10), 123(16), 112(14), 95(6), 84(19), 70(76), 55(7), and 43(22) (Figure 3). The compound was suggested as being pumiliotoxin 237A (Daly et al., 1999). The mass spectrum and GC retention time of the natural peak B were identical to those of synthetic pumiliotoxin 237A. Therefore, the chemical structure of peak B was identified as pumiliotoxin 237A (or its optical isomer). Based on the similarity in mass spectra between peak B of *Scheloribates* sp. and major peak

A (t_R = 15.71 min) of *S. azumaensis* (Figure 3), the structure of peak A was again confirmed to be pumiliotoxin 251D.

The extract from *Scheloribates* sp. comprised more than 10 peaks other than pumiliotoxin **237A**. Structures of three alkaloids were examined, based on GC/MS analyses (Figure 4) and fragmentation patterns as proposed (Jain et al., 1995; Daly et al., 1999) (Figure 5). Compound C (peak C, t_R = 11.41 min) had an M^+ ion at *m*/*z* (%): 193(6) and base ion at *m*/*z* 150(100) with a diagnostic ion at *m*/*z* 70(24). The structure of compound C was suggested to be 8-deoxypumiliotoxin 193H (6-(2′-methylpropylidene)-8-methyl-1-azabicyclo[4.3.0]-nonane), as reported (Jain et al., 1995; Daly et al., 1999). The mass spectrum of compound D (peak D, t_R = 13.97 min) showed an M^+ ion at *m*/*z* (%): 221(2) and base ion at *m*/*z* 180(100) with a diagnostic ion at *m*/*z* 124(15). The spectrum supported the structure of a 6,8-diethyl-5-propenylindolizidine (6,8-diethyl-5-propenyl-1-azabicyclo[4.3.0]nonane). The mass fragmentation is almost identical to that of indolizidine 223A (6,8-diethyl-5-propylindolizidine), reported by Garraffo et al. (1997) as an alkaloid often found in poison frogs. Toyooka et al.

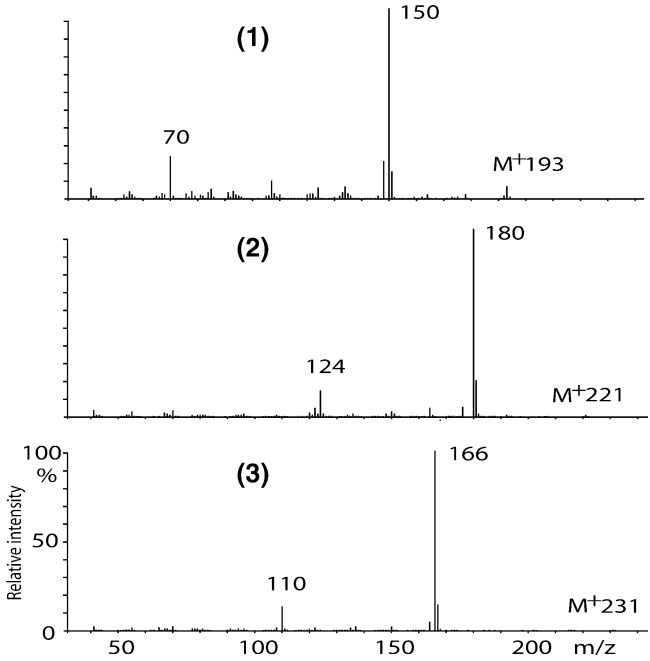

FIG. 4. Mass spectra of three components of *Scheloribates* sp. (1) Peak C (8-deoxypumiliotoxin 193H, t_R = 11.40 min). (2) Peak D (6,8-diethyl-5-propenylindolizidine, t_R = 13.95 min). (3) Peak E (1-ethyl-4-pentenynylquinolizidine, t_R = 15.58 min).

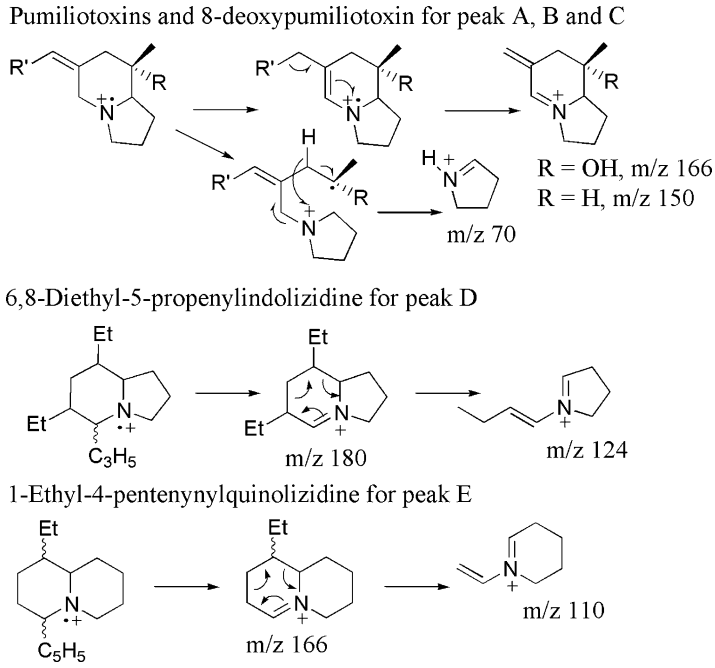

FIG. 5. Mass fragmentation pathways proposed for alkaloids from oribatid mites.

(2002) corrected the configuration at the C-6 of indolizidine 223A by synthesis. Compound E (peak E, t_R = 15.57 min) gave an M^+ ion at *m/z* (%): 231(1) and base ion at *m/z* 166(100) with a diagnostic ion at *m/z* 110(13). The structure was tentatively suggested to be a 1, 4-disubstituted quinolizidine 231A (1-ethyl-4-pentenynylquinolizidine), which was reported as a poison frog alkaloid (Jain et al., 1996; Daly et al., 1999). Structures other than the four compounds (peaks B, C, D, and E) in Figure 2(3) remain obscure, although they too are considered to be alkaloids, based on GC/MS analyses.

DISCUSSION

No alkaloids have been detected in species belonging to Mesostigmata (Sakata et al., 1997), Astigmata (Kuwahara, 2004), or in three cohorts of glandulate Oribatida (Sakata et al., 1995, 2003; Raspotnig et al., 2001, 2005a,b; Sakata and Norton, 2001; Shimano et al., 2002). Some compounds identified among Acari are known as alarm, sex, or aggregation pheromones, and most of them may function as defense allomones (Kuwahara, 2004).

In contrast, the hexane extracts from the present two oribatid mites (*S. azumaensis* and *Scheloribates* sp.) were composed mostly of alkaloids. Pumiliotoxin 237A was detected in the extract of *Scheloribates* sp., and the structure was identified by synthesis. The presence of pumiliotoxin 251D, detected in *S. azumaensis*, was concluded by an MS Library search, GC/FT-IR, and comparison of the GC/MS fragmentation patterns with those of pumiliotoxin 237A.

Three major components (peaks 1′, 2′, and 3′) other than peak A in the former species were suggested to be 2-(2-pentenyl)-2-cyclopenten-1-one, precoccinelline 193C, and a coccinelline-type alkaloid, respectively, based on GC/MS analysis. This is the first discovery of coccinellid alkaloids distributed in animals other than ladybird beetles and frogs. Likewise, structures of three peaks (peaks C, D, and E) in the latter species were elucidated as 8-deoxypumiliotoxin 193H, a 6,8-diethyl-5-propenylindolizidine, and a 1,4-disubstituted quinolizidine 231A (1-ethyl-4-pentenynylquinolizidine). Many compounds (more than seven peaks, not designated) in the latter were similarly suggested to be alkaloids via GC/MS, but their structures remain obscure. Although these compounds have yet to be identified by routine natural product chemistry, we can claim at the moment that pumiliotoxins 251D and 237A were present in *Scheloribates* mites, possibly functioning as defense allomones.

The presence of volatile alkaloids is also suggested in two other unidentified *Xylobates* species belonging to Xylobatidae, Oribatida (Sakata, personal communication). The stain made by crushing these mites on filter paper was turned orange by Dragendolff reagent, indicating the component(s) may be alkaloid(s). Thus, alkaloids might be widely distributed among *Scheloribates* species and/or species belonging to the same cohort. Although pumiliotoxin **251D** content was not determined in *S. azumaensis*, it may be more than 100 ng based on its recorder response.

Pumiliotoxins and other alkaloids are adult-specific components and are not detectable at the larval or nymphal stage, although all stages depend on the same diet. Secretions from nymphs and larvae consisted only of non-alkaloids, that is, "Astigmata compounds". This suggests that pumiliotoxins and other alkaloids are biosynthesized by adult oribatid mites. This immature/adult dichotomy in chemistry seems to be a general characteristic among the so-called "higher" oribatid mites Brachypylina (Raspotnig et al., 2005a).

Ants and mites are the two major prey items consumed by *D. pumilio* and related poison frogs (Lieberman 1986; Donnelly, 1991; Jones et al., 1999). It may be that many Brachypylina species are distributed upon the soil surface, and that several *Scheloribates* species (their body sizes vary from 330 to 980 μm; Hammer, 1958, 1967) are large enough to serve as prey items for frogs (body size 2–3 cm). The aposematic coloration and toxicity of poison frogs are linked with dietary specialization (Vences et al., 1998, 2003; Vences and Kniel,

1998; Summers and Clough, 2001; Santos et al., 2003; Summers, 2003). No aposematism has developed, however, in those alkaloid-containing Oribatida, possibly because their predators may be achromatopsic (defective in color vision). The present two *Scheloribates* are dark reddish brown and have a smooth body surface. Two other unidentified *Xylobates* species (as mentioned above) possess black bodies with bristles. There is no particular difference noticed from other oribatid species possessing "Astigmata compounds".

Alkaloids such as pyrrolidines, piperidines, decahydroquinolines, indolizidines, and quinolizidines are distributed in myrmicine ants (as summarized by Daly et al., 2000). More recently, Saporito et al. (2004) have identified pumiliotoxins 307A and 323A in formicine ants of the genera *Brachymyrmex* and *Paratrechina*. Certain species of ants are known as predators of Oribatida (Masuko, 1994). It is conceivable that ants may accumulate those dendrobatid alkaloids by consuming alkaloid-biosynthetic Oribatida. Currently, however, there is no evidence of sequestration by ants.

The genus *Scheloribates* is distributed worldwide, and is now perhaps the first of many alkaloid-containing genera of the Brachypylina cohort of the suborder Oribatida. If a food chain is established connecting such mites with frogs as predators, it will not be surprising to find that frogs are sequestering these mite alkaloids.

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A QUANTITATIVE SURVEY OF MYCOSPORINE-LIKE AMINO ACIDS (MAAS) IN INTERTIDAL EGG MASSES FROM TEMPERATE ROCKY SHORES

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Abstract—Mycosporine-like amino acids (MAAs) have been reported as functional chemical sunscreens in a variety of marine organisms, but their role in development of marine embryos and larvae remains largely unexplored. In this study, we quantified MAAs from intertidal egg masses of 46 species of mollusks, two species of polychaetes, and one species of fish from southeastern Australia. We aimed to elucidate potential patterns of occurrence and variation based on egg mass maturity, adult diet, spawning habitat, phylogeny, and viability. Our analyses revealed that maturity and spawning habitat did not affect MAA composition within egg masses. In contrast, adult diet, phylogeny, and viability affected MAA composition. Herbivores had higher levels of certain MAAs than carnivores; similarly, viable egg masses had higher levels of some MAAs than inviable ones. MAA composition varied according to the taxonomic group, with nudibranchs and anaspids showing different MAA composition compared to that of neogastropods, sacoglossans, and polychaetes. Basommatophoran egg masses had more porphyra-334 than the other taxa, and anaspids had more mycosporine-2-glycine than the other groups. MAAs occurred in relatively high concentrations in intertidal molluskan egg masses when compared to adult mollusks and other common intertidal organisms. Despite the complexity of factors affecting MAA composition, the prevalence of MAAs in some species is consistent with protection afforded to offspring against negative effects of UV radiation.

Key Words— Gastropod, egg mass, larvae, marine invertebrate, chemical sunscreen, intertidal, mycosporine–glycine.

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INTRODUCTION

Surface ultraviolet radiation (UVR) has been shown to deleteriously affect reproduction, development, growth, and behavior of many organisms including marine invertebrates (Haeder et al., 1998). Biologically significant UVR comprises UV-B (280–315 nm) and UV-A (315–400 nm) (Cockell and Knowland, 1999), with UV-B absorbed strongly by the ozone layer (Paul and Gwynn-Jones, 2003). Although UVR is attenuated in the water column, biologically significant levels of UV-B may still penetrate to 20 m or more (Booth and Morrow, 1997). Hence, organisms in shallow water or intertidal habitats are expected to be especially vulnerable. Recent research confirms that encapsulated larvae of intertidal mollusks are at high risk of UVR damage (Przeslawski et al., 2004).

Larvae and embryos may reduce or eliminate exposure to UVR in several ways. Offspring may simply avoid UVR as a result of adult spawning behavior. Many free-spawning invertebrates, for example, release gametes nocturnally so that fertilized eggs undergo early developmental stages in darkness (e.g., Pagano et al., 2004). Other organisms, particularly gastropods, enclose offspring within leathery capsules or gelatinous egg masses (see Przeslawski, 2004a). The embryos are essentially sessile for the duration of their encapsulation and reliant on adult spawning habitat for protection against UVR. Many gastropods only spawn under boulders or in other fully shaded microhabitats, but some species consistently deposit their egg masses in habitats exposed to sunlight (Benkendorff and Davis, 2004). The developing embryos within these egg masses are potentially exposed to high intensities of UVR with no visible protection. Nevertheless, gastropod embryos of species that consistently spawn in full sunlight are less vulnerable to the negative effects of UVR than embryos from species that only spawn in shaded habitats (Przeslawski et al., 2004).

Alternatively, some encapsulated embryos may be protected from UVR through chemical sunscreens, such as mycosporine-like amino acids (MAAs). MAAs are a suite of 19 compounds with absorption maxima at 310–360 nm. They are produced *de novo* via the shikimate pathway in algae, fungi, and bacteria (Bentley, 1990), and animals are assumed to acquire MAAs through diet or symbioses with these organisms (Shick and Dunlap, 2002). MAAs have been found in adults from a variety of organisms including algae, fish, cnidarians, mollusks, and echinoderms (reviewed by Shick and Dunlap, 2002). The concentration and composition of MAAs can vary in organisms according to latitude (Bosch et al., 1994), altitude (Tartarotti et al., 2001), depth (Gleason and Wellington, 1995), sex (Michalek-Wagner, 2001), and species (Xiong et al., 1999). MAAs have also been reported from the eggs and larvae of several invertebrates including urchins, corals, ascidians, and gastropods (reviewed by Karentz, 2001). Only two molluskan egg masses were covered in this review, so

Przeslawski (2004b) conducted a preliminary investigation on a further 46 gastropod egg masses. This revealed that the total MAA content varies significantly according to taxonomic group and adult diet, but it is still not clear how MAA composition varies with these and other factors. Larvae are generally considered the most vulnerable life stage, and so MAA composition in such species may be especially important to the overall success of a population.

In this study, we collected benthic egg masses from a range of intertidal invertebrates and quantified their MAA composition. We aimed to identify common MAAs in intertidal egg masses and to determine which factors account for variation in MAA composition and abundance. Here, we investigate the effects of egg mass maturity, adult diet, spawning habitat, taxonomic group (order), and embryo viability. We predicted that herbivores would have a richer complement of MAAs at higher concentrations in their spawn than carnivores, as they have a more direct link to *de novo* MAA sources. We also anticipated that egg masses of species that routinely spawn in habitats exposed to full sun would contain a larger number of MAAs at higher concentrations than those spawned in shaded habitats. In addition, we collected adults of several shelled species and predicted that adults would show fewer MAAs at lower concentrations than spawn due to the need for photoprotection of vulnerable developmental stages.

METHODS AND MATERIALS

We undertook a quantitative survey of MAA composition and concentration in egg masses from 49 intertidal organisms representing 46 gastropod species, two unidentified polychaete species, and one gobiesocid fish (Table 1). Most egg masses were collected from intertidal habitats along the Illawarra coast November 2001–February 2004. Some samples were collected from adults kept in laboratory aquaria, and egg capsules of *Nucella lapillus* were collected from Cornwall, England. Egg masses were identified to species level based on observations of laying adult or previous research where possible (Rose, 1985; Smith et al., 1989; Benkendorff, 1999). Potential spatial and temporal variation in MAA composition was examined on a subset of the egg masses. We restricted these analyses to species with three or more replicates. Spatial differences in MAA composition were analyzed for *Dolabrifera brazieri*, *Dicathais orbita*, *Placida* cf. *dendritica*, and *Siphonaria denticulata*. Temporal differences were examined in egg masses of *Agnewia tritoniformis*, *Conus papilliferus*, *Dendrodoris fumata*, *D. brazieri*, *P.* cf. *dendritica*, and *S. denticulata*. Nested analysis of similarities (ANOSIMs) revealed no significant spatial ($R = 0.049$, $P = 0.273$) or temporal ($R = 0.17$, $P = 0.114$) variations in MAA concentrations for any of the species examined. Therefore, egg mass data for all species collected from different sites and times were pooled in the remaining analyses.

Both capsular and gelatinous egg masses were collected. Egg masses from herbivores represented 16 species, and carnivores represented 30 species; the diet of three species was unknown (Table 1). We collected egg masses of six species from full sun habitats (exposed rock platforms), 13 species from partial sun (algae beds, sand, or vertical rock faces), and 30 species from shaded habitats (under boulders, overhangs, or caves) (Table 1). Egg masses were examined under a dissecting microscope (40× magnification) to determine development, and they were classed accordingly into one of four developmental stages: (1) undeveloped egg masses contained eggs that had not yet developed to the trochophore stage; (2) egg masses with intermediate development contained eggs with trochophores or early veligers; (3) mature egg masses had late stage veligers, crawling juveniles and/or showed signs of hatching; (4) inviable egg masses contained no viable eggs and often showed coloration changes associated with damage or stress (Pechenik, 1983; Przeslawski et al., 2004). After examination, egg masses were cleaned by agitation in filtered seawater for 30 sec, followed by gentle blotting to remove excess water. Egg masses were then lyophilized, and dry weight was recorded. Samples were stored at −80°C until extractions were performed. No degradation of MAAs was detected in an 18-mo storage period (data not presented), as also noted in previous studies (Dunlap and Chalker, 1986; Karsten et al., 1998).

Preliminary tests were performed to determine the ideal extraction conditions for these samples as recommended by Tartarotti and Saommaruga (2002). Samples were extracted using various temperatures (4, 20, and 40°C), sample weights (2, 10, 20, and 50 mg) and solvent concentrations (60% and 80% MeOH); and optimal extractions occurred at 20°C with a 20-mg sample in 80% methanol. Based on the preliminary extraction test, three serial extractions were performed on 20 mg dry weight of each egg mass in 0.5 ml 80% HPLC-grade MeOH for 1 hr at room temperature (≈20°C). Following extraction of each sample, the supernatant was pooled, and the absorption spectra from 250 to 450 nm of the extract was taken in a quartz cuvette by a scanning spectrophotometer (Shimadzu UV-1601). Extraction efficiency was tested on egg masses of *Bembicium nanum*, *Bursatella leachii*, and *S. denticulata*; and, we recovered over 95% of total MAAs after three of 6 extractions for each species.

MAAs were separated by reverse-phase high performance liquid chromatography (RP-HPLC) on a Phenosphere C8 column (5 μm, 4.6 internal diam × 250 mm) with guard (Phenomenex) at a flow rate of 0.8 ml/min. The aqueous mobile phase was 39.9:0.1:60 (water/acetic acid/methanol). Nine MAAs were identified and quantified using maximum wavelength absorption and cochromotography with prepared standards representing the most common MAAs (Karentz, 2001; Shick and Dunlap, 2002): standards for mycosporine–glycine and palythinol were prepared from *Palythoa tuberculosa*; mycosporine-2-glycine and mycosporine taurine from *Anthopleura elegantissima*; shinorine and

TABLE 1. LIST OF SPECIES AND ASSOCIATED EGG MASS CHARACTERISTICS USED IN THIS STUDY

Taxa	Species	*N*	Habitat	Diet	Structure
Phylum Mollusca: Class Gastropoda					
Superorder Neritopsina					
Neritoidea (SF)	*Nerita atramentosa*	2	Full sun	Herbivore	Capsule
Superorder Caenogastropoda					
Littorinimorpha (IO)	*Bembicium nanum*[a,b]	18	Full sun	Herbivore	Gel
Littorinimorpha (IO)	*Cabestana spengleriii*[a,b]	5	Shade	Carnivore	Capsule
Littorinimorpha (IO)	*Conuber* sp.[a]	10	Full sun	Carnivore	Gel
Littorinimorpha (IO)	*Cypraea erosa*	1	Shade	Herbivore[c]	Capsule
Littorinimorpha (IO)	*Ranella australasia*	1	Shade	Carnivore	Capsule
Neogastropoda (IO)	*Agnewia tritoniformis*	5	Shade	Carnivore	Capsule
Neogastropoda (IO)	*Bedeva* sp.	1	Shade	Carnivore	Capsule
Neogastropoda (IO)	*Cominella eburnea*[a,b,d]	5	Shade	Carnivore	Capsule
Neogastropoda (IO)	*Conus papilliferus*[a]	6	Shade	Carnivore	Capsule
Neogastropoda (IO)	*Dicathais orbita*[a,b]	8	Shade	Carnivore	Capsule
Neogastropoda (IO)	*Lepsiella reticulata*[b]	2	Shade	Carnivore	Capsule
Neogastropoda (IO)	*Morula marginalba*	2	Shade	Carnivore	Capsule
Neogastropoda (IO)	*Mitra badia*	1	Shade	Carnivore	Capsule
Neogastropoda (IO)	*Mitra carbonaria*[a]	13	Shade	Carnivore	Capsule
Neogastropoda (IO)	*Nucella lapillus*[b]	2	Partial sun	Carnivore	Capsule
Superorder Heterobranchia					
Cephalaspidea (O)	*Bulla quoyii*	1	Partial sun	Herbivore	Gel
Cephalaspidea (O)	*Bullina lineata*	5	Partial sun	Carnivore	Gel
Cephalaspidea (O)	*Hydatina physis*[a]	8	Partial sun	Carnivore	Gel
Sacoglossa (O)	*Aplysiopsis formosa*	2	Partial sun	Herbivore	Gel
Sacoglossa (O)	*Oxynoe viridis*[a]	6	Partial sun	Herbivore	Gel
Sacoglossa (O)	*Placida* cf. *dendritica*[a]	9	Partial sun	Herbivore	Gel
Notaspidea (O)	*Berthellina citrina*	1	Shade	Carnivore	Gel
Notaspidea (O)	*Pleurobranchus peronii*	2	Shade	Carnivore	Gel
Notaspidea (O)	*Pleurobranchus* sp.	2	Shade	Carnivore	Gel
Anaspidea (O)	*Aplysia juliana*[b]	9	Partial sun	Herbivore	Gel
Anaspidea (O)	*Aplysia sydneyensis*[a]	14	Partial sun	Herbivore	Gel
Anaspidea (O)	*Aplysia parvula*	2	Partial sun	Herbivore	Gel
Anaspidea (O)	*Bursatella leachii*[a,b]	6	Partial sun	Herbivore	Gel
Anaspidea (O)	*Dolabella auricularia*[b]	2	Partial sun	Herbivore	Gel
Anaspidea (O)	*Dolabrifera brazieri*[a,b]	16	Shade	Herbivore	Gel
Anaspidea (O)	*Stylocheilus striatus*[a]	8	Partial sun	Herbivore	Gel
Nudibranchia (O)	*Aeolidiella foulisi*	1	Shade	Carnivore	Gel
Nudibranchia (O)	*Austraeolis ornata*	5	Shade	Carnivore	Gel
Nudibranchia (O)	*Dendrodoris carneola*	1	Shade	Carnivore	Gel
Nudibranchia (O)	*Dendrodoris fumata*[a]	7	Shade	Carnivore	Gel
Nudibranchia (O)	*Dendrodoris nigra*	1	Shade	Carnivore	Gel
Nudibranchia (O)	*Doriopsilla miniata*	1	Shade	Carnivore	Gel
Nudibranchia (O)	*Goniodoris meracula*	3	Shade	Carnivore	Gel
Nudibranchia (O)	*Hoplodoris nodulosa*	4	Shade	Carnivore	Gel
Nudibranchia (O)	*Hypselodoris obscura*	3	Shade	Carnivore	Gel

TABLE 1. CONTINUED

Taxa	Species	*N*	Habitat	Diet	Structure
Phylum Mollusca: Class Gastropoda					
Superorder Heterobranchia					
Nudibranchia (O)	*Platydoris galbanus*	3	Shade	Carnivore	Gel
Nudibranchia (O)	*Plocampherus imperialis*	1	Shade	Carnivore	Gel
Nudibranchia (O)	*Rostanga arbutus*	3	Shade	Carnivore	Gel
Basommatophora (O)	*Siphonaria denticulata*[a]	14	Full sun	Herbivore	Gel
Basommatophora (O)	*Siphonaria zelandica*	6	Full sun	Herbivore	Gel
Phylum Annelida: Class Polycheata					
Unknown order	Unknown polychaete 1	3	Shade	Unknown	Gel
Unknown order	Unknown polychaete 2	4	Full sun	Unknown	Gel
Phylum Chordata: Class Osteicthyes					
Gobiesociformes (O)	*Aspasmogaster costatus*[e]	2	Shade	Unknown	Gel

N = number of viable egg masses collected. Taxa refer to superfamily (SF), infraorder (IO), or order (O)

[a]Species used in analysis of maturity.

[b]Inviable egg masses of this species also collected.

[c]This species is either an omnivore or herbivore (Beesley et al., 1998). For statistical analyses, we have classified it as an herbivore since it likely ingests some algae.

[d]This species is tentatively identified based on crawling juveniles that emerged from capsules.

[e]Tentative identification based repeated sighting of adults on and near egg masses.

porphyra-334 from *Porphyra tenera*; palythine from *Mastocarpus stellatus*; and palythene and asterina-330 from the ocular lens of *Plecropomus leopardus*. Concentration was calculated based on standards and primary calibration of equipment at the Australian Institute of Marine Science and expressed in nmol/mg sample dry weight.

To provide a standardized comparison between egg masses and other organisms, we collected several intertidal organisms from full sun habitats ($N = 1$): *Actinia tenebrosa* (anemone), *Haliclona* sp. (sponge), *Hormosira banksii* (phaeophyte), and *Ulva lactuca* (chlorophyte). Adult gastropods from the following species were also collected ($N = 4$): *B. nanum*, *S. denticulata*, and *S. zelandica*. These specimens were removed from their shells and prepared and analyzed as described above with the exception that >20 mg dry weight of the whole adult (including gonads and *in vivo* eggs) were used in extractions.

Nonmetric multidimensional scaling (nMDS) plots of the data were used to clarify the distribution of MAAs in egg masses based on maturity, spawning habitat, adult diet, phylogeny (order), and viability. Nested ANOSIMs were conducted on data subsets encompassing all species, where $N \geq 3$ (Table 1). Samples without MAAs were given a nominal value of 0.0001 nmol/mg for mycosporine–glycine, to enable the calculation of Bray–Curtis similarity index for MDS plots and ANOSIMs. We used PRIMER v. 5 (Plymouth Routines in Multivariate Ecological Research) for all multivariate analyses. In addition,

two-way or nested ANOVAs were conducted on all species in JMP v. 4. These were performed separately for each MAA examined using egg mass maturity, adult diet, spawning habitat, order, and viability as factors in the analysis. Each species was considered independent and was appropriately nested or crossed with these factors (see Westoby et al., 1995a,b). Most data were skewed and/or had unequal variances, and data were log-transformed (Zar, 1998) to ensure that the assumptions of ANOVA were met. Tukey's HSD tests were used *post hoc* to reveal significant relationships. $\alpha = 0.05$ for all statistical analyses unless otherwise specified.

RESULTS

Spectrophotometry indicated the presence of UVR-absorbing compounds in many species as evidenced by maximal absorbance at UVR wavelengths (Figure 1). Of the 49 species tested, we detected and quantified MAAs in 43 viable egg masses (Table 2). We did not detect MAAs in the egg masses of the polychaetes or in egg masses from four species of mollusks (*Ranella australasia*, *Cominella eburnea*, *Mitra carbonaria*, and *Aplysiopsis formosa*) (Table 2). The mean total concentration of MAAs was over 20 nmol/mg in the egg masses

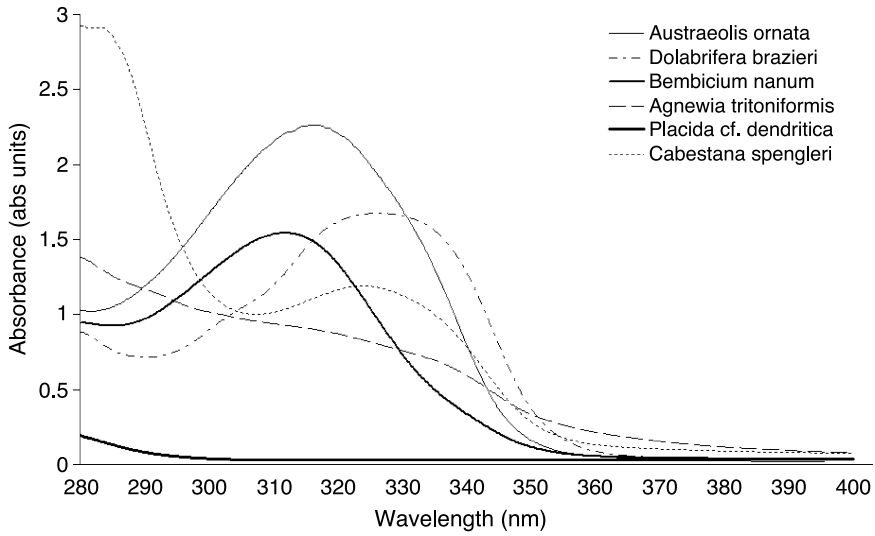

FIG. 1. UVR absorption of methanol extracts of egg masses from various species. All extracts are diluted 1:1 in 80% aqueous methanol except *Cabestana spengleri* and *Placida* cf. *dendritica*, which are undiluted.

TABLE 2. MAA CONCENTRATIONS OF VIABLE EGG MASSES USED IN THIS STUDY (MEAN ± SEM, NMOL/MG DRY WEIGHT)

Species	Myc-gly	Shinorine	Porphyra	Myc-2-gly	Palythene	Palythine	Asterina	Palythinol	Total
Phylum Mollusca: Class Gastropoda									
Superorder Neritopsina									
N. atramentosa	0.68 ± 0.02	0.21 ± 0.03	0.05 ± 0.05	0.01 ± 0.01	0.00	0.00	0.00	0.00	0.95 ± 0.14
Superorder Caenogastropoda									
B. nanum	4.41 ± 0.59	0.68 ± 0.08	0.90 ± 0.16	0.15 ± 0.05	0.02 ± 0.02	0.36 ± 0.29	0.03 ± 0.01	0.01 ± 0.01	6.57 ± 2.28
C. spenglerii	0.61 ± 0.18	0.31 ± 0.13	0.46 ± 0.11	0.13 ± 0.04	0.00	1.98 ± 0.20	0.10 ± 0.03	0.00	3.58 ± 0.90
Conuber sp.	0.24 ± 0.13	0.08 ± 0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.29 ± 0.63
C. erosa	2.20	0.77	0.32	0.07	0.00	0.06	0.00	0.00	3.41
R. australasia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A. tritoniformis[a]	2.78 ± 0.86	1.02 ± 0.13	0.44 ± 0.05	0.09 ± 0.03	0.00	0.43 ± 0.39	0.00	0.15 ± 0.08	4.91 ± 2.56
Bedeva sp.	1.91	0.47	0.10	0.00	0.00	0.48	0.09	0.08	3.13
C. eburnea	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C. papilliferus	0.16 ± 0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17 ± 0.03
D. orbita[a]	2.72 ± 0.61	1.48 ± 0.36	0.30 ± 0.07	0.12 ± 0.01	0.00	0.03 ± 0.03	0.00	0.00	4.65 ± 2.16
L. reticulata[*]	1.08 ± 0.36	0.33 ± 0.33	0.00	0.00	0.00	0.00	0.00	0.00	1.41 ± 0.97
M. marginalba	2.45 ± 0.85	3.51 ± 1.09	0.21 ± 0.01	0.00	0.00	0.18 ± 0.03	0.00	0.21 ± 0.04	6.56 ± 2.65
M. badia	0.00	0.00	0.00	0.00	0.00	1.05	0.00	0.00	1.05
M. carbonaria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
N. lapillus	2.96 ± 0.23	0.40 ± 0.17	0.12 ± 0.01	0.04 ± 0.04	0.00	0.78 ± 0.01	0.00	0.00	4.31 ± 0.48
Superorder Heterobranchia									
B. quoyii	1.10	2.89	1.00	0.18	0.00	1.35	0.16	0.00	6.68
B. lineata	1.20 ± 0.19	1.77 ± 0.21	0.41 ± 0.10	0.06 ± 0.01	0.00	1.45 ± 0.39	0.29 ± 0.26	0.00	5.17 ± 1.60
H. physis	0.78 ± 0.18	0.68 ± 0.11	0.20 ± 0.13	0.01 ± 0.00	0.00	0.91 ± 0.16	0.02 ± 0.01	0.00	2.60 ± 1.49
A. formosa	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
O. viridis	0.45 ± 0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.45 ± 0.21
P. cf. *dendritica*	0.30 ± 0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.30 ± 0.17
B. citrina	0.00	0.00	0.00	0.00	0.00	0.23	0.00	0.00	0.23

P. peronii	2.84 ± 2.12	0.21 ± 0.03	0.09 ± 0.03	0.11 ± 0.02	0.00	1.78 ± 1.54	0.46 ± 0.46	0.00	5.49 ± 5.94
Pleurobranchus sp.	0.64 ± 0.30	0.06 ± 0.06	0.03 ± 0.03	0.03 ± 0.03	0.00	0.28 ± 0.28	0.00	0.13 ± 0.13	1.15 ± 0.30
A. juliana	0.53 ± 0.18	0.72 ± 0.42	0.48 ± 0.31	0.09 ± 0.08	0.00	0.08 ± 0.07	0.04 ± 0.02	0.08 ± 0.05	2.02 ± 2.89
A. sydneyensis	2.43 ± 0.47	3.77 ± 0.73	1.38 ± 0.29	0.45 ± 0.17	0.28 ± 0.22	2.76 ± 0.66	0.03 ± 0.01	0.42 ± 0.11	11.50 ± 7.05
A. parvula	0.60 ± 0.01	1.41 ± 0.87	4.41 ± 1.45	0.14 ± 0.06	0.00	0.98 ± 0.73	0.12 ± 0.05	0.00	7.65 ± 4.44
B. leachii[a]	2.65 ± 0.66	1.76 ± 0.42	0.63 ± 0.11	0.55 ± 0.14	0.33 ± 0.09	0.63 ± 0.12	0.39 ± 0.12	0.00	6.94 ± 3.69
D. auricularia	0.62 ± 0.35	1.90 ± 0.18	1.71 ± 0.73	0.11 ± 0.06	1.39 ± 0.08	3.62 ± 0.01	0.00	0.95 ± 0.69	10.30 ± 2.72
D. brazieri[a]	2.19 ± 0.16	2.52 ± 0.21	3.91 ± 0.54	0.30 ± 0.05	1.19 ± 0.31	2.16 ± 0.35	0.57 ± 0.09	0.14 ± 0.10	12.97 ± 4.83
S. striatus	2.17 ± 0.44	2.27 ± 0.55	0.67 ± 0.12	0.47 ± 0.08	2.48 ± 0.50	2.95 ± 1.25	0.00	3.88 ± 0.89	16.78 ± 2.39
A. foulisi	0.06	0.02	0.05	0.00	0.00	0.06	0.00	0.08	0.28
A. ornata	2.15 ± 0.47	5.80 ± 1.93	1.87 ± 0.50	0.35 ± 0.11	0.00	9.67 ± 2.49	0.60 ± 0.20	0.12 ± 0.12	20.56 ± 11.52
D. carneola	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25
D. fumata	1.24 ± 0.31	0.67 ± 0.17	0.31 ± 0.07	0.05 ± 0.01	0.00	2.64 ± 0.76	0.00	0.26 ± 0.10	5.16 ± 3.62
D. miniata	0.17	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.56
D. nigra	0.29	0.06	0.03	0.01	0.00	0.17	0.00	0.00	0.25
G. meracula	0.00	0.10 ± 0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.10 ± .03
H. nodulosa[a]	0.55 ± 0.35	0.38 ± 0.24	0.29 ± 0.20	0.03 ± 0.03	0.00	0.70 ± 0.42	0.00	0.11 ± 0.04	2.06 ± 2.51
H. obscura[a]	0.54 ± 0.08	0.22 ± 0.08	0.10 ± 0.05	0.02 ± 0.01	0.00	0.53 ± 0.30	0.00	0.09 ± 0.05	1.50 ± 0.68
P. galbanus	3.07 ± 0.39	2.46 ± 0.92	0.96 ± 0.20	0.14 ± 0.04	0.00	6.08 ± 0.97	0.00	1.64 ± 0.49	14.35 ± 4.35
P. imperialis	4.72	4.78	1.53	0.29	0.00	7.06	0.50	0.00	18.89
R. arbutus[a]	0.00	0.19 ± 0.19	0.00	0.00	0.00	0.00	0.32 ± 0.32	0.00	0.51 ± 0.69
S. denticulata[a]	2.39 ± 0.45	1.73 ± 0.27	3.44 ± 0.89	0.02 ± 0.01	0.50 ± 0.32	1.79 ± 0.47	0.45 ± 0.17	0.00	10.32 ± 6.90
S. zelandica[a]	0.97 ± 0.21	1.04 ± 0.26	4.95 ± 1.38	0.01 ± 0.00	0.00	0.60 ± 0.26	0.05 ± 0.02	0.00	7.62 ± 4.76
Phylum Annelida: Class Polycheata									
Unkn polychaete 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unkn polychaete 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phylum Chordata: Class Osteicthyes									
A. costatus[a]	0.00	0.00	0.00	0.00	0.50 ± 0.03	0.00	0.00	0.00	0.50 ± 0.03

See Table 1 for sample sizes.

[a]An unknown peak was detected in at least one egg mass from this species.

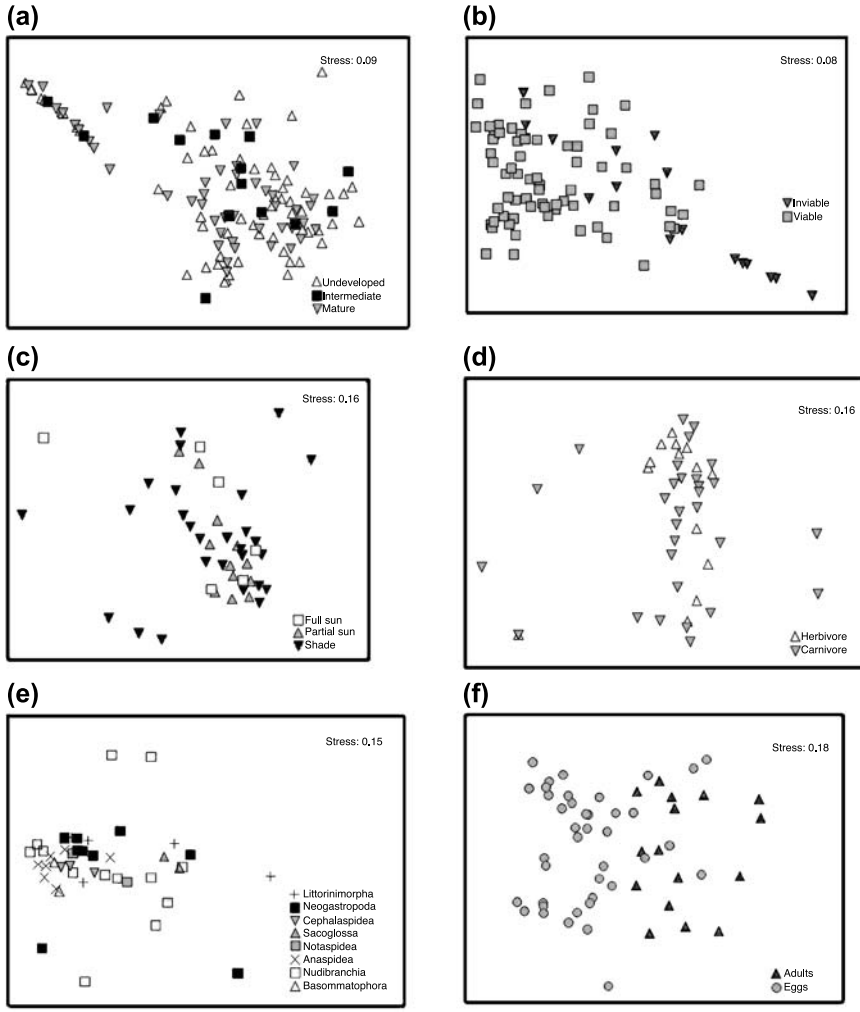

FIG. 2. Differences in the composition and relative abundance of MAAs in the egg masses of selected species as revealed by nMDS plots constrasting: (a) *Bembicium nanum*, *Siphonaria denticulata*, and *Siphonaria zelandica* adults and their egg masses; (b) effects of maturity on viable egg masses from 16 species; (c) effects of viability on egg masses from 10 species; (d) effects of spawning habitat on viable egg masses from 49 species (means shown); (e) effects adult diet on viable egg masses from 45 species with known diet (means shown); and (f) gastropod phylogeny on viable egg masses from 45 species (means shown).

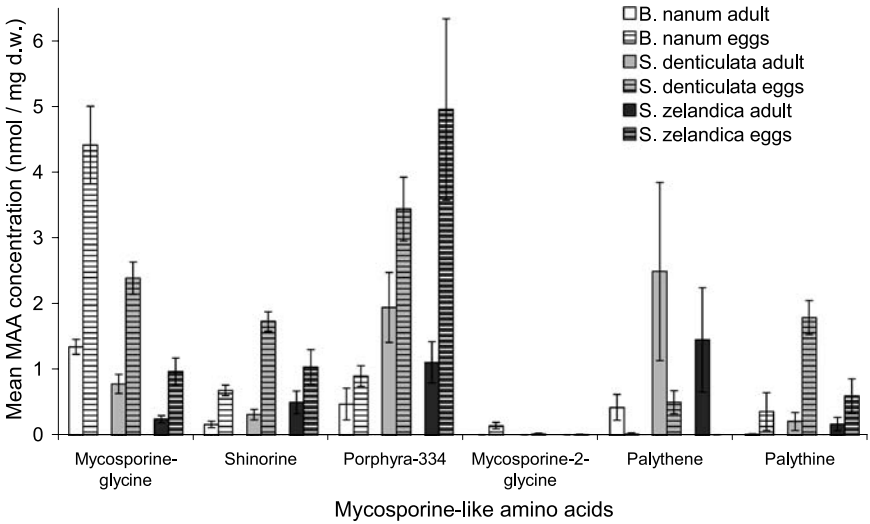

FIG. 3. MAA compositions of adults and egg masses of four species. Asterina-330 and palythinol are not included because only trace amounts were detected in these species (<0.05 nmol/mg), and no significant relationships were observed. Error bars are standard error of mean.

of *Austraeolis ornata* and was greater than 15 nmol/mg in the gelatinous masses from two other species of opisthobranch (*Stylocheilus striatus* and *Plocamopherus imperialis*). The other intertidal organisms examined showed a similar range in total MAA concentration to the mollusks, with the brown algae *Hormosira banksii* showing no detectable levels of MAAs, whereas low levels were found in the green algae *Ulva lactuca* (1.24 nmol/mg d.w.) and the sponge *Haliclona* sp. (2.18 nmol/mg d.w.). The anemone *Actinia tenebrosa* had moderately high levels of total MAAs (9.05 nmol/mg d.w.).

Eight of the nine MAAs analyzed in this study were detected in the egg masses examined. Overall, mycosporine glycine was the most common MAA, occurring in egg masses from 38 species (Table 2). Palythene was the least common, occurring in just eight species (Table 2). Palythine occurred at the highest concentration; one egg mass of the nudibranch *Austraeolis ornata* contained 16.833 nmol/mg palythine. Mycosporine–taurine was not found in any sample collected.

Unidentified HPLC peaks were detected in viable egg masses of the following species (approximate retention time in minutes, λ_{max}): *Agnewia tritoniformis*, *Lepsiella reticulata*, and *Dicathais orbita* (4.5, 307 nm); *Rostanga arbutus*, *Hoplodoris nodulosa*, and *Hypselodoris obscura* (6.3, 296 nm); *B.*

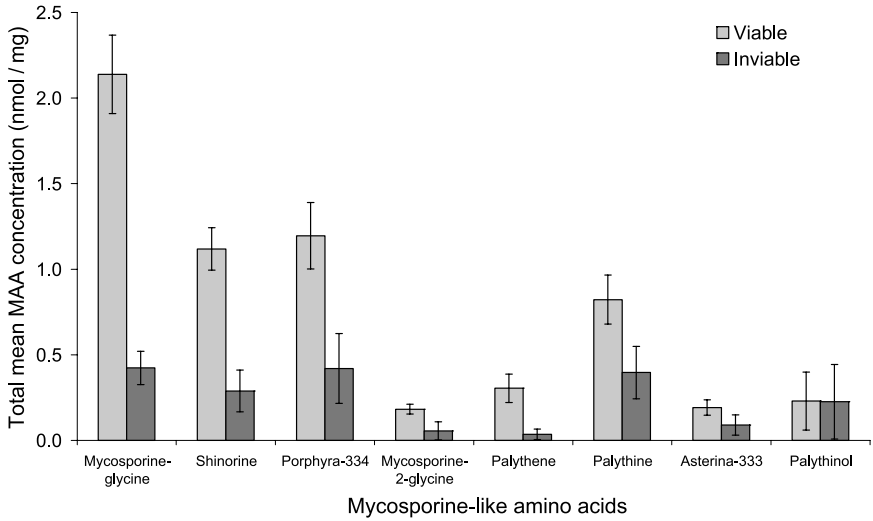

FIG. 4. MAA concentration in viable (N = 81) and inviable (N = 22) egg masses of *Bembicium nanum*, *Cabestana spengleri*, *Mayena australis*, *Cominella eburnea*, *Conus papiilliferus*, *Dicathais orbita*, *Lepsiella reticularis*, *Nucella lapillus*, *Aplysia juliana*, *Bursatella leachii*, *Dolabella auricularia*, and *Dolabrifera brazieri*. Error bars are standard error of mean.

leachii (12.2, 346 nm); *Siphonaria denticulata* (7.1, 334 nm), *Siphonaria zelandica* (2.9, 319 nm); and *A. costatus* (4.2, 325 nm). These peaks did not match with any of the MAA standards used and, therefore, could not be positively identified or quantified for analysis.

Comparison with Adults. An nMDS plot of viable egg masses and adults of species for which we had replicates revealed distinct clusters (Figure 2a), suggesting that MAA composition of egg masses was different from that of adults. ANOSIMs on each species confirmed differences between egg masses and adults of *B.nanum* (R = 0.556, P = 0.02), *S. denticulata* (R = 0.343, P = 0.016), and *S. zelandica* (R = 0.504, P = 0.019). The egg masses of these species contained higher concentrations of mycosporine–glycine (F = 23.5012, $P < 0.001$), shinorine (F = 21.214, P = 0.04), mycosporine-2-glycine (F = 11.6937, P = 0.001), and palythine (F = 8.385, P = 0.006) than the adults as revealed by a two-factor ANOVA (Figure 3). In contrast, adults had more palythene than egg masses (F = 40.873, P <0.001). An interaction between species and life stage was detected for palythinol concentrations (F = 51.336, $P < 0.001$), and Tukey's HSD tests revealed that *B. nanum* adults had more palythinol than the egg masses (Figure 3).

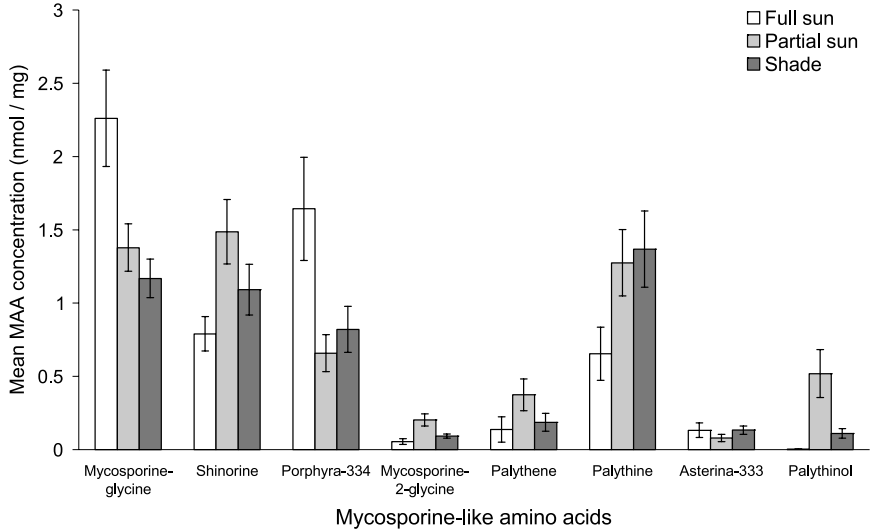

FIG. 5. Effects of spawning habitats on MAA concentration in all viable egg masses collected in this study. Habitats varied in spectral exposure and encompassed full sun (n = 54), partial sun (N = 76), and shade (N = 107). Error bars are standard error of mean.

Egg Mass Maturity and Viability. MAA composition did not vary as egg masses matured for all 16 species of mollusks tested. There was no apparent difference in MAA content between undeveloped, intermediate, and mature egg masses as revealed by an nMDS plot (Figure 2b) and a nested ANOSIM on data subsets including species where $N \geq 3$ (R = 0.079, P = 0.072) (refer to Table 1 for species tested). Thus, viable egg masses at all stages of development were pooled for each species in remaining analyses.

An nMDS ordination comparing MAA composition in viable and inviable egg masses of 10 species in which MAAs were present revealed minimal segregation of the samples (Figure 2c; see Table 1 for species tested). ANOSIMs were conducted separately on the five species where $N \geq 3$ for both viable and inviable egg masses, and these revealed species-specific effects. MAA concentration was significantly different between viable and inviable egg masses for two species: *B. nanum* (R = 0.927, P = 0.001) and *L. reticulata* (R = 0.274, P = 0.001). Similar trends were seen for *D. orbita* (R = 0.914, P = 0.06) and *D. brazieri* (R = 0.393, P = 0.054), although they were not significant at α = 0.05. No apparent differences were observed for *C. papilliferus* (R = 0.074, P = 0.274). Two-factor ANOVAs on the transformed data for these species revealed significant interactions between viability and species on concentrations of mycosporine–glycine (F = 10.03, $P < 0.001$), shinorine (F = 5.470, P = 0.001),

porphyra-334 ($F = 5$, $P < 0.001$), mycosporine-2-glycine ($F = 5.8418$, $P < 0.001$), and palythene ($F = 3.907$, $P = 0.007$). Tukey's HSD tests revealed that viable egg masses of *B. nanum* and *D. orbita* had significantly more mycosporine–glycine, shinorine, porphyra-334, and mycosporine-2-glycine than inviable egg masses; and viable *D. brazieri* egg masses had significantly more mycosporine-2-glycine and palythene than inviable egg masses (Figure 4).

Spawning Habitat and Adult Diet. An nMDS plot of MAA composition revealed no clear separation between egg masses deposited in full sun habitats compared to those deposited in shaded habitats (Figure 2d). Similarly, a nested ANOSIM failed to detect effects based on spawning habitat ($R = -0.048$, $P = 1$). Overall, the concentrations of mycosporine–glycine and porphyra-334 were highest in egg masses from species spawning in full sun, whereas palythine, palythinol, and shinorine concentrations were lowest in egg masses from these species (Figure 5). However, nested ANOVAs on the concentration of each MAA supported the multivariate results, showing no significant differences between spawning habitats.

A multivariate analysis of MAA composition in egg masses showed no overall effect of adult diet. The nMDS plot reveals no distinct separation of samples according to diet (Figure 2e), and a nested ANOSIM confirmed no significant differences between herbivores and carnivores ($R = -0.285$, $P = 1$). However, nested ANOVAs on individual MAAs did reveal some significant differences

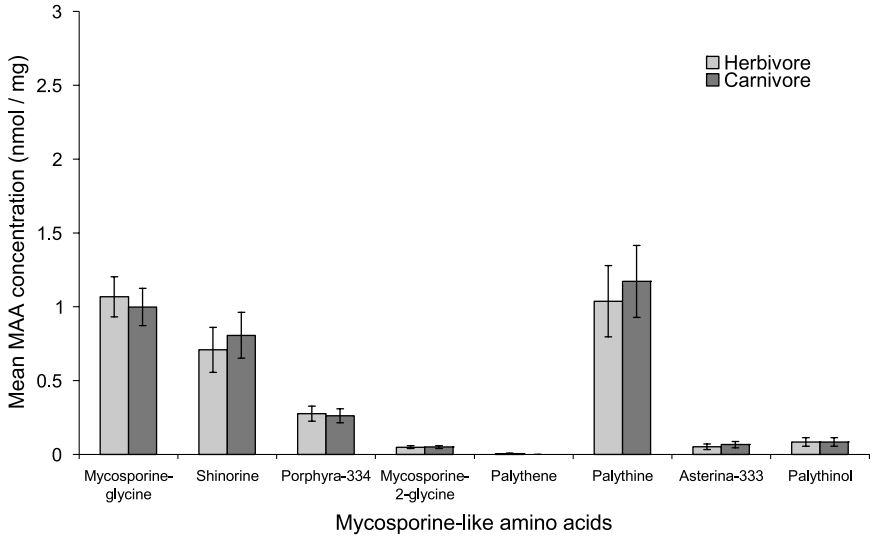

FIG. 6. Effects of adult diet on MAA concentration for all viable egg masses in which adult diet is known to be either herbivore ($N = 114$) or carnivore ($N = 112$) (refer to Table 1). Error bars are standard error of mean.

according to diet. Herbivores had significantly higher levels of porphyra-334 ($F = 5.24$, $P = 0.027$) and palythene ($F = 11.95$, $P = 0.001$), with no palythene recorded in any egg masses from carnivores (Figure 6). Although not statistically significant, similar trends were also detected for mycosporine–glycine ($F = 3.860$, $P = 0.056$) (Figure 6).

Gastropod Phylogeny. The taxonomic grouping (order) of gastropods correlated with MAA content in egg masses. An nMDS plot showed some taxonomic clusters, particularly within anaspids (Figure 2f). Considerable variation was observed within many orders (e.g., Nudibranchia and Neogastropoda), as evidenced by the large spread of points on the nMDS (Figure 2f) and relatively large error bars for individual MAA concentrations (Figure 7). A nested ANOSIM on all orders with two or more species indicated less variation within than between taxonomic groups in their MAA composition ($R = 0.082$, $P = 0.098$). Pairwise tests showed that the MAA content of anaspid egg masses was different from that of sacoglossans ($R = 0.818$, $P = 0.048$), and possibly neogastropods ($R = 0.248$, $P = 0.063$). The MAA composition of nudibranch egg masses appeared different from that of neogastropods ($R = 0.244$, $P = 0.057$) and sacoglossans ($R = 0.396$, $P = 0.056$). Although not significant

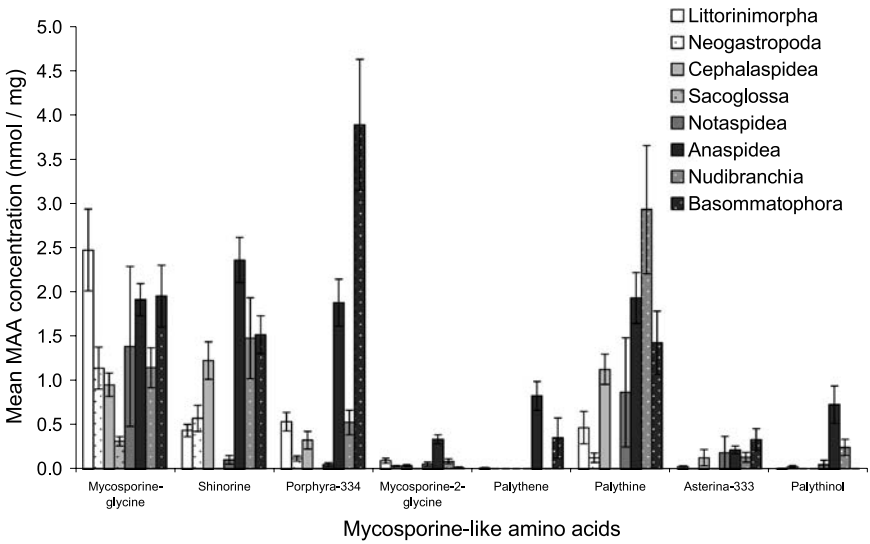

FIG. 7. Effects of gastropod order on MAA concentration in viable egg masses collected in this study: Littorinimorpha ($N = 4$), Neogastropoda ($N = 10$), Cephalaspidea ($N = 3$), Sacoglossa ($N = 3$), Notaspidea ($N = 3$), Anaspidea ($N = 7$), Nudibranchia ($N = 12$), and Basommatophora ($N = 2$), where n indicates number of species. Error bars are standard error of mean.

at $\alpha = 0.05$, nonsignificant results here should be interpreted cautiously because of the large intra- and interspecific variation in MAA complements (Figure 2f).

Nested ANOVAs of individual compounds confirmed significant phylogenetic differences in the quantity of shinorine ($F = 3.910$, $P < 0.001$), porphyra-334 ($F = 3.302$, $P < 0.001$), mycosporine-2-glycine ($F = 3.302$, $P < 0.001$), palythene ($F = 5.415$, $P < 0.001$), and palythine ($F = 2.956$, $P < 0.001$). Sacoglossans had significantly less shinorine than anaspids, cephalaspids, nudibranchs, and basommatophorans; and similarly, they had less palythine than anaspids (Figure 6). Anaspids had significantly more porphyra-334 and mycosporine-2-glycine than sacoglossans and neogastropods; and they had more palythene than all other orders except for the basommatophorans (Figure 7). Basommataphorans had significantly more porphyra-344 than sacoglossans (Figure 7).

DISCUSSION

This study is the first quantitative survey of mycosporine-like amino acid composition in eggs or extraembryonic structures. It confirms that MAAs are prevalent in the intertidal egg masses of many gastropods on temperate rocky shores. Unfortunately, it is difficult to directly compare MAA concentration of egg masses used here with organisms from other studies because of the persistent use of several different measures of concentration across the literature (Karentz, 2001). However, those studies that reported MAA concentration in the units used here (nmol/mg) show that nonsymbiotic animals have similar ranges of MAA concentrations to those exhibited in this study (McClintock and Karentz, 1997; Banaszak et al., 1998). For example, larvae of the urchin *Strongylocentrotus droebachiensis* contain 4–8 nmol/mg total MAAs (Adams and Shick, 2001), which represents the middle range of the concentrations we detected (Table 2). Indeed, direct comparisons with other intertidal organisms in the present study such as *Hormosira banksii*, *Ulva lactuca*, and *Haliclona* sp. confirm that MAA concentrations in egg masses of some species are relatively high.

Egg masses of *B. nanum*, *S. denticulata*, and *S. zelandica* are laid in habitats where they are directly exposed to UVR. They contained more MAAs than the whole adults examined (Figure 3), which is consistent with the suggestion that MAAs have a photoprotective role in these species. Other intertidal invertebrates have been found to sequester MAAs in spawn to minimize UV-induced damage to offspring in a potentially hostile environment. This trend has been recorded in the sea urchin *Strongylocentrotus droebachiensis* (Adams et al., 2001) and the sea hare *Aplysia dactylomela* (Carefoot et al., 1998; Carefoot et al., 2000). It remains unknown if species that spawn exclusively in shade sequester MAAs in eggs.

The relatively high concentrations of MAAs in some egg masses suggest that embryos of certain species are protected from the damaging effects of UVR. This is consistent with previous research in which embryos of species that consistently spawn in habitats exposed to full sun were more resistant to the damaging effects of UVR than embryos of species that only spawn in shaded habitats (Przeslawski et al., 2004). However, contrary to our prediction, we have found no clear pattern in MAA composition relating to spawning habitat (Figures 2d and 5). Furthermore, a direct comparison of total MAA concentration in egg masses of species used in both the present study and in a previous one by Przeslawski et al. (2004) revealed no significant correlation between MAA concentration and the difference in embryonic mortality between full spectrum and UV-blocked treatments ($R = 0.004$, $P = 0.795$). This suggests that MAAs are not the sole source of protection afforded to encapsulated intertidal embryos.

There are many other ways for marine organisms to mitigate the deleterious effects of UVR (Bandaranayake, 1998). Antioxidants may play an important role in minimizing UVR damage in marine organisms (reviewed by Dunlap et al., 1999). Indeed, the most common MAA observed in this study, mycosporine–glycine, is also a moderate antioxidant (Dunlap and Yamamoto, 1995). The potential importance of this compound is supported by comparisons with adults and inviable egg masses. Eggs had significantly higher concentrations of mycosporine–glycine than adults (Figure 3); and viable egg masses had higher concentrations than inviable egg masses (Figure 4). Furthermore, egg masses from species spawning in full sun contained the highest levels of mycosporine–glycine relative to species that spawned in partial or full shade (Figure 3). Mycosporine–glycine is the only MAA found in this study that absorbs maximally at 310 nm (Figure 1), the range of UVR (UVB) that is most biologically damaging (Paul and Gwynn-Jones, 2003). Thus, embryos encapsulated in environments exposed to UVR may use mycosporine–glycine in a dual protective role as a UV-B sunscreen and an antioxidant. Egg masses routinely deposited in habitats exposed to full sun may also possess other protection against damage caused by UVR. Other metabolites such as carotenoids may provide photoprotective antioxidant functions; such compounds have already been found in holothurian eggs (Bandaranayake, 1998) and warrant further investigation. In addition, high levels of the DNA repair enzyme photolyase have been found in several adult mollusks, including *B. leachii* (Carlini and Regan, 1995), a species used in this study. The capability of encapsulated intertidal embryos to repair UVR-induced DNA damage is currently unknown.

MAA composition in gastropod egg masses shows enormous phylogenetic variation (Figure 7), and phylogeny may well overwhelm the influence of all other factors examined in this study. Even unknown compounds detected in this study elicited phylogenetic patterns. For example, the same unknown peak was seen only in egg masses of three species of neogastropods; and a different

unknown peak, possibly deoxygadusol (see Shick and Dunlap, 2002), was found only in three species of nudibranchs. Previous research on sea anemones has similarly revealed that differences in MAA concentration primarily reflect phylogeny rather than environmental factors (Shick et al., 2002). Alternatively, phylogeny may be related to an ecological factor not considered in the present study; and indeed, variation based on phylogenetic and ecological factors is not often mutually exclusive (Westoby et al., 1995b).

Phylogeny and diet can often be confounded, but the effects of phylogeny are unlikely to be influenced by diet in the present survey. In general, egg masses from herbivores had higher levels of some MAAs than those from carnivores (Figure 6); but when analyzed according to order, egg masses of carnivorous nudibranchs had significantly more MAAs than egg masses from most herbivorous orders (Figure 7). Nevertheless, MAAs in eggs or larvae are likely dependent on adult diet, and studies on single species of mollusks and echinoderms reveal that eggs have higher MAA content if they are deposited by adults that consumed food rich in MAAs compared to adults that ate food containing few or no MAAs (Adams et al., 2001; Carefoot et al., 1998, 2000). The present study indicates that this diet dependence may extend to differences in MAAs between trophic levels (Figure 6).

Nevertheless, analysis of MAA composition based on adult diet was not as definitive as previous analyses of total MAA concentration in which herbivores unilaterally showed higher concentrations of MAAs than carnivores (Przeslawski, 2004b). In the present study, we found minimal differences in MAA composition between trophic levels (Figure 2e) and significant differences in only two of the eight MAAs detected (Figure 6). These results underscore the value of MAA composition analysis compared to the potentially overly simplistic interpretations arising from univariate analysis of total MAA concentration. For example, in the previous univariate analyses, it was suggested that herbivores have more direct links to sources of MAAs, and bioaccumulation does not occur in a broad range of species (Przeslawski, 2004b). However, the potential impact of MAA bioaccumulation cannot be ignored and has previously been recorded in a trophic chain including phytoplankton, herbivorous pteropods, and carnivorous pteropods (Whitehead et al., 2001). Indeed, the high concentrations of some MAAs in certain nudibranch egg masses are consistent with MAA bioaccumulation from their prey and associated zooxanthellae (Table 2). Furthermore, analyses of total MAA concentration cannot account for the different strategies employed by organisms based on MAA composition. Some species, such as *Siphonaria denticulata* used in this study, may incorporate several compounds at low or moderate concentrations (Table 2). Other species, such as *Bembicium nanum*, may use higher concentrations of a single MAA to confer protection (Table 2).

Viable egg masses had a richer complement of MAAs and had higher concentrations of individual compounds than inviable egg masses (Figure 4).

These results support previous univariate analysis of total MAA concentration in these egg masses (Przeslawski, 2004b). No previous study has investigated MAA composition in inviable eggs, and the mechanisms behind the relationship here are unclear. It is unknown if lower MAA concentration in these species resulted in the lack of viable embryos or if it was a consequence of inviability. The egg masses in this study were collected *in situ* after spawning so the history of the spawning adult and egg mass prior to collection was not known.

MAA concentration of egg masses varied tremendously within species (Table 2, Figure 1). Indeed, previous research has shown that unique MAA compositions can be used to identify clones within a species of coral (Diamond, 1986). The intraspecific variation in the present study may have limited the detection of significant differences according to habitat, diet, and taxonomic order. Notably, however, the present survey has generally incorporated more replicates than most other surveys of MAAs. Indeed, previous surveys have reported MAA concentration based on single samples for each organism (e.g., Karentz et al., 1991; McClintock and Karentz, 1997) or a low number of replicates (e.g., Bosch et al., 1994; Büdel et al., 1997; Teai et al., 1997; Xiong et al., 1999). This lack of replication may yield biased estimates of MAA concentrations; and results should be treated with caution, particularly in cases where MAA composition shows high intraspecific variation.

Although the magnitude of their effectiveness as sunscreens for organisms remains unclear, MAAs are likely important to developing encapsulated embryos. MAAs may play a role in osmotic regulation and developmental regulation (reviewed by Shick and Dunlap, 2002), and such functions would certainly be vital to encapsulated embryos developing in the intertidal. Unfortunately, there is a paucity of empirical research on alternate functions of MAAs to photoprotection in marine invertebrates. In contrast, there is a wealth of evidence that MAAs minimize UV-induced abnormality and mortality (reviewed by Karentz, 2001). Previous research shows MAA concentration is logarithmically correlated to developmental success in sea urchin embryos because of reduction in UVR exposure (Adams and Shick, 1996). No similar studies have been conducted on embryos of the species used in this study, but MAAs may indeed confer protection to embryos from species that consistently spawn in full sun habitats. However, MAA composition does not seem to be simply based on exposure to UVR, and instead likely represents complex and potentially confounding influences from both phylogenetic and ecological factors.

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APPLICATION OF THE ELECTRONIC NOSE TO THE CLASSIFICATION OF RESISTANCE TO WESTERN FLOWER THRIPS IN CHRYSANTHEMUMS†

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Abstract—A metal oxide sensor-based electronic nose was tested for its ability to discriminate among chrysanthemum cultivars with varying degrees of resistance to western flower thrips (WFT), based on volatile chemicals released from cut leaves. Cultivars that were susceptible, intermediate, or resistant to WFT [based on mean cultivar rank (MCR)] were used as standards, and were correctly classified (> 90%) by using discriminant function analysis. Several cultivars with unknown resistance were classified based on the standards, and were used as standards in a subsequent trial to classify other unknowns. The results of this study demonstrate some agreement between the WFT resistance categories as designated by the electronic nose and results of feeding bioassays (MCR), suggesting that this technique may serve as a useful screening tool for WFT resistance.

Key Words—Chrysanthemum, western flower thrips, electronic nose, resistance.

INTRODUCTION

Thrips are destructive to ornamental plants because of the physical damage caused by their feeding on tissues of developing and mature flowers and leaves. In addition, thrips can vector diseases such as tomato spotted wilt virus, which

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affects a wide range of plants (Broadbent et al., 1990). The western flower thrips (WFT), *Frankliniella occidentalis* (Pergande), has been the major insect pest of greenhouse floriculture in Canada since the late 1980s (Broadbent et al., 1987). Thrips adults and larvae are thigmotactic, preferring to hide in complex plant parts, flower buds, and other folded tissues, where they are difficult to detect and to reach with insecticides. Control of WFT has become even more difficult because of resistance to conventional insecticides (Broadbent and Pree, 1997; Immaraju et al., 1992). Alternate means of controlling thrips are clearly required.

Host-plant resistance to WFT, particularly in chrysanthemum, is recognized as having great potential to reduce reliance on pesticides for control of this pest (Van Dijken, 1992; De Jager et al., 1993). A study of chrysanthemum cultivars revealed wide variation in their degree of resistance or susceptibility to WFT (Broadbent et al., 1990). Variation in pollen production (De Jager and Butôt, 1993) and difference in color of blooms (Van Dijken et al., 1993) were found to have little impact on this interaction. Secondary metabolites of chrysanthemum have been suggested to be the primary factor in resistance (De Jager et al., 1995, 1996). Fung et al. (1999) also suggested a role for secondary metabolites in the chrysanthemum–thrips interaction. They concluded that primary metabolites such as amino acids did not contribute to the activity of chrysanthemum leaf sap against WFT.

The electronic nose (EN) is an analytical instrument designed to mimic the human nose (Gardner and Bartlett, 1994; Mielle, 1996; Bartlett et al., 1997; Dickinson et al., 1998). There are a number of different technologies available, but generally, an EN consists of a semiselective sensor array that reacts with volatile chemicals in the sample headspace. In our laboratory, we have used a commercial EN (Fox 3000, AlphaMOS, Toulouse, France), which consists of 12 metal oxide sensors, each of which has a particular specificity for a class of compounds, such as hydrocarbons, aldehydes, amines, or aromatics, depending on the type and amount of semiconducting film that has been applied (Table 1). As headspace gases from a sample are passed over the sensors, the reaction between oxygen, the film, and volatile molecules results in a change in conductivity of the sensor through oxidation (increases conductance) or reduction (decreases conductance) reactions to form a pattern (Mielle, 1996). The patterns are then processed and analyzed using advanced pattern recognition techniques such as principal component analysis (PCA) or discriminant function analysis (DFA). A database of known samples can then be created, and the EN can then be used to identify unknown samples by comparison with the database with a high degree of accuracy (AlphaMOS, 1997). We have used the Fox 3000 EN in a number of applications including the development of orange juice flavor (Farnworth et al., 2002), the detection of spoilage in packaged minimally processed lettuce (Odumeru et al., 2003), and the optimization of beer aging (McKellar et al., 2002).

TABLE 1. LIST OF SENSORS IN THE FOX 3000 ELECTRONIC NOSE

Sensors	Description	Specificity
SY/LG	Fluoride/chlorine	Fluoride/chlorine
SY/gCT	Nonpolar volatiles	Methane, propane
SY/Gh	Aromatic	Alcohol and aromatic
SY/G	Ammonia/sulfur	Amines/ammonia
SY/AA	Nonpolar volatiles	Hydrocarbon, methane, propane
P10/2	Nonpolar volatiles	
SY/gCTl	Organic solvents	Polar, ethanol, alcohol
P10/1	Nonpolar volatiles	
PA2	Organic solvents	
T30/1	Organic solvents	
P40/1	Fluoride/chlorine	
T70/2	Food aroma	Alcohol, volatiles

It is reasonable to hypothesize that thrips resistance might be related to volatile chemicals released by leaves. Thus, the purpose of the present study was to determine whether the EN could discriminate between resistance categories of chrysanthemums using samples of cut leaves.

METHODS AND MATERIALS

Chrysanthemum Plants. Rooted cuttings of all chrysanthemum cultivars were provided by Yoder Canada (Leamington, Ontario, Canada) and potted and grown in the greenhouse at the Southern Crop Protection and Food Research Centre of Agriculture and Agri-Food Canada (AAFC) in Vineland Station, Ontario. Mature (6 wk old) plants were shipped to the Food Research Program (Guelph, Ontario) and were watered and maintained in the lab while being analyzed by the electronic nose.

Two trials were conducted, with 11 cultivars tested in trial 1 and 13 in trial 2. In trial 1, five samples were taken from each of two replicate plants, whereas for trial 2, three samples were taken from each of three replicate plants. The exception was Icecap in trial 2, which was represented by two sets of three plants ($N = 18$ in total).

Operation of the Electronic Nose. The first pair of fully opened leaves from the top of each replicate plant was taken for sampling. Five leaf disks (#6 punch; 1-cm diam) were taken from the apex of each lobe of a leaf. If the leaf was too small to allow five samples, the other paired leaf was also used. The leaf disks were placed individually into 10-ml autosampler vials (Fisher Scientific) and capped with silicon/Teflon autosampler vial caps (London Scientific). Each vial was loaded into an autosampler (HS50, CTC Analytics, Switzerland) and heated

at 35°C for 5 min. Headspace (2500 µl) was removed from each vial by the autosampler and injected into the electronic nose with a 10-min delay between samples.

Data Analysis. Raw output from each of the 12 sensors was analyzed by DFA using UNISTAT® Version 5.5 (UNISTAT Limited, London, UK), a Microsoft® Excel add-in. DFA typically is used to determine which continuous variables (here the sensor outputs) discriminate between two or more naturally occurring groups. DFA provides orthogonal (independent) linear discriminant functions such that the first one provides the most overall discrimination among groups, the second provides the second most, and so on. In practice, two discriminant functions (here designated LD1 and LD2) are generally sufficient to describe most of the variation. The intention is to minimize the within-group differences and maximize the between-group differences. The common practice is to use discriminant functions to predict group membership of samples that were not used in the development of the original model. In the absence of such data, cross-validation can be performed. In this approach, each sample is removed sequentially from the model, and the model reformulated without that sample. The classification of the removed sample is then tested against the new model. Both unknown samples and cross-validations are presented in this study.

RESULTS

Preliminary results comparing different leaves on the same plant suggested that discrimination between susceptible, medium, and resistant cultivars was more effective using the first leaf, or pair of leaves, from the top of the plants (data not shown). This leaf was used in all further experiments.

The cultivars tested in trial 1 are listed in Table 2. Six (two each of susceptible, medium, and resistant) of the 11 cultivars were selected as standards, based on mean cultivar rank (MCR). The MCR is a ranking system used to compare thrips resistance based on the amount of feeding damage on leaves (Broadbent et al., 1990). A particular classification was deemed correct when the majority of the replicates were correctly classified. When the classification determined by one or more of the replicate plants differed from the results when the plant replicates were pooled, the pooled classification was taken as correct.

Overall, the six standards were correctly classified by cross-validation, with only a few incorrect classifications (Table 2). The percent correct for the individual plants and combined data were all > 90%; however, a lower percent correct was obtained when the data were pooled. Of the unknowns, Dark Bronze Charm was consistently classified as medium in resistance. Snowdon was deemed susceptible, although for plant A, more of the replicates were classified as either

TABLE 2. CLASSIFICATION OF CHRYSANTHEMUM CULTIVARS BY THRIPS RESISTANCE (TRIAL 1)

Cultivar		Combined	Plant A	Plant B
Percent correct		91.7	96.7	100.0
N	Initial assignment[a]	10	5	5
Amber	S	S[b]	S	S
Dark Bronze Charm	U	M (1S)	M (1S)	M (1S)
Florida Marble	S	S	S	S
Palisade	M	M (1R)	M	M
Polaris	M	M (3S)	M	M
Snowden	U	S (3R)	1S-2M-2R	S
Super White	R	R (1S)	R (1S)	R
Super Yellow	R	R	R	R
Vero	U	S (3R)	R (1S)	S (1R)
Yellow Favor	U	R (3M)	S (1R)	R (2M)
Yellow Vero	U	S (4R)	R (2S)	S (1R)

[a] Thrips resistance based on mean cultivar rank (Broadbent et al., 1990). S, susceptible; M, medium; R, resistant; U, unknown.
[b] First capital letter indicates the majority classification of the replicates. Numbers and classifications in parenthesis are those replicates that were misclassified by cross-validation.

medium or resistant. Of the other unknowns, Vero and Yellow Vero were susceptible, and Yellow Favor was resistant; however, this classification was reversed for plant A (Table 2).

Figure 1 shows the scatter diagram for the three resistance categories using pooled data from trial 1. Standards of known resistance from Table 2 were plotted as open symbols, with the closed symbols representing the unknowns (Figure 1a). The axes labels represent the first and second linear discriminant, with the percent variation explained in parentheses. Scatter diagrams are often difficult to interpret; thus, the centroid for each category was plotted with standard deviation bars in the *x*- and *y*-direction (Figure 1b). Unknown samples are adjacent to the standards of the same resistance category, with greater variation found with the unknowns as compared to the standards.

The ability of the electronic nose to distinguish among the individual chrysanthemum cultivars was assessed by initially assigning all replicate samples to the known cultivar and testing the prediction by cross-validation. When the data from all plants were pooled, > 80% of the samples were correctly classified into cultivar (Table 3). Higher correct classifications were found when individual plants were analyzed separately. Table 3 also shows that, for the pooled and plant B data, incorrectly classified samples were classified as another cultivar in the same resistance category in 76 and 62.5% of cases, respectively. This suggests

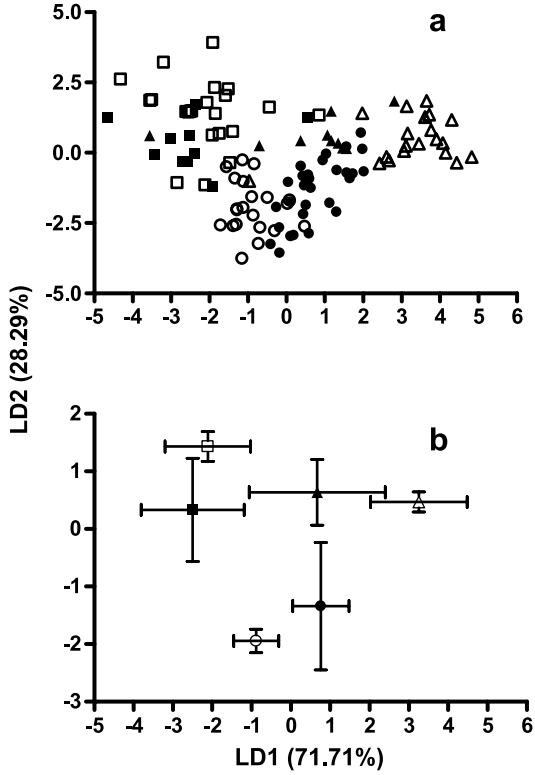

FIG. 1. Scatter diagram (a) and error (SD) bar plot (b) for western flower thrips resistance categories of chrysanthemums from trial 1 (pooled plants A and B). Standards of known resistance (open symbols) were used to classify unknowns (closed symbols) into susceptible (○), intermediate (□), or resistant (△). Values on axes are the percent variation explained by that linear discriminant.

that cultivar misclassification may be due, in part, to similarities between cultivars with respect to thrips resistance.

A second trial was performed in which 13 different cultivars were examined. One of these, Icecap, was represented by two sets of three plants, and the data for this cultivar were pooled. Susceptible standards were Snowdon and Vero, unknowns from trial 1. The medium standards (Polaris and Palisade) were the same as those from trial 1. The two resistant standards from trial 1 (Super White and Super Yellow) were also used as standards in this trial, as was the other resistant cultivar (Yellow Favor), unknown in trial 1.

Classification of the standards using the pooled data sets for the three plant replicates was >88% by cross-validation (Table 4). Up to 100% correct

TABLE 3. CLASSIFICATION OF CHRYSANTHEMUM CULTIVARS INTO VARIETIES (TRIAL 1)

	All plants	Plant A	Plant B
Number of replicates analyzed	110	55	55
% Correct	81.3	94.6	86.0
Number incorrect	21	3	8
% Incorrect	18.75	5.26	14.04
% Incorrect from same category	76.19	33.33	62.5

classification was obtained with plant replicates A and C. Classification of unknowns appeared to be less successful in this trial. Chardonnay was correctly classified as resistant; however, classification of the other unknowns was less clear. Fiery Barbara and May Shoesmith were designated resistant, although they were susceptible in two of the three plant replicates. Red Delano was designated susceptible, although it was resistant and medium in one each of the three plant replicates. Classification of Icecap was difficult; whereas it was susceptible in the combined data set, there was no clear classification in any of the plant replicates.

TABLE 4. CLASSIFICATION OF CHRYSANTHEMUM CULTIVARS BY THRIPS RESISTANCE (TRIAL 2)

Cultivar		Combined	Plant A	Plant B	Plant C
Percent correct	Initial	88.9	100.0	90.5	100.0
N[c]	assignment[a]	9	3	3	3
Chardonnay	U	R (3S)[b]	R (1S)	R (1S)	S
Fiery Barbara	U	R (1S-1M)	S (1M)	S (1R)	R (1S)
Icecap	U	S (3M-6R)	S-R	3M-2S-1R	S-M
May Shoesmith	U	R (3S)	S	S (1R)	R (1S)
Palisade	M	M	M	M	M
Polaris	M	M (1S)	M	M	M
Red Delano	U	S (3M)	R (1M)	S (1M)	M
Snowden	S	S (3R)	S	S (1R)	S
Super White	R	R	R	R	R
Super Yellow	R	R (3S)	R	R (1S)	R
Vero	S	S	S	S	S
Yellow Favor	R	R	R	R	R
Yellow Reagan	U	M (1S)	R (1M)	M	M (1S)

[a] Thrips resistance based on mean cultivar rank (Broadbent et al., 1990). S, susceptible; M, medium; R, resistant; U, unknown.

[b] First capital letter indicates the majority classification of the replicates. Numbers and classifications in parenthesis are those replicates that were misclassified by cross-validation.

[c] $N = 18$ for Icecap.

The difficulties experienced in classification of unknowns are reflected in the scatter diagrams for plant C, as an example (Figure 2). Whereas the standards showed little variation, large error bars were found with the unknowns.

Variation among cultivars appeared to be related to factors other than thrips resistance, as is indicated in Table 5. Classification into cultivars was poor for the combined trial 2 data set, with only 59% correct. Of the 55 incorrectly classified samples, only one third were from the same resistance category, no improvement over random assignment. Two of three misclassified plant A samples and one of two misclassified plant C samples were correctly placed in the same resistance category.

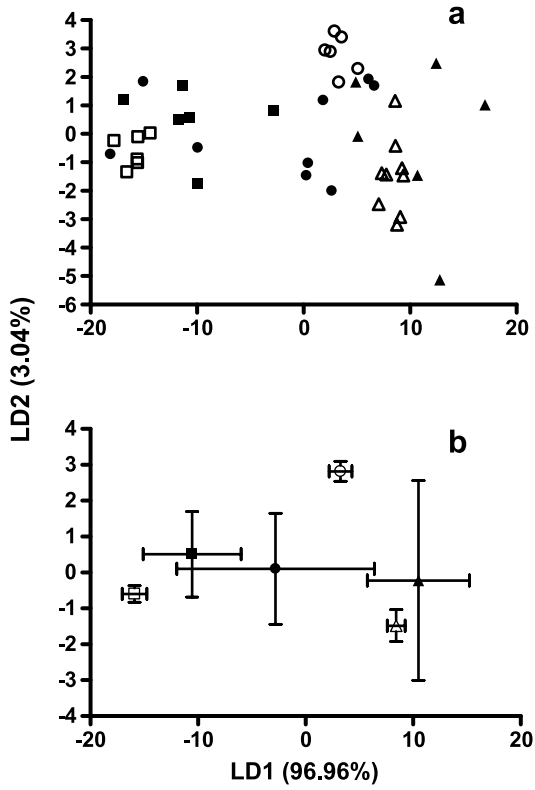

FIG. 2. Scatter diagram (a) and error (SD) bar plot (b) for western flower thrips resistance categories of chrysanthemums in trial 2 (plant C). Standards of known resistance (open symbols) were used to classify unknowns (closed symbols) into susceptible (○), intermediate (□), or resistant (△). Values on axes are the percent variation explained by that linear discriminant.

TABLE 5. CLASSIFICATION OF CHRYSANTHEMUM CULTIVARS INTO VARIETIES (TRIAL 2)

	All Plants	Plant A	Plant B	Plant C
Number of replicates analyzed	126	42	42	42
% Correct	58.7	98.3	100.0	95.2
Number incorrect	55	3	0	2
% Incorrect	43.65	7.14	0	4.76
% Incorrect from same category	34.55	66.67	0	50

Table 6 gives a summary of the results of the two trials, with the associated MCR values for cultivars for which they are available. Three of the cultivars in trial 2, which were treated as unknowns (Icecap, Chardonnay, and May Shoesmith), had been classified using the MCR system (Broadbent et al., 1990). Icecap was classified by the EN as susceptible, and the corresponding MCR was relatively high compared to the values for known medium resistant (Table 6). Chardonnay, designated resistant, had a MCR below 10, consistent with other resistant cultivars. In contrast, May Shoesmith had a MCR of 15.6, putting it in the medium region; however, the EN classified this cultivar as resistant.

TABLE 6. SUMMARY CLASSIFICATION OF CHRYSANTHEMUM CULTIVARS BY THRIPS RESISTANCE

	Trial 1		Trial 2		
Cultivar	Standards[b]	Classification[c]	Standards	Classification	MCR[a]
Amber	S				28.8
Red Delano				S	
Florida Marble	S				37.1
Yellow Vero		S			
Vero		S	S		
Icecap				S	21.3
Snowden		S	S		
Dark Bronze Charm		M			
Yellow Reagan				M	
Polaris	M		M		15.9
Palisade	M		M		18.6
Chardonnay				R	9.4
Yellow Favor		R	R		
Fiery Barbara				R	
May Shoesmith				R	15.6
Super White	R		R	R	5.1
Super Yellow	R		R	R	4.0

[a] Mean cultivar rank (Broadbent et al., 1990).
[b] Used as standards in this trial: S, susceptible; M, medium; R, resistant.
[c] Classified by model in this trial.

A stepwise DFA was carried out to determine which of the 12 sensors were contributing the most to the classification into resistance categories. The SY/LG sensor, which detects fluorine/chlorine-containing compounds, was selected in all three of the analyses listed in Table 2. The other sensor most often selected (in two of the analyses) was SY/gCT, which detects nonpolar solvents such as methane and propane. For trial 2, the sensors most often selected were SY/gCTI (three of four analyses in Table 4), which detects organic solvents such as ethanol, and SY/G (two of four analyses in Table 4), which detects amines and ammonia.

DISCUSSION

This study has demonstrated that the EN has some capability to classify chrysanthemum cultivars into western flower thrips resistance categories using volatiles released from leaf disks. Standard cultivars that had been designated susceptible, medium, and resistant based on thrips feeding damage were well classified by cross-validation. Unknowns were less well classified, particularly in trial 2. When classification of plants into cultivars was attempted, misclassified cultivars were often assigned to the same resistance category, suggesting that cultivars in the same category are sufficiently similar to confound cultivar classification (at least in trial 1). Some difficulty was experienced in maintaining the quality of plants prior to sampling because of delays in delivery, which may have influenced the EN results. However, overall, there was fairly good agreement between unknowns in trial 2 and the MCR, suggesting that the EN can serve as an effective screening tool for thrips resistance.

The ability of the EN to discriminate among thrips-resistant cultivars suggests that volatile chemicals given off by the leaves are involved in resistance. Some evidence has been presented to implicate plant secondary metabolites in thrips resistance. De Jager et al. (1995) found that 76% of the variation in resistance of chrysanthemum cultivars was explained by the phytochemicals in leaf sap. This was later attributed to the synergistic effect of unidentified secondary metabolites fractionated from chrysanthemum leaves (De Jager et al., 1996). More recently, Tsao et al. (2003) extracted a new unsaturated fatty acid isobutylamide, *N*-isobutyl-(2*E*,4*E*,10*E*,12*Z*)-tetradecatetraen-8-ynamide, from chrysanthemum leaves, which correlated with thrips resistance (Tsao et al., 2005).

The EN is able to provide a "fingerprint'' of the volatiles released from cut chrysanthemum leaves, but is not able to identify the compounds involved in resistance. The relationship between volatiles detected by the EN and the putative resistance chemicals isolated from leaves is presently unknown. Recent findings (J.C. Young et al., unpublished results) suggest that thrips resistance in chrysanthemums is complex. Volatile chemicals given off by cut leaves were analysed

by solid-phase microextraction and GC/MS, and it was found that specific volatiles could be correlated with resistant and susceptible cultivars. Further studies to relate specific volatiles to the EN responses are warranted.

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CUTICULAR HYDROCARBONS OF BUFFALO FLY, *Haematobia exigua*, AND CHEMOTAXONOMIC DIFFERENTIATION FROM HORN FLY, *H. irritans*

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Abstract—We determined the quantity and chemical composition of cuticular hydrocarbons of different strains, sexes, and ages of buffalo flies, *Haematobia exigua*. The quantity of cuticular hydrocarbons increased from less than 1 μg/fly for newly emerged flies to over 11 μg/fly in 13-d-old flies. The hydrocarbon chain length varied from C_{21} to C_{29}, with unbranched alkanes and monounsaturated alkenes the major components. Newly emerged flies contained almost exclusively C_{27} hydrocarbons. Increasing age was accompanied by the appearance of hydrocarbons with shorter carbon chains and an increase in the proportion of alkenes. 11-Tricosene and 7-tricosene were the most abundant hydrocarbons in mature *H. exigua*. Cuticular hydrocarbons of *H. exigua* are distinctly different from those of horn flies, *Haematobia irritans*. The most noticeable differences were in the C_{23} alkenes, with the major isomers 11- and 7-tricosene in *H. exigua* and (*Z*)-9- and (*Z*)-5-tricosene in *H. irritans*, respectively. Cuticular hydrocarbon analysis provides a reliable method to differentiate the two species, which are morphologically difficult to separate. The differences in cuticular hydrocarbons also support their recognition as separate species, *H. exigua* and *H. irritans*, rather than as subspecies.

Key Words—Cuticular hydrocarbons, *Haematobia*, buffalo fly, horn fly, Diptera, chemotaxonomy, gas chromatography, mass spectrometry, alkenes.

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INTRODUCTION

The buffalo fly, *Haematobia exigua* de Meijere (Diptera: Muscidae) abounds in the Oriental and Australian regions (Seddon, 1967; Williams et al., 1985). *H. exigua* is an obligate blood feeder spending most of its time on the host (MacQueen and Doube, 1988) and constitutes one of the major animal health problems to dairy and beef producers in northern Australia. The horn fly, *Haematobia irritans* (L.) a close relative of *H. exigua*, is found in the Americas, North Africa, and Europe, and is also considered a major arthropod pest of pastured cattle in the US and Canada (Drummond et al., 1986; Kunz, 1986).

Morphological differentiation of the adult fly species is difficult, and Pont (1973) indicated that the various larval stages were probably indistinguishable. While Zumpt (1973) recognized the two as subspecies of *H. irritans*, Skidmore (1985) subsequently recognized them as two separate species, *H. exigua* and *H. irritans*, although acknowledging their close relationship. Species and subspecies status are currently being applied to these flies by various authors. One approach to species identification that could clarify this situation is cuticular hydrocarbon profiling (Lockey, 1991), which has been successfully applied to a number of problem species (Carlson et al., 1993; Page et al., 1997; Brown et al., 1998; Broza et al., 2000; Howard et al., 2003). The cuticular hydrocarbons of *H. irritans* have been identified (Mackley et al., 1981), with the major components being odd-numbered, straight-chain C_{21}–C_{29} saturated hydrocarbons and monounsaturated C_{23}, C_{25}, and C_{27} alkenes. Double bond positions were established through oxidative cleavage followed by gas chromatography-mass spectrometry (GC-MS) of the resulting aldehydes.

In this study, we report the identification of *H. exigua* cuticular hydrocarbons and variations in their composition and quantity depending on the sex and age of the fly. We employ a more elegant and direct route to the determination of double bond positions through the formation and GC-MS analysis of dimethyl disulfide (DMDS) adducts (Scribe et al., 1988; Carlson et al., 1989). Considering the morphological and behavioral similarities between *H. irritans* and *H. exigua*, the resulting information is valuable and useful for taxonomic purposes.

METHODS AND MATERIALS

Flies. *H. exigua* were obtained from a colony of a field strain at the DPI Oonoonba Veterinary Laboratory, Townsville (DPI strain), and from the closed laboratory colony at CSIRO Entomology, Indooroopilly (CSIRO strain), Queensland, Australia. Both colonies were cultured on bovine hosts (Stegeman et al., 1996), and the CSIRO strain flies were collected from cattle kept in an

insectary. Pupae submitted from Townsville were kept in cardboard cylinders lined with filter paper at 27°C and 70–80% RH, and emerged flies were fed with bovine blood (Anderson, 1995). *H. irritans* were obtained from the colony of Prof. Butler at the University of Florida, Gainesville, FL, USA.

Hydrocarbon Extraction. One ml of hexane (UltimAR, Mallinckrodt) containing 5 μg *n*-tetracosane [internal standard (IS)] was added to 20 flies that had been immobilized (−20°C or carbon dioxide). After standing for 5 min, the hexane solution was passed through a silica gel plug (1 g) confined in a Pasteur pipette that was then washed with 5 × 1 ml hexane. The combined hexane solutions were dried with anhydrous sodium sulfate, filtered, evaporated to dryness, and reconstituted with 100 μl hexane. Single fly extractions were carried out analogously using 20-fold lower IS concentration and reconstituted with 5 μl hexane.

Chemicals. (*Z*)-9-Tricosene was obtained from ISP Fine Chemicals (Columbus, OH, USA).

Methylthiolation of Alkenes. DMDS adducts were prepared according the method of Carlson et al. (1989) with a reaction time of 16 hr at 40°C.

Analysis and Identification. Gas chromatography (GC) was carried out on an HP5890 gas chromatograph equipped with a capillary column (DB-5, J&W; 30 m × 0.25 mm, 0.25 μm film thickness) using nitrogen as the carrier gas (column pressure, 100 kPa) and with a flame ionization detector (FID). The oven temperature was increased (5°C/min) from 200 to 260°C (280°C for DMDS adducts) where it was maintained for 5 min. Data acquisition and processing were performed with an HP3392A integrator with peak area used for the quantitation of components (relative to tetracosane). Coupled GC-MS was performed on an HP5890 GC (same column as described above) with a VG Trio 2000 mass spectrometer. Ionization was by electron impact (EI), and the total ion current was accumulated from *m*/*z* 35 to 400 (500 for DMDS adducts) over 1 sec. Selected ion mass spectrometry of high-intensity mass fragments was used for the detection or confirmation of components at low abundance. The stereochemistry of (*Z*)-9-tricosene was confirmed by comparison of its GC retention time with the standard sample.

RESULTS

Cuticular hydrocarbon extracts from newly emerged, 2–3, and 13-d-old female *H. exigua* were analyzed by GC-FID (Figure 1). Relevant hydrocarbon peaks are designated with a reference number (for key, see Table 1). All alkanes were completely resolved from the alkenes. At least partial, but often complete, separation of homologous alkenes with double bonds at 5, 7,

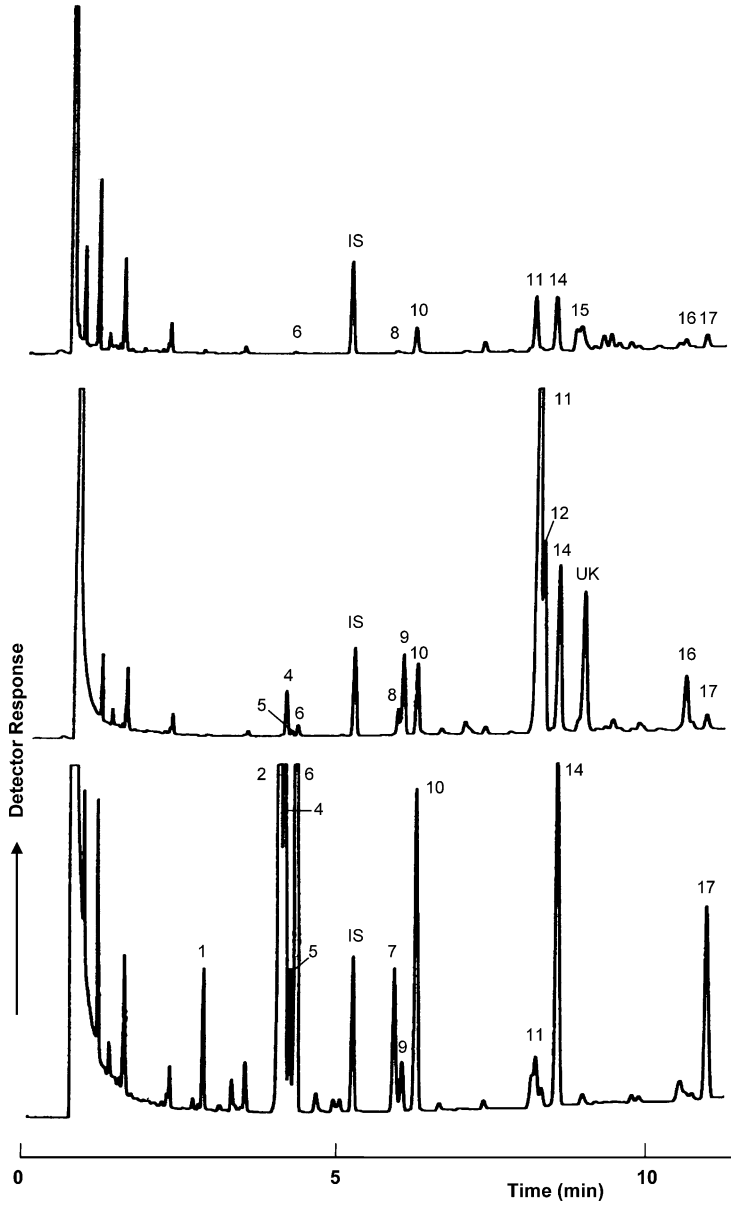

FIG. 1. Gas chromatography-flame ionization detector traces of cuticular hydrocarbon extracts from female *Haematobia exigua*; Top: newly emerged; center: 2–3 d old; bottom: 13 d old (IS = internal standard, UK = unknown; for assignments of reference numbers, see Table 1).

TABLE 1. QUANTITIES OF CUTICULAR HYDROCARBONS EXTRACTED FROM *Haematobia exigua* AND *Haematobia irritans* (μg/FLY)

		Species/strain/age/sex								
		H. exigua							*H. irritans*	
		DPI					CSIRO		U Florida	
		ne		2–3 d		13 d	3 d		unknown age	
Ref. no.	Cuticular hydrocarbons	F	M	F	M	F	F	M	F	M
1	C_{21}	D	D		D	0.15	0.09	0.05	0.04	D
2	11-C_{23}:1			T		4.73	2.32	0.77		
3	Z9-C_{23}:1						T	T	0.85	0.16
4	7-C_{23}:1			0.12	0.34	1.53	0.74	2.03		
5	5-C_{23}:1			D	0.05	0.21	0.12	0.33	0.81	0.20
6	C_{23}	D	D	D	0.09	2.73	0.71	1.03	0.20	0.09
7	11-C_{25}:1					0.26	0.06	D		
8	9-C_{25}:1	D	D	0.08	0.07				0.39	0.20
9	7-C_{25}:1	T		0.25	0.13	0.09	D	D		
10	C_{25}	0.08	0.08	0.22	0.22	0.57	0.12	0.20	0.24	0.14
11	9-C_{27}:1	0.21	0.14	2.73	1.90	0.17	D		0.25	0.12
12	7-C_{27}:1	D	D	0.64	0.42	D	D	D		
13	5-C_{27}:1	D								
14	C_{27}	0.19	0.14	0.62	0.48	0.76	0.17	0.14	0.29	0.17
15	C_{27}-Me	0.21	0.03							
16	9-C_{29}:1	D	D	0.18	0.11	D			0.07	0.02
17	C_{29}	D	D	D	D	0.43	0.12	0.19	0.11	0.05
Total alkanes		0.48	0.25	0.84	0.79	4.64	1.21	1.61	0.88	0.45
Total alkenes		0.21	0.14	4.00	3.02	6.99	3.24	3.13	2.37	0.70

F = female, M = male, ne = newly emerged, D = detected but not quantified (small quantities), T = trace quantities (selected ion monitoring).

and 9 positions was achieved, but the 9 and 11 alkenes coeluted. The hydrocarbon chain length varied from C_{21} to C_{29}, with a distinct shift in relative quantities to shorter hydrocarbons with increasing age of the flies (Figure 1, Table 1).

From newly emerged flies (DPI strain), only small quantities of hydrocarbons (<1 μg) were extracted, and these were mostly C_{27} alkenes and alkanes (Table 1). The flies, which were kept in cardboard cylinders and maintained on bovine blood, contained larger amounts of hydrocarbons at older ages, reaching over 11 μg per female fly at 13 d. With increasing age (13 d), the DPI strain had a higher proportion of shorter carbon chains (79% C_{23}) and a higher proportion of alkenes (60%). The CSIRO strain, which had been kept on cattle postemergence, contained at the age of 3 d a similar amount of cuticular hydrocarbons to the DPI strain of the same age, but its hydrocarbon pattern was more

similar to that of 13-d-old DPI flies. Eighty and twenty percent of the CSIRO (3 d) and DPI flies (13 d) were gravid, respectively.

Unbranched, odd-numbered C_{21} to C_{29} alkanes were recovered from most *H. exigua*. Heptacosane was the most abundant alkane in young flies, whereas tricosane was found in the highest quantities in mature flies. A series of methyl branched C_{27} alkanes was detected only in newly emerged flies.

The position of the double bond in C_{23} to C_{29} monounsaturated alkenes was established through derivatization with DMDS, GC-MS analysis of the adducts, and correlation of GC retention times of parent compounds and adducts. The presence of abundant fragments (often at or close to 100% abundance) corresponding to cleavage at the original site of unsaturation, now carrying thiomethyl groups at each end, defined the location of the double bond. For instance, four adducts of C_{23} alkenes showing intense ions at *m*/*z* 201/215, 173/243, 145/271, and 117/299 indicated the presence of 11-, 9-, 7-, and 5-tricosene, respectively. The most abundant hydrocarbon in mature (3 and 13 d old) female *H. exigua* was 11-tricosene, followed by 7-tricosene, which together constituted about 90% of all alkenes. 7-Tricosene was the most abundant hydrocarbon in mature (3 d old) male CSIRO flies. Other C_{23} alkenes included 5-tricosene and (*Z*)-9-tricosene, with the latter being detected only in the CSIRO strain in trace quantities. In the early stages of the DPI flies' development, C_{27} and C_{25} alkenes dominated the extract, with 7- and 9-heptacosene and 7-pentacosene as the major components.

The cuticular hydrocarbon content of individual *H. exigua* was consistent in quantity and composition within a sex/age group (Table 2). The standard errors of the means of the major components from replicated hydrocarbon extraction from individual flies were small (typically 5%). Furthermore, the mean values from single fly extractions closely match the normalized values from the batch extractions.

TABLE 2. QUANTITIES OF CUTICULAR HYDROCARBONS EXTRACTED FROM INDIVIDUAL FEMALE AND MALE *Haematobia exigua* [MEAN (SE)][a]

	Quantity (μg/fly)	
Cuticular hydrocarbon	Female	Male
11-C_{23}:1	2.3 (0.15)	0.82 (0.05)
7-C_{23}:1	0.79 (0.06)	2.02 (0.10)
C_{23}	0.70 (0.06)	1.11 (0.06)
C_{25}	0.13 (NA)	0.25 (0.03)
C_{27}	0.17 (0.01)	0.16 (0.01)

[a] CSIRO flies, 3 d old, $N = 5$, NA = not applicable.

Single fly extracts of female and male *H. irritans* yielded as major components (*Z*)-9-tricosene, 5-tricosene, 9-pentacosene, pentacosane, 9-heptacosene, and heptacosane (Table 1).

DISCUSSION

Analyses of *H. irritans* cuticular hydrocarbons were consistent with those published previously (Mackley et al., 1981; Milstrey, 1983), but were obtained in a more efficient fashion. This was a consequence of the superior resolving power of modern capillary GC columns compared with packed columns, avoiding the necessity for prior column-chromatographic separation of alkenes from alkanes. Structure elucidation of the hydrocarbons was achieved on the basis of GC retention times, methylthiolation, and mass spectrometry of the parent compounds and their adducts. Resolution of all unsaturated positional isomers was obtained with the exception of the 9 and 11 isomers, where the parent compounds as well as the DMDS adducts partially coeluted. However, these isomers could be distinguished by selected ion monitoring of the DMDS adducts, which showed clear differences in the major fragments obtained by EI ionization. The stereochemistry of the double bond could be assigned unambiguously only for (*Z*)-9-tricosene, the only unsaturated reference hydrocarbon that we were able to obtain. It is likely that all other alkenes share the *Z* configuration, based on their GC retention times and precedence from the structures of cuticular hydrocarbons from related flies (Mackley et al., 1981).

The cuticular hydrocarbon profile from newly emerged *H. exigua* was similar to the profile reported from a colony of newly emerged *H. irritans* (Milstrey, 1983). Normal C_{25} and C_{27} and methyl branched-C_{27} hydrocarbons provided the bulk of the alkanes, and 9-heptacosene and 9-pentacosene largely made up the alkenes. A few additional components not detected previously in *H. irritans* (Milstrey, 1983) were observed in *H. exigua*, including 9-nonacosene, 5- and 7-heptacosene, and 7-pentacosene; however, they were present only in small or trace quantities. Branched C_{27} alkanes were present in newly emerged *H. exigua* and *H. irritans*, but were not detected in any other age category in either species. The quantity of alkanes and alkenes extracted was lower in newly emerged *H. exigua* than in *H. irritans* (Milstrey, 1983). Similar to *H. irritans*, there was no marked difference in the composition of cuticular hydrocarbons between newly emerged female and male *H. exigua* (Table 1), although the quantity of the methyl branched C_{27} alkanes was lower in male flies.

Significant qualitative and quantitative changes in hydrocarbon composition were observed 2–3 d after the emergence of *H. exigua*. Shorter carbon chains were more prominent and alkenes constituted about 80% of the total hydrocarbons, compared to less than 35% in newly emerged flies (Table 1). Similar age-related

changes in hydrocarbon content have also been observed for *H. irritans* (Milstrey, 1983). However, qualitative differences between the composition of unsaturated hydrocarbons in *H. exigua* and *H. irritans* were obvious. Extraction of 2- to 3-d-old *H. exigua* kept in a cardboard cylinder since emerging (DPI strain, Table 1) yielded 5- and 7-tricosene, 7- and 9-pentacosene, 7- and 9-heptacosene (major component) and 9-nonacosene. *Haematobia irritans* (3–4 d old) yielded (*Z*)-5- and (*Z*)-9-tricosene (major components), (*Z*)-9-pentacosene and (*Z*)-9-heptacosene (Mackley et al., 1981; Milstrey, 1983), and 9-nonacosene (this study, see below). Thus, none of the (*Z*)-9-tricosene, which was the major alkene component in *H. irritans* (except newly emerged), was found in the DPI *H. exigua*. Conversely, 7-tricosene, 7-pentacosene, and 7-heptacosene were detected in *H. exigua* but not in *H. irritans*. The extraction of 13-d-old female DPI flies yielded a total of 11 μg cuticular hydrocarbons per fly, with the alkenes comprising 60% of these. Further evidence for the shift to shorter carbon chain length with age is provided by the detection of 11-tricosene and 11-pentacosene in the extracts of 13-d-old females. 11-Tricosene was the major component and, together with 7-tricosene and tricosane, contributed 79% to the total hydrocarbons. An inspection of a subsample of this population indicated that 20% of the female flies were gravid.

Extraction of CSIRO *H. exigua*, kept on cattle for 3 d postemergence, yielded a similar amount of hydrocarbons to the caged flies of the same age (Table 1). However, the resultant hydrocarbon profile was much more similar to that of 13-d-old DPI flies. All mature *H. exigua* had predominantly C_{23} alkenes, with 11- and 7-tricosene as the most abundant isomers. The 3-d-old female CSIRO flies were 80% gravid, and the close resemblance of their hydrocarbon profiles to those of the DPI flies (13 d old, 20% gravid) indicates that the observed change in the hydrocarbon profile is related to developments other than the attainment of sexual maturity. The CSIRO flies also showed quantitative differences between the sexes, with 11-tricosene and 7-tricosene as the major components in female and male *H. exigua*, respectively. However, results from this study may not fully reflect sexual dimorphism, as the flies were collected from mixed-sex populations, unlike the *H. irritans* study where pupae were individually contained (Milstrey, 1983). Transfer of cuticular hydrocarbons between female and male flies through mating has been documented (Scott et al., 1988). The CSIRO fly extracts also contained small amounts of (*Z*)-9-tricosene, the major alkene component of *H. irritans*. However, (*Z*)-9-tricosene was only detected in *H. exigua* with selected ion mass spectrometry, which provided higher sensitivity than total ion current or flame ionization detectors.

Variability in the quantity of major cuticular hydrocarbons extracted from individual *H. exigua* was low (Table 2). All major components were detected in all samples, and the standard errors of the means were all below

10% and typically around 5%. Thus, single fly extractions provide hydrocarbon profiles that are characteristic of the population with a reasonable degree of certainty.

To complete our study, we extracted one female and one male *H. irritans* and determined the structure of the unsaturated hydrocarbons by methylthiolation followed by GC-MS. We confirmed (except for the stereochemistry) the previous findings, obtained by ozonolysis of the parent components, that (*Z*)-9-tricosene, (*Z*)-5-tricosene, (*Z*)-9-pentacosene, pentacosane, (*Z*)-9-heptacosene, and heptacosane are the major cuticular hydrocarbons found in *H. irritans* (Mackley et al., 1981; Milstrey, 1983). Our *H. irritans* extracts also contained small quantities of 9-nonacosene, which was not previously reported, probably due to the lower sensitivity resulting from packed rather than capillary GC columns. Although the ages of the *H. irritans* samples were unknown, the resulting hydrocarbon profile indicated that they were at least a few days old.

Results presented in this study and comparison with previous reports (Mackley et al., 1981; Milstrey, 1983) demonstrate that cuticular hydrocarbon profiles of *H. exigua* and *H. irritans* are almost identical in newly emerged flies, but can readily be distinguished in flies older than 2 d (Table 3). This dissimilarity is independent of sex, feeding regimen (*in vivo* or *in vitro*), or sexual maturity. For chemotaxonomic differentiation, a simple analysis of C_{23} alkenes of flies aged 2 d or more provides an unambiguous identification, with each species possessing unique positional isomers (rows in bold italics in Table 3). The major alkenes were the 11- and 7-tricosene in *H. exigua* (both not detected

TABLE 3. RELATIVE QUANTITIES OF CUTICULAR HYDROCARBONS IN EXTRACTS FROM MATURE *Haematobia exigua* AND *Haematobia irritans*

Cuticular hydrocarbon	*H. exigua*	*H. irritans*[a]
C_{21}	✓	✓
11-C₂₃:1	✓✓	***nd***
Z9-C₂₃:1	*	✓✓
7-C₂₃:1	✓✓	***nd***
5-C₂₃:1	✓	✓✓
C_{23}	✓	✓
11-C_{25}:1	✓	nd
9-C_{25}:1	✓	✓
7-C_{25}:1	✓	nd
C_{25}	✓	✓
9-C_{27}:1	✓	✓
7-C_{27}:1	✓	nd
C_{27}	✓	✓

[a] Mackley et al. (1981), Milstrey (1983), this study.
✓ = present, ✓✓ = major component (>25% of total alkenes), nd = not detected, * = trace amounts in some fly extracts, rows in bold italics denote key differences between species.

in *H. irritans*), and the (*Z*)-9- and (*Z*)-5-tricosene in *H. irritans*. Such a chemotaxonomic differentiation is more reliable and robust than the difficult differentiation based on morphological characters (Zumpt, 1973).

Cuticular hydrocarbons are utilized by insects as species recognition cues (Carlson et al., 1998; Howard et al., 2003). Clear differences in the cuticular hydrocarbon profiles of *H. exigua* and *H. irritans* thus provide support for their recognition as separate species. Additional cuticular hydrocarbon profiling and molecular, genetic, morphological, and cross-breeding studies of specimens of both species across their extensive distributions would be desirable to further explore species integrity.

In summary, we determined the qualitative and quantitative composition of cuticular hydrocarbons of different strains, sexes, and ages of *H. exigua*. We demonstrated that the hydrocarbon profile of mature *H. exigua* unambiguously differs from that of *H. irritans*, and that by determining the identity of a few key compounds in cuticular hydrocarbon extracts, fly species can be distinguished.

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SEX PHEROMONE OF THE DOGWOOD BORER, *Synanthedon scitula*

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Abstract—The sex pheromone of female dogwood borers (DWB) *Synanthedon scitula* (Harris) (Lepidoptera: Sesiidae) was determined to be an 88:6:6 ternary blend of (*Z,Z*)-3,13-octadecadienyl acetate (*Z,Z*-3,13-ODDA), (*E,Z*)-2,13-octadecadienyl acetate (*E,Z*-2,13-ODDA), and (*Z,E*)-3,13-octadecadienyl acetate (*Z,E*-3,13-ODDA) by gas chromatography–electroantennographic detection (GC–EAD) and gas chromatography–mass spectrometry (GC–MS). The major sex pheromone component, *Z,Z*-3,13-ODDA, was attractive as a single component. A blend of *Z,Z*-3,13-ODDA with 1–3% of *E,Z*-2,13-ODDA (binary blend) was more attractive than the single component. A third component, *Z,E*-3,13-ODDA, was sometimes observed in GC–EAD analyses, and enhanced attraction to the binary blend in some field bioassays. Lures containing 1 mg of binary and ternary blends attracted 18 and 28 times more male DWB moths, respectively, than caged virgin females in field trials. Attraction was strongly antagonized by addition of as little as 0.5% of *E,Z*-3,13-octadecadienyl acetate (*E,Z*-3,13-ODDA). In a period of 12 wk in 2004, more than 60,000 males were captured in sticky traps baited with synthetic pheromone blends in six apple orchards in Virginia, West Virginia, and North

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Carolina. Lure longevity trials showed that ~76% of the pheromone remained in rubber septum lures after 12 wk in the field.

Key Words—Dogwood borer, *Synanthedon scitula*, sex pheromone, (*Z,Z*)-3,13-octadecadienyl acetate, (*E,Z*)-2,13-octadecadienyl acetate, (*Z,E*)-3,13-octadecadienyl acetate, (*E,Z*)-3,13-octadecadienyl acetate, pheromone antagonist.

INTRODUCTION

The dogwood borer (DWB), *Synanthedon scitula* (Harris) (Lepidoptera: Sesiidae), is an important pest of at least 19 species of fruit, nut, and ornamental trees in the eastern United States and Canada (Engelhardt, 1932; Eichlin and Duckworth, 1988; Bergh and Leskey, 2003). Since the 1980s, DWB has become an increasingly important economic pest of apple (Riedl et al., 1985; Warner and Hay, 1985; Kain and Straub, 2001) largely due to the increased use of clonal, size-controlling rootstocks in high-density orchards (Marshall and Andrews, 1994). Commercially available pheromone lures for DWB rely on generalized sex pheromone components identified from other sesiid species (Tumlinson et al., 1974; Nielsen et al., 1975, 1979). This has led to inconsistencies and variability in the results from published research on DWB conducted in apple orchards, native habitats, and managed urban landscapes (reviewed in Bergh and Leskey, 2003). Furthermore, different lures marketed for monitoring DWB vary considerably in their attractiveness to and selectivity for DWB (Bergh et al., 2004). A species-specific sex pheromone identified from DWB is needed for reliable, standardized detection and monitoring of this pest.

The objectives of this study were to determine the composition of the sex pheromone of DWB and to develop a species-specific and effective pheromone lure for monitoring. Species-specific sex pheromone baited traps would be useful for comparing the seasonal life history and relative abundance of DWB in its different habitats, and accurate information on population density would facilitate management decisions in apple orchards and ornamental nurseries. The failure of previous attempts to manage DWB using mating disruption (Pfeiffer and Killian, 1999) may have been due, in part, to an inadequate characterization of its sex pheromone. Mating disruption products for the congeneric peach tree borer, *Synanthedon exitiosa* (Say), and lesser peach tree borer, *S. pictipes* (Grote and Robinson), are effective (Gentry and Snow, 1984; Pfeiffer et al., 1991; Agnello and Kain, 2002) and commercially available, and identification of the DWB sex pheromone may enable development of this management approach for DWB as well. It will also enable the investigation of pheromone-based, premating reproductive isolation mechanisms among several sympatric sesiid congeners. This article describes the determination of the sex pheromone composition of the DWB, and field trapping studies that examined and compared the responses

of males to different geometric isomers, blend ratios, pheromone antagonists, and virgin females. The release rates and longevities of binary and ternary pheromone lures were quantified under field conditions.

METHODS AND MATERIALS

Insects. Larvae (~100) of DWB were excavated from burrknot tissue of apple trees (Jefferson and Berkeley Counties, WV, USA and Frederick County, VA, USA) with obvious signs of infestation, including frass and entry wounds, in October and November of 2002 and 2003. Larvae were brought to the laboratory and reared on general-purpose lepidopteran diet (Bioserv, Frenchtown, NJ, USA) in an incubator at 25°C (16L:8D) until pupation. Pupae were sexed according to the characteristics described by Leskey and Bergh (2003) and held individually in 1-oz clear plastic cups (Jet Plastica Industries, Hatfield, PA, USA) with a small piece of moistened cotton dental wick and topped with plastic caps. Pupae were sent to Beltsville, MD, USA in January 2002 and 2003. Upon arrival, the pupae were kept in an insectary at 25°C and 16L:8D photoperiod until adult emergence. Absorbent cotton moistened with 8% sugar water was provided as a food source for emerged moths. Female moths were transferred to an effluvial collection device for collection of volatiles.

Effluvial Collections. Volatiles were collected using six groups of 1- to 13-d-old virgin females (3–10 females per group) at room temperature and 16L:8D photoperiod. The moths were introduced separately into three 1-l, four-necked glass containers (Zhang et al., 1994). Air was drawn into the container through 6- to 14-mesh activated charcoal (Fisher Scientific, Pittsburgh, PA, USA) and out of the container through two traps (15 cm × 1.5-cm o.d.) containing Super Q (200 mg each; Alltech Associates, Inc., Deerfield, IL, USA) by vacuum (~1 l/min). Female moths were fed a 10% sugar solution on cotton balls and aerated continuously for 3–7 d at room temperature and 16L:8D photoperiod. The adsorbent traps were changed every 48 hr. Adsorbents were eluted with methylene chloride (4 × 0.5 ml, spectrometry grade, EMD Chemicals Inc., Gibbstown, NJ, USA) and the eluates (2 ml/sample) were concentrated to ~20 μl under a nitrogen stream and stored at −30°C until analysis.

Pheromone Gland Extractions. Pheromone gland extracts were obtained during photophase from the six groups of 2- to 13-d-old virgin females that had been used in aerations. A female abdomen was compressed gently until the ovipositor everted from the abdominal tip. The ovipositor was then excised with microscissors into a conical glass vial containing ~100 μl methylene chloride–methanol (3:1), and the glands were soaked for at least 2 hr at room temperature. The extracts were removed, and the glands were reextracted with 100 μl methylene chloride–methanol (Zhang and Polavarapu, 2004). The

combined solution was concentrated to ~20 μl under a nitrogen stream and stored at −30°C until analysis.

Electrophysiological Recordings and Mass Spectrometry. The coupled gas chromatography–electroantennographic detection (GC–EAD) system used was as previously described (Zhang et al., 1997; Zhang and Polavarapu, 2003). For GC–EAD analysis, an Hewlett-Packard (HP) 6890 gas chromatograph equipped with a 60 m × 0.25-mm i.d., 0.25-μm film-thickness DB-WAXETR capillary column (J&W Scientific Inc., Folsom, CA, USA; 120°C for 2 min, 10°C/min to 250°C, hold for 10 min) or a 60 m × 0.25-mm i.d., 0.25-μm film-thickness DB-5 capillary column (J&W Scientific Inc., 100°C for 2 min, 15°C/min to 250°C, hold for 10 min) in splitless mode with hydrogen carrier gas (1.4 ml/min) was used.

Gas chromatography–mass spectrometry (GC–MS) was conducted on an HP 6890 GC coupled to an HP 5973 mass-selective detector using an identical DB-WAXETR capillary column (120°C for 2 min, 15°C/min to 230°C, hold for 25 min), but with helium as carrier gas. A 70-eV electron beam was used for sample ionization.

Chemicals. (*Z,Z*)-3,13-Octadecadienyl acetate (~90% purity, Bedoukian Research, Danbury, CT, USA) was purified by flash chromatography using 15% $AgNO_3$ on silica gel 60 (230–400 mesh, EM Science, Gibbstown, NJ, USA), eluted with 2:10 CH_2Cl_2:hexane. The fractions with <0.3% of the impurity, *E, Z*-3,13-ODDA, were combined. All other pheromone standards were purchased from PheroBank (Wageningen, the Netherlands), and purities of chemicals were checked on a 60-m polar DB-WAXETR GC capillary column before preparing lures for field studies (>97% purity).

Field Trapping Tests. Red natural rubber septa (5 mm, Wheaton, Millville, NJ, USA) loaded with the desired rates of *Z,Z*-3,13-ODDA and the blends in ~20 μl of hexane solution were used for field trials with hexane controls. The same amount of solvent (hexane) was loaded on the septum for the blank control. After loading, the solvent was allowed to evaporate in a fume hood for 30 min. Lures were then wrapped in aluminum foil, stored in 20-ml plastic vials and shipped by express carrier on the same day. Upon arrival, the lures were kept in a freezer at −10°C until deployed.

All field tests were conducted in commercial apple orchards in West Virginia, Virginia, and North Carolina using Pherocon 1C (North Carolina and West Virginia) or Delta sticky traps (Virginia) (Trécé, Salinas, CA, USA). Traps were placed in trees at ~1.2 m above the ground (Riedl et al., 1985). At each location, experimental lures were randomized within each of three to five rows (depending on the number of replicates per test) that were separated by at least one buffer row, and traps were spaced at a minimum of ~20-m intervals within a row. Traps were rotated among positions within each row at weekly intervals for the duration of each test. The number of dogwood borer and other male sesiid moths captured were recorded weekly.

In the first comparison, the following lures were deployed: the single component (100% *Z,Z*-3,13-ODDA), the binary blend (*Z,Z*-3,13-ODDA/*E,Z*-2,13-ODDA = 94:6), a second two-component blend (*Z,Z*-3,13-ODDA/*Z,E*-3,13-ODDA = 94:6), the ternary blend (*Z,Z*-3,13-ODDA/*E,Z*-2,13-ODDA/*Z,E*-3,13-ODDA = 88:6:6), and the control. To evaluate the effect on trap catch of addition of a third component, 0.5, 1, 3, or 10% of *Z,E*-3,13-ODDA was added to the base binary blend. Similarly, 0.5, 1, 3, or 10% of *E,Z*-2,13-ODDA was added to a blend consisting of *Z,Z*-3,13-ODDA/*Z,E*-3,13-ODDA (94:6) to evaluate the addition of *E,Z*-2,13-ODDA on trap capture. In another comparison, 0.5, 1, 2, or 3% *E,Z*-3,13-ODDA was added to the ternary blend. Caged virgin females (2- to 4-d-old and one female per cage) were deployed in traps to provide a natural source of pheromone for comparison with traps baited with the binary and ternary blends over a 3-wk period.

Lure Analyses. The binary and ternary lures exposed in the field during 6, 8, and 12 wk were collected and placed individually into 20 ml hexane in a 25-ml vial and soaked for 48 hr. GC analyses of pheromone lures were conducted with an HP 6890 GC equipped with a DB-5 capillary column by injecting 1 μl of each extract. Pure *Z,Z*-3,13-ODDA was used as the standard, and remaining pheromone concentrations were obtained by comparison with this standard analyzed under the same conditions.

Statistics. Data from control traps were omitted from the statistical analyses because of zero trap catches. Unless otherwise specified, trap catch data from each test were log transformed (log $X + 1$) to normalize the variance before analysis. Means were compared by one-way analysis of variance (ANOVA) followed by Ryan–Einot–Gabriel–Welsch range test (SPSS 10.0 for Windows; George and Mallery, 2002) for significance at $\alpha = 0.05$.

RESULTS

Analysis of Sex Pheromone Components in Female Effluvial Collections and Gland Extracts. A typical coupled GC–EAD graph is shown in Figure 1, exhibiting two EAD responses in female gland extract (Figure 1A) and three EAD responses in effluvial extract (Figure 1B). The mass spectrum of the component in the gland extracts that elicited the largest EAD response (peak 1) exhibited the highest mass ion at *m*/*z* 308 (1) along with a comparatively strong mass fragment at *m*/*z* 248 (22) and other fragments: *m*/*z* 219 (6), 149 (14), 135 (27), 121 (24), 109 (37), 96 (66), 81 (85), 67 (80), and 55 (81). It closely matched the spectra of authentic 3,13-ODDA and 2,13-ODDA. Among the EAD responses, peaks 1 and 2 coincided with *Z,Z*-3,13-ODDA and *E,Z*-2,13-ODDA, respectively, in GC retention times on both capillary columns (Table 1), suggesting that *Z,Z*-3,13-ODDA and *E,Z*-2,13-ODDA were likely candidates for

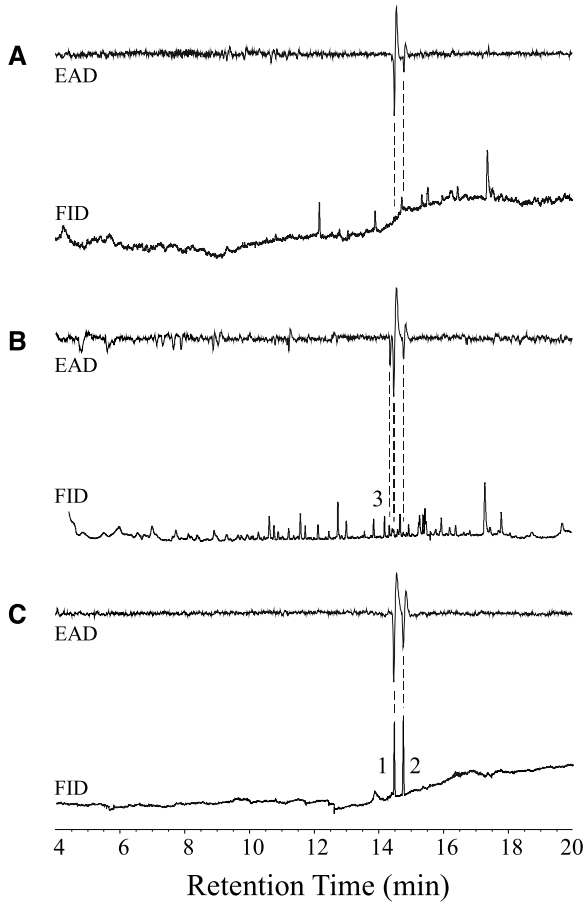

FIG. 1. Simultaneous EAD responses of an adult male dogwood borer (DWB) antenna and FID chromatograms of (A) a combined gland extract from two virgin female DWBs (3- and 5-d-old), (B) effluvia trapped for 48 hr from seven virgin female DWBs (3- to 13-d-old), and (C) synthetic *Z,Z*-3,13-ODDA and *E,Z*-2,13-ODDA (1 ng each). Identification of peaks: 1, *Z,Z*-3,13-ODDA; 2, *E,Z*-2,13-ODDA; and 3, *Z,E*-3,13-ODDA.

the insect-produced compounds (peaks 1 and 2). The identity of the component corresponding to the greatest EAD response (peak 1) was confirmed by co-injection with a synthetic standard of *Z,Z*-3,13-ODDA. To confirm the identity of peak 2, identify the third GC–EAD active peak, and determine the ratio of the three components that elicited EAD responses, a combined extract of the glands of 20 females was analyzed by GC–MS. Even with selected ion monitoring of the *m*/*z* 248 (M − 60) ion, only two peaks were deleted, at the retention times of

TABLE 1. RETENTION TIMES OF EAD-ACTIVE COMPOUNDS OBTAINED FROM GLAND AND EFFLUVIAL EXTRACTS OF FEMALE DWB AND SYNTHETIC 3,13- AND 2,13-OCTADECADIENYL ACETATES

Compounds	Retention time (min)		EAD response to 10 ng dose (mV, mean ± SD)[a]
	DB-WAXETR	DB-5	
From females			
(*Z,Z*)-3,13-Octadecadienyl acetate	14.48	13.82	
(*E,Z*)-2,13-Octadecadienyl acetate	14.75	13.93	
Synthetic			
(*E,E*)-3,13-Octadecadienyl acetate	14.39	13.81	0.13 ± 0.03 ab
(*Z,E*)-3,13-Octadecadienyl acetate	14.42	13.80	0.24 ± 0.04 a
(*E,Z*)-3,13-Octadecadienyl acetate	14.44	13.83	0.30 ± 0.09 a
(*Z,Z*)-3,13-Octadecadienyl acetate	14.48	13.82	0.30 ± 0.10 a
(*Z,E*)-2,13-Octadecadienyl acetate	14.46	13.84	0.03 ± 0.03 b
(*Z,Z*)-2,13-Octadecadienyl acetate	14.53	13.86	0.05 ± 0.04 b
(*E,E*)-2,13-Octadecadienyl acetate	14.68	13.90	0.04 ± 0.02 b
(*E,Z*)-2,13-Octadecadienyl acetate	14.75	13.93	0.20 ± 0.13 ab

[a]Means followed by different letters are significantly different at $\alpha = 0.05$ ($N = 3$, $df = 7.16$, $F = 8.30$, $P < 0.001$).

Z,Z-3,13-ODDA and *E,Z*-2,13-ODDA, and with a ratio of 94:6. The identity of the second component eliciting an EAD response (peak 2) was confirmed by co-injection with a synthetic standard of *E,Z*-2,13-ODDA in GC–MS. Authentic standards of synthetic *Z,Z*-3,13-ODDA and *E,Z*-2,13-ODDA also elicited strong antennal responses (Figure 1C). However, the third component of the effluvial extract that sometimes elicited EAD responses was not detected in gland extracts via selected ion monitoring. GC–EAD analyses of the four synthetic geometric isomers of 3,13-ODDA revealed that all elicited significant EAD responses (Table 1) and that the retention times of *Z,E*-3,13-ODDA were identical to those of peak 3 on both capillary columns. Therefore, the minor component in the effluvial extract (peak 3) that elicited antennal responses was tentatively determined to be *Z,E*-3,13-ODDA, and the ternary blend ratio was estimated to be 88:6:6 (*Z,Z*-3,13-ODDA/*E,Z*-2,13-ODDA/*Z,E*-3,13-ODDA).

Field Trapping Tests. The numbers of male moths caught in initial field studies by using traps baited with septa containing 1 mg of the single major component, two 2-component blends, and the ternary blend are summarized in Figure 2. Catches of male moths in orchards in three states all showed that the major sex pheromone component, *Z,Z*-3,13-ODDA, was attractive as a single component. The binary blend of *Z,Z*-3,13-ODDA with 6% *E,Z*-2,13-ODDA was more attractive than the single component. Addition of the second minor component, *Z,E*-3,13-ODDA, to create the ternary blend, further enhanced attraction (Figure 2).

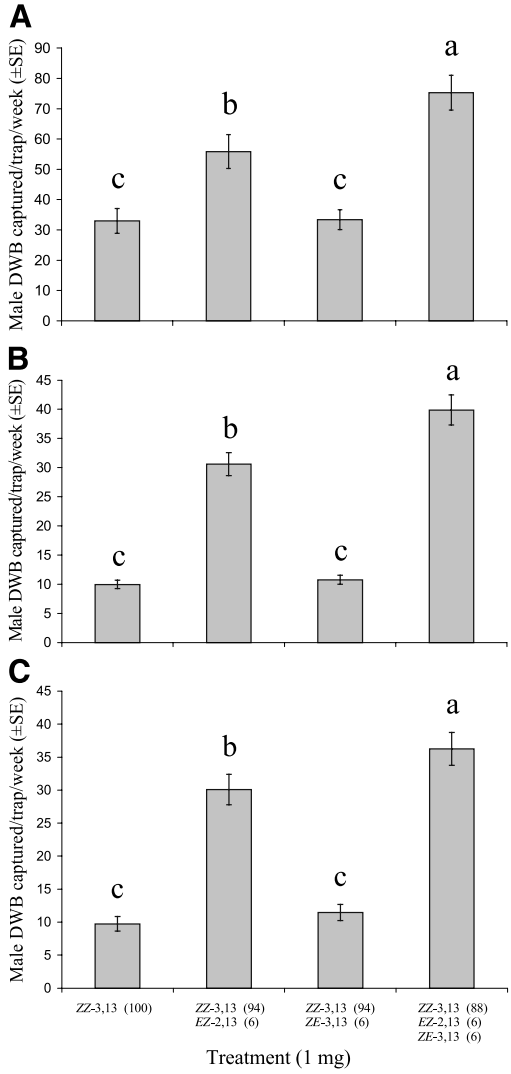

FIG. 2. Male DWB captured in traps with different DWB pheromone blends conducted (A) from May 18 to July 8, 2004, in North Carolina (total number of male DWBs trapped was 14,101, $N = 70$, $df = 3{,}276$, $F = 63.3$), (B) from June 2 to August 25, 2004, in Virginia (total number of male DWBs trapped was 11,917, $N = 130$, $df = 3{,}516$, $F = 112.1$), and (C) from June 1 to August 23, 2004, in West Virginia (total number of male DWBs trapped was 10,506, $N = 120$, $df = 3{,}476$, $F = 223.0$). Bars superscripted by different letters are different (arcsin $\sqrt{p}$ transformed, where p is the original proportion; $P < 0.05$).

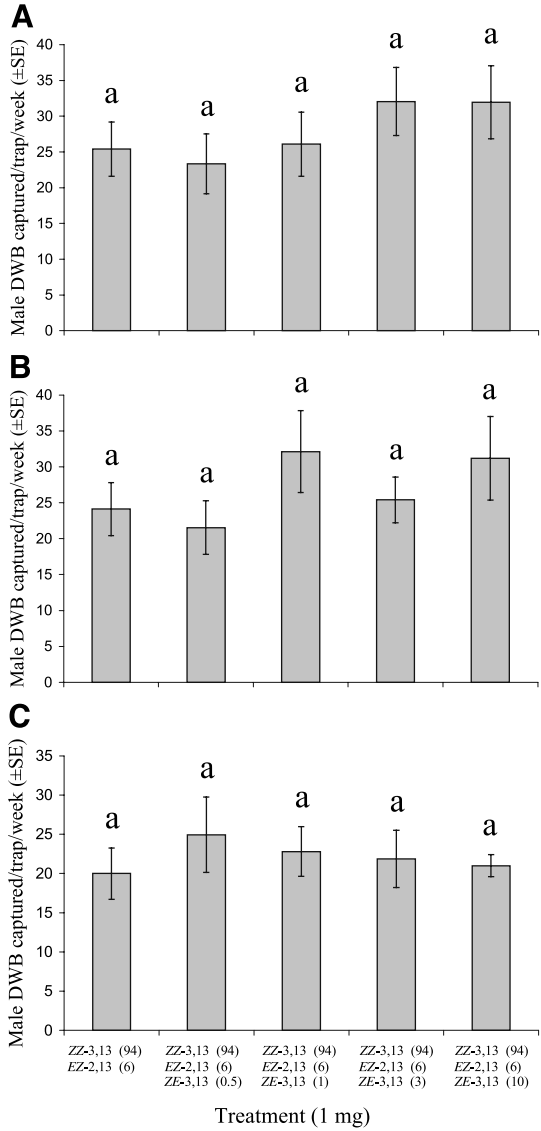

FIG. 3. Male DWB captured in traps baited with DWB pheromone ratio 1. Tests conducted (A) from July 13 to August 24, 2004, in North Carolina (total number of male DWBs trapped was 2,915, $N = 21$, $df = 4,100$, $F = 0.79$, $P = 0.53$), (B) from July 8 to August 5, 2004, in Virginia (total number of male DWBs trapped was 2,016, $N = 15$, $df = 4,70$, $F = 1.02$, $P = 0.41$), and (C) from June 21 to July 26, 2004, in West Virginia (total number of male DWBs trapped was 1,659, $N = 15$, $df = 4,70$, $F = 0.30$, $P = 0.88$).

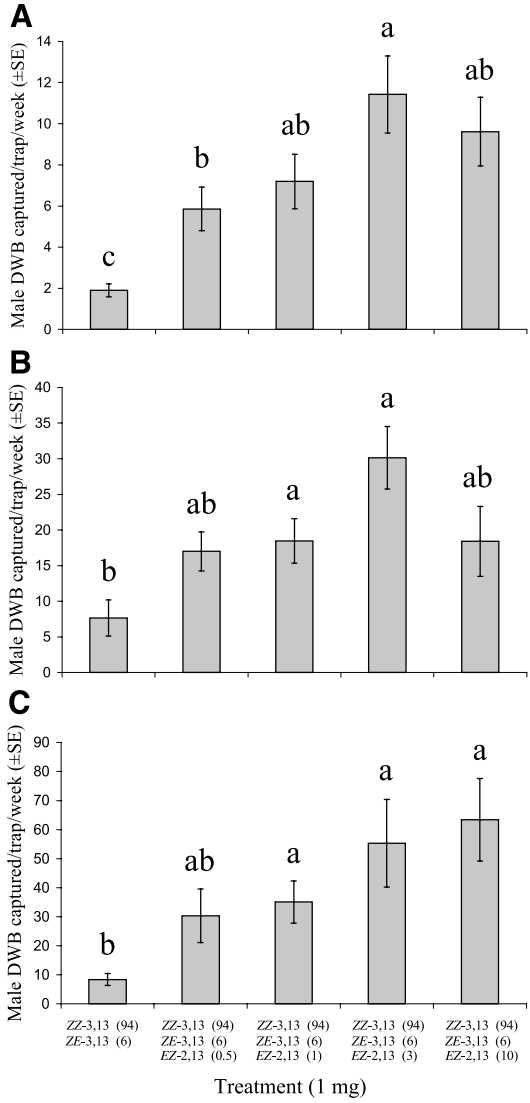

FIG. 4. Male DWB captured in traps baited with pheromone ratio 2. Tests conducted (A) from July 13 to August 24, 2004, in North Carolina (total number of male DWB trapped was 724, $N = 21$, $df = 4,100$, $F = 9.73$), (B) from July 7 to August 11, 2004, in Virginia (total number of male DWB trapped was 1,375, $N = 15$, $df = 4,70$, $F = 6.01$), and (C) from June 24 to July 23, 2004, in West Virginia (total number of male DWB trapped was 2,887, $N = 15$, $df = 4,70$, $F = 6.58$). Bars superscripted by different letters are different ($P < 0.05$).

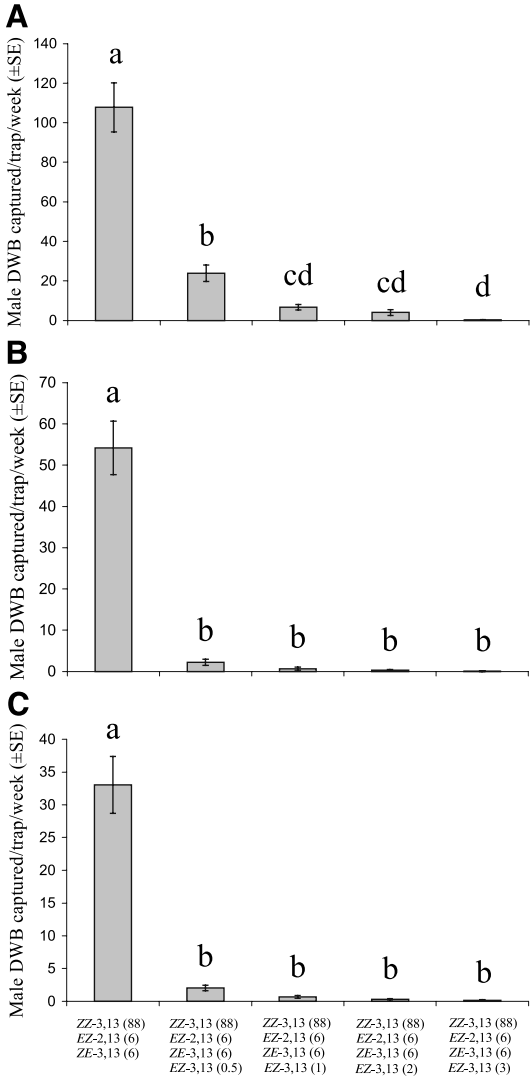

FIG. 5. Male DWB captured in trails of pheromone antagonists conducted (A) from July 14 to August 18, 2004, in North Carolina (total number of male DWB trapped was 2,568, $N = 18$, $df = 4,85$, $F = 83.85$), (B) from July 15 to August 12, 2004, in Virginia (total number of male DWB trapped was 861, $N = 15$, $df = 4,70$, $F = 156.7$), and (C) from June 24 to July 23, 2004, in West Virginia (total number of male DWB trapped was 1,086, $N = 30$, $df = 4,145$, $F = 181.4$). Bars superscripted by different letters are different ($P < 0.05$).

However, in a follow-up trial, addition of 0.5 to 10% of *Z,E*-3,13-ODDA to the binary blend of *Z,Z*-3,13-ODDA and *E,Z*-2,13-ODDA (94:6) had no effect (Figure 3). In contrast, when 0.5 to 10% of *E,Z*-2,13-ODDA was added to a binary blend of *Z,Z*-3,13-ODDA and *Z,E*-3,13-ODDA (94:6), higher numbers of males were attracted to most of the ternary blends compared to the binary blend (Figure 4). In a subsequent test, the addition of as little as 0.5% of the geometric isomer, *E,Z*-3,13-ODDA, to the most attractive ternary blend (*Z,Z*-3,13-ODDA/*E,Z*-2,13-ODDA/*Z,E*-3,13-ODDA = 88:6:6) strongly antagonized the attraction of male DWB to traps (Figure 5).

In the final field test, we compared the attractiveness of binary and ternary blends to virgin females. Rubber septa loaded with 1 mg doses of binary and ternary blends attracted more males than traps baited with one virgin female (Figure 6).

Our pheromone blends showed greatly improved species specificity in comparison to results reported from the use of commercially available lures (Rogers and Grant, 1990; Davidson et al., 1992; Braxton and Raupp, 1995; Pfeiffer and Killian, 1999; Bergh et al., 2004). In the most recent comparison of commercially available lures, the Scenturion dogwood borer lure was the most attractive and species specific, with the percentage of DWB captured ranging

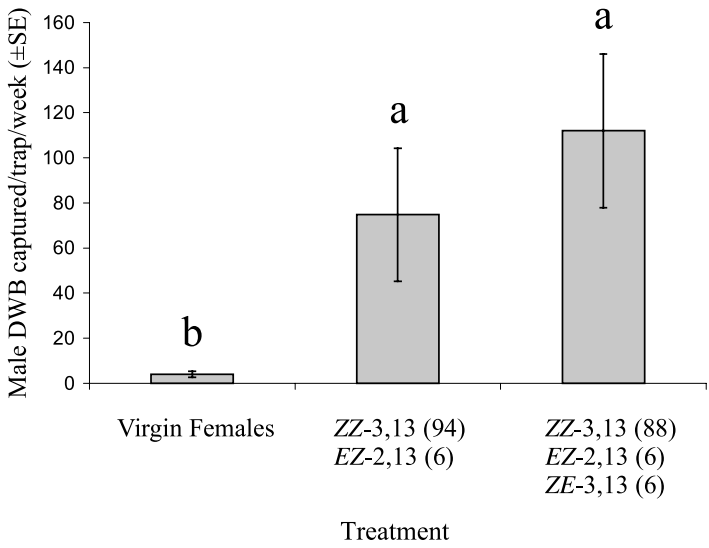

FIG. 6. Male DWB captured in traps baited with synthetic pheromone or virgin females from August 16 to September 13, 2004, in West Virginia (total number of male DWB trapped was 763, $N = 4$, $df = 2,9$, $F = 25.72$). Bars superscripted by different letters are different ($P < 0.05$).

TABLE 2. MEAN AMOUNT OF PHEROMONE COMPONENTS REMAINING IN LURES AFTER EXPOSURE UNDER FIELD CONDITIONS[a]

Orchard	Blend	Amount remaining (mg)/septum (mean ± SD in %)		
		6 wk[b]	8 wk[c]	12 wk[d]
1	Binary	0.87 ± 5.5		
2	Binary		0.79 ± 7.9	
1	Ternary	0.86 ± 9.4		
2	Ternary		0.84 ± 6.2	
3	Binary			0.72 ± 3.8
4	Binary			0.75 ± 1.7
3	Ternary			0.81 ± 2.6
4	Ternary			0.77 ± 5.1

[a] Original dose was 1.0 mg/septum. Values listed represent sum of remaining components.
[b] Exposed from May 18 to June 29, 2004, in North Carolina.
[c] Exposed from May 18 to July 9, 2004, in North Carolina.
[d] Exposed from June 2 to August 25, 2004, in Virginia.

from 33 to 86% of all Sesiidae captured (Bergh et al., 2004). In contrast, about 92% of all moths captured with our single-component or two-component blend (*Z,Z*-3,13-ODDA/*Z,E*-3,13-ODDA = 94:6) were DWB males. Even greater levels of specificity were observed with our binary (*Z,Z*-3,13-ODDA/*E,Z*-2,13-ODDA = 94:6) and ternary blends (*Z,Z*-3,13-ODDA/*E,Z*-2,13-ODDA/*Z,E*-3,13-ODDA = 88:6:6), with which over 97% of moths caught were DWB males (Leskey et al., 2005).

Lure longevity and release rate were evaluated using lures loaded with 1 mg of the binary and ternary blends and exposed in the field for 6, 8, and 12 wk. GC analyses indicated that 87% of the pheromone remained in the rubber septum lures exposed for 6 wk in the field (May 18 to June 29, 2004) in North Carolina, 82% of the pheromone remained in lures exposed for 8 wk (8 May to July 9, 2004) in Virginia, and 76% of the pheromone remained in lures exposed for 12 wk (June 2 to August 25, 2004) in Virginia (Table 2).

DISCUSSION

Following the discovery of *E,Z*- and *Z,Z*-3,13-ODDA as components of the sex pheromones of lesser peach tree borer and peach tree borer, respectively (Tumlinson et al., 1974), the geometrical isomers and corresponding alcohols of 3,13-ODDA have been reported as the main pheromone components of many sesiid species (Nielsen et al., 1979; Tumlinson, 1979; Snow et al., 1985). A positional isomer, *E,Z*-2,13-ODDA, was later identified as a component of the sex pheromone of other Sesiidae, including grape root borer, *Vitacea*

polistiformis (Harris), maple clearwing, *S. acerrubri* (Engelhardt), and squash vine borer, *Melittia satyriniformis* (Hübner) (Schwarz et al., 1983; Snow et al., 1987).

Z,Z-3,13-ODDA was reported as a sex attractant for DWB, based on a combination of electroantennogram tests and the results of field trapping tests in which many compounds were screened (Nielsen et al., 1975, 1979). However, specific identification of the DWB sex pheromone has not been reported previously. Based on GC–EAD analyses of effluvial collections, gland extracts, and synthetic isomers of 3,13- and 2,13-ODDA, and field trapping tests, we have confirmed that *Z,Z*-3,13-ODDA is the main pheromone component of DWB. As a single component, it attracts male DWB, in concurrence with the results of Nielsen et al. (1975). However, we also showed that two other components increased attraction of male DWB to Z,Z-3,13-ODDA. When *Z,Z*,-3,13-ODDA was combined with *E,Z*-2,13-ODDA in a 94:6 blend (the natural ratio), moth captures were greater than with *Z,Z*-3,13-ODDA alone or with the ternary blend of *Z,Z*-3,13-ODDA in combination with *Z,E*-3,13-ODDA (94:6). Our findings explain the results reported in previous studies, in which DWB moths were captured in traps used for evaluating seasonal flight activity of grape root borer, which contained lures with a blend of 99:1 (*E,Z*-2,13-ODDA/*Z,Z*-3,13-ODDA) (Alm et al., 1989; Snow et al., 1991). The ternary blend (*Z,Z*-3,13-ODDA/*E,Z*-2,13-ODDA/*Z,E*-3,13-ODDA = 88:6:6) proved to be more attractive than any other treatment across all locations in one trial (Figure 2), but in other trials (Figures 3 and 6), it was no better than the 94:6 binary blend of *Z,Z*-3,13-ODDA:*E,Z*-2,13-ODDA.

Thus, two pheromone components, *Z,Z*-3,13-ODDA and *E,Z*-2,13-ODDA, appear to be essential for maximal attraction of male DWB. When as little as 1% *E,Z*-2,13-ODDA was added to a 94:6 (*Z,Z*-3,13-ODDA/*Z,E*-3,13-ODDA) blend, captures increased at all locations (Figure 4). However, we did not observe a similar response when *Z,E*-3,13-ODDA was added to a 94:6 (*Z,Z*-3,13-ODDA/*E,Z*-2,13-ODDA) blend (Figure 3). We believe that *Z,E*-3,13-ODDA may indeed synergize the response to the binary blend (*Z,Z*-3,13-ODDA/*E,Z*-2,13-ODDA) based on the finding that in one set of trials, more male moths were caught at all locations with this ternary blend than with the binary blend (Figure 2). However, male DWB may not have been able to discriminate among treatments in our trial that evaluated the addition of 0.5–10% *Z,E*-3,13-ODDA to the binary blend (*Z,Z*-3,13-ODDA/*E,Z*-2,13-ODDA) (Figure 3), because the distances between stimuli were approximately 20 m, whereas in our other field trial that compared the binary and ternary blends with our other treatments, the distance between these two attractive stimuli was as much as 100 m.

E,Z-3,13-ODDA, the major component of lesser peach tree borer sex pheromone (Tumlinson et al., 1974), has been described as a potential antagonist to

the attraction of male DWB (Karandinos et al., 1977; Greenfield, 1978; Warner and Hay, 1985), whereas Snow et al. (1985) concluded that it had no effect on DWB response to *Z,Z*-3,13-ODDA. Here, we document the strongly antagonistic effect of *E,Z*-3,13-ODDA on the response of male DWB to its sex pheromone. In field trials, as little as 0.5% *E,Z*-3,13-ODDA added to our ternary blend strongly reduced trap captures at all locations (Figure 5). The importance of this particular compound in terms of niche separation is clear. In EAG studies, male DWB antennae responded as strongly to *E,Z*-3,13-ODDA as to *Z,Z*-3,13-ODDA (Nielsen et al., 1979). Interestingly, when *Z,Z*-3,13-ODDA, which is also a component of the peach tree borer sex pheromone, was added to *E,Z*-3,13-ODDA, the pheromone of lesser peach tree borer, addition of as little as 0.5% of *Z,Z*-3,13-ODDA completely antagonized the response of lesser peach tree borer males (Tumlinson et al., 1974; Karandinos et al., 1977; Mclaughlin et al., 1977). Understanding the role of attractants, antagonists, and synergists among isomers of 3,13-ODDA and 2,13-ODDA will aid in understanding mechanisms of reproductive isolation among sympatric, congeneric sesiid species.

Measurement of the amount of pheromone remaining in field-aged lures showed that the compounds that compose the DWB sex pheromone are released slowly from red rubber septa, with most of a 1-mg applied dose being recovered after 12 wk of field exposure (Table 2). The experiment was terminated only because of the seasonal decline in DWB populations, rather than due to a decline in the lure attractiveness, and we believe that lures formulated with 1 mg of pheromone or less (Leskey et al., 2005) will remain effective for the duration of the annual period of emergence and flight activity of DWB.

In summary, our synthetic lures were extremely attractive to male DWB. Traps baited with the ternary blend containing *Z,Z*-3,13-ODDA, *E,Z*-2,13-ODDA, and *Z,E*-3,13-ODDA (88:6:6) captured ~28 times more male DWB than traps baited with caged, virgin female DWB. The identification of the DWB pheromone represents a major advance in several respects. First, a reliable and standardized pheromone-based monitoring system can now be delivered to growers, providing them with the means to determine seasonal flight activity and infestation levels in orchards and nurseries, and enabling more informed management decisions. Second, the use of an improved, species-specific blend of compounds should advance our ability to investigate the effectiveness of mating disruption and attract-and-kill management tactics for this pest. Third, the behavioral antagonism shown by the addition of *E,Z*-3,13-ODDA to the ternary blend suggests strong reproductive isolation barriers between sympatric sesiid species. Characterization of the DWB sex pheromone will enable further investigation of premating mechanisms of reproductive isolation among DWB, peach tree borer, and lesser peach tree borer, based on their different behavioral responses to isomers of ODDA.

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EFFECTS OF GELDANAMYCIN ON HATCHING AND JUVENILE MOTILITY IN *Caenorhabditis elegans* AND *Heterodera glycines*

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Abstract—Several *Streptomyces* species are known to produce metabolites that inhibit plant pathogens. One such compound is geldanamycin (GA), a benzoquinone ansamycin originally isolated from *Streptomyces hygroscopicus*. We examined the effect of geldanamycin on egg hatch and juvenile motility in *Caenorhabditis elegans* and in two populations of the plant-parasitic nematode *Heterodera glycines*. When *C. elegans* eggs were exposed to geldanamycin, both hatch and motility were reduced by GA doses between 2 and 50 μg/ml. The *H. glycines* inbred populations TN17 and TN18 exhibited low dose stimulation of hatch and motility, whereas levels occurring at higher GA doses were at or below control levels. These experiments represent the first demonstration of geldanamycin effects in *C. elegans* and *H. glycines* and suggest that the heat shock chaperone Hsp90, the known molecular target of geldanamycin, may be involved in nematode egg hatch and motility. This study also indicates that geldanamycin-producing strains of *Streptomyces* may be useful as biocontrol agents for nematodes.

Key Words—Soybean cyst nematode, *Heterodera glycines*, *Caenorhabditis elegans*, hatching, motility, behavior, eggs, geldanamycin, *Streptomyces*, Hsp90, biocontrol.

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INTRODUCTION

Several species of *Streptomyces* have proven effective at reducing plant-parasitic nematode damage (reviewed in Siddiqui and Mahmood, 1999). In addition, metabolites produced by *Streptomyces* spp. were demonstrated to be nematicidal (Nair et al., 1995) or to inhibit nematode hatching (Lee et al., 1996) or motility (Mura et al., 1999). The ability to control bacterial or fungal plant pathogens has been demonstrated for the strains *Streptomyces violaceusniger* YCED-9 (Trejo-Estrada et al., 1998a,b) and *Streptomyces hygroscopicus* spp. *geldanus* EF-76 (Beauséjour et al., 2003), but neither has been tested on nematodes. Both of these strains produce the antibiotic geldanamycin (GA), a specific inhibitor of the heat shock protein chaperone Hsp90 (Whitesell et al., 1994). The impact of pure GA on nematodes was explored in a recent report by David et al. (2003). In that study, plate-grown *Caenorhabditis elegans* were exposed to a single, very high dose of GA, but no discernable effects on the nematodes were detected. We theorized that absorption or ingestion of GA by nematodes might be higher in a liquid culture system than on agar plates, and that the response to GA in plant-parasitic nematodes might differ from *C. elegans*. Thus, the objective of the current work was to determine the effect of GA exposure on *C. elegans* and the soybean cyst nematode *Heterodera glycines* in liquid culture bioassays.

METHODS AND MATERIALS

Test Organisms. *C. elegans* wild-type strain (var. Bristol-N2) and *Escherichia coli* strain OP50 were obtained from the *Caenorhabditis* Genetics Center (St. Paul, MN, USA). *H. glycines* strains TN17 and TN18 are inbred lines that were selected on the basis of relative hatching phenotypes. TN17 was selected for "fast hatching" and TN18 for "slow hatching" as defined by the relative number of eggs hatching early and late during a 2-wk period (Terry Niblack, personal communication). Despite nomenclature preferences that differ in the nematology subdisciplines, for simplicity we refer to immature forms of both *C. elegans* and *H. glycines* as "juveniles" (J2).

Nematode Culturing. *C. elegans* was routinely plate-propagated as previously described (Lewis and Fleming, 1995). *H. glycines* strains TN17 and TN18 were maintained on soybean (*Glycine max* (L.) Merr. cv. Essex) as previously described (Nitao et al., 1999).

Nematode Bioassays. Microwell assay procedures were similar to those described previously (Nitao et al., 1999). Assays were conducted in sterile 96-well tissue culture plates (polystyrene, flat bottom wells; Corning, New York, NY, USA). Each trial consisted of 10 replicate wells per treatment, with ~100

eggs/well (exact counts of eggs/well were determined), and the experiment was repeated for each treatment. Geldanamycin (Sigma-Aldrich, St. Louis, MO, USA) was prepared in dimethyl sulfoxide (DMSO) at 18 mM and subsequently diluted in deionized water as required. Controls and all treatments contained a final concentration of no more than 0.1% DMSO.

Caenorhabditis elegans. N2 embryos were harvested from gravid adults as described (Lewis and Fleming, 1995) and resuspended in M9 buffer. Aliquots (50 µl) of egg suspension were placed in each well of 96-well microtiter dishes, and the exact number of eggs per well was determined. GA dissolved in DMSO, or DMSO alone as control, was added to all eggs, and the plates were incubated in the dark at 20°C. GA concentrations tested were 0, 1, 2, 5, 10, 25, 50, and 100 µg/ml. Because GA is known to be light-sensitive (Queitsch et al., 2002) and unstable in aqueous solution (Wiesgigl and Clos, 2001), the compound was handled with care to minimize the possibility of degradation. Numbers of hatched eggs and of motile, hatched juveniles were counted after 21 hr. Motility of hatched juveniles was defined as any unprovoked movement within a 5-sec interval during which nematodes were observed under an inverted microscope.

Heterodera glycines. Eggs were collected from greenhouse-grown soybean [*Glycine max* (L.) Merr. cv. Essex] and surface-disinfested with 0.5% sodium hypochlorite (Nitao et al., 1999). Eggs were placed in 96-well microtiter plates as described above, treated with GA and/or DMSO, and incubated in the dark at 30°C, a temperature within the optimum growth range for *H. glycines* (Alston and Schmitt, 1988). Egg hatch and motility of second-stage juveniles were counted after incubation for 4 d.

Statistical Analysis. Data were analyzed using SAS® v9.1.3 for Windows (SAS Institute Inc., Cary, NC, UAS). Estimates of the percent hatched and percent motile for each nematode type at each concentration were obtained for each of two trials by summing recorded counts over the 10 wells observed per trial: % Hatched Eggs = 100% (# active + # inactive − # larvae)/# eggs; % Motile J2 = 100% (# active)/(# active + # inactive). For each of the three nematode populations, observed counts associated with each duplicate trial at each of the seven observed concentrations (i.e., $N = 14$ data points) were used to conduct all statistical analyses. For each nematode population, a nonlinear dose-response regression was fit to percent hatched and percent motile vs. GA concentration. A log-normal equation was fitted to the *H. glycines* TN17 and TN18 data, whereas a more general exponential equation was fitted to *C. elegans* data (Ratkowsky, 1990). R^2 is the ratio of the regression sum of squares to the total sum of squares (RegSS/TotSS), which reflects the proportion of the total data variability the regression equation has explained. Significant differences in hatching and motility observed at various GA concentrations vs. no GA were determined by conducting pairwise comparisons within an ANOVA

framework; specifically, a logistic regression using SAS® v9.1.3 Proc GLIMMIX with a logit link function and a binomial distribution were used to model percent hatched eggs and percent motile J2 for each nematode population. All statistical comparisons were conducted at the 95% level of significance.

RESULTS

To establish whether GA would interfere with nematode hatching or motility, we exposed *C. elegans* eggs to GA over a 100-fold range of concentrations, including 1, 2, 5, 10, 25, 50, and 100 μg/ml. Pairwise comparisons of percent hatched eggs or percent motile J2 among GA doses and the control are shown in Table 1. The relationships between percent egg hatch and percent juvenile motility vs. GA concentration were plotted (Figure 1). Statistical tests for linearity of the data failed; subsequently, nonlinear equations of exponential form exhibited the best fit to the data among all possibilities examined.

$$\%\,\text{Hatched Eggs} = 100\%[0.77 - 0.038 \cdot \%C_{GA} \cdot \exp(-0.038 \cdot \%C_{GA})]\; r^2 = 32.1\%$$

$$\%\,\text{Motile J2} = 100\%[0.498 - 0.038 \cdot \%C_{GA} \cdot \exp(-0.038 \cdot \%C_{GA})]\; r^2 = 37.5\%$$

Eggs that were exposed to GA concentrations of between 2 and 100 μg/ml exhibited hatch rates that were significantly lower than the control, but each was

TABLE 1. EFFECT OF GELDANAMYCIN ON EGG HATCH AND MOTILITY OF *Caenorhabditis Elegans*

GA conc. (μg/ml)	% Hatched eggs	% Motile J2
0	79.6 ± 7.1a	59.5 ± 7.1 a
2	60.9 ± 10.3 bc	28.1 ± 6.0 c
5	58.6 ± 10.5 cd	38.4 ± 7.0 b
10	54.8 ± 10.7 de	17.1 ± 4.3 d
25	47.6 ± 10.8 f	25.2 ± 5.6 c
50	51.1 ± 10.8 ef	19.3 ± 4.7 d
100	63.9 ± 10.0 b	34.8 ± 6.7 b
Total no. of eggs or J2	13,875	9,160

Means followed by different letters are different ($\alpha = 0.05$) by pairwise comparisons conducted on logit-transformed binomial response data in a logistic regression ($N = 14$; duplicate trials at each concentration).
Reported means ± standard errors were back-transformed to percentages from logit-scale estimates.

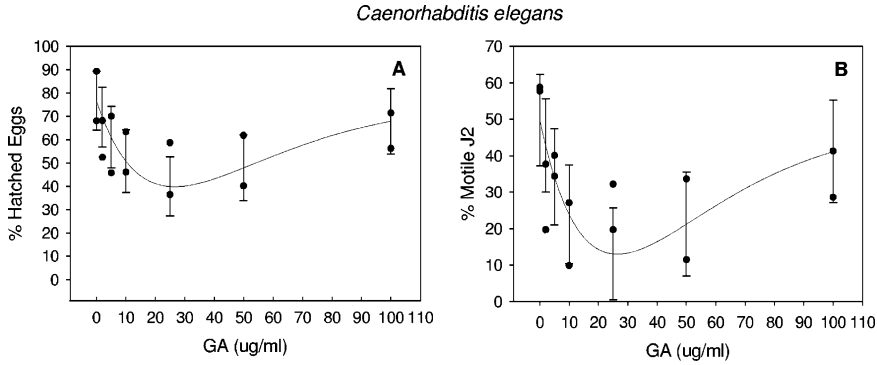

FIG. 1. Effect of geldanamycin (GA) on hatching and motility in *Caenorhabditis elegans*. Duplicate trials were used to fit a nonlinear dose-response regression to % hatched eggs and % motile juvenile values vs. GA concentration. Closed circles at each dose represent data means for the separate trials. Error bars were based upon the variation between duplicate trials pooled across the observed GA concentrations ($N = 14$) and indicate 95% confidence intervals for individual estimates of % hatched or % motile. (A) Percentage of *C. elegans* eggs that hatched after incubation for 20 hr in various concentrations of GA or DMSO control. (B) Percentage of *C. elegans* hatched juveniles that were motile after 20 hr incubation with GA or DMSO control.

similar to the hatch rate of at least one other GA concentration (Table 1). The dose showing the greatest decrease in egg hatch (47.6%) compared to control (79.6%) was 25 μg/ml GA. However, the suppressive effect on hatch rate reversed between 25 and 100 μg/ml GA, with the highest concentration (63.9% hatch) showing a similar effect to 2 μg/ml (60.9%) (Figure 1A). Geldanamycin also reduced the motility of *C. elegans* juveniles that hatched from treated eggs (Table 1, Figure 1B). Motility responses paralleled the hatch responses, with all GA levels showing significantly lower motility than the controls (Table 1). The greatest reductions in motile J2 were observed at 10 and 50 μg/ml GA (17.1% and 19.3% vs. 59.5% control). According to the nonlinear regression model, 25 μg/ml GA were shown to cause the greatest reduction in J2 motility. Curves constructed with nonlinear regression equations were U-shaped for both hatching and motility.

We also measured the effect of GA on egg hatch and juvenile motility in TN17 and TN18, two inbred populations of *H. glycines*. These strains were chosen on the basis of their selected hatching phenotypes (TN17, fast hatching; TN18, slow hatching), to determine whether they would respond differently to GA exposure. The hatch rate for each population was determined after 4 d of incubation in various GA doses suspended in DMSO or in the control treatment of DMSO alone. The dose–response relationships for TN17 and TN18 were

nonlinear. Log-normal equations were fit to the data to generate the curves shown in Figure 2.

TN17

$$\%\text{Hatched} = 100\% \cdot \left[0.098 + 0.086 \cdot \exp\left(-0.5 \cdot \left\{\log_e\left[(\%C_{GA} + 1)/5.3\right]/1.1\right\}^2\right)\right]$$

$$r^2 = 55.5\%$$

$$\%\text{Motile} = 100\% \cdot \left[0.20 + 0.38 \cdot \exp\left(-0.5 \cdot \left\{\log_e\left[\%C_{GA} + 1)/4.5\right]/1.7\right\}^2\right)\right]$$

$$r^2 = 65.5\%$$

TN18

$$\%\text{Hatched} = 100\% \cdot \left[-0.15 + 0.32 \cdot \exp\left(-0.5 \cdot \left\{\log_e\left[(\%C_{GA} + 1)/7.7\right]/3.7\right\}^2\right)\right]$$

$$r^2 = 31.7\%$$

$$\%\text{Motile} = 100\% \cdot \left[-0.05 + 0.36 \cdot \exp\left(-0.5 \cdot \left\{\log_e\left[\%C_{GA} + 1)/3.1\right]/2.2\right\}^2\right)\right]$$

$$r^2 = 89.8\%$$

Pairwise comparisons of percent hatched *H. glycines* eggs or percent motile J2 at each GA dose compared to control are shown in Table 2. For TN17, GA stimulated egg hatch between 2 and 25 μg/ml, but at 50 and 100 μg/ml, the percent egg hatch was similar to the control (Figure 2A). For TN18, a similar stimulation of egg hatch was observed, with both curves exhibiting an asymmetric inverse U shape (Figure 2C). None of the GA doses tested reduced the hatch rate to levels lower than the controls.

For TN17, percent motile J2 was higher than control at 2 and 5 μg/ml and declined to near control levels at 10–50 μg/ml GA (Figure 2B). At 100 μg/ml, the percent motile J2 was significantly lower (27.6%) than control (43.1%). For TN18, GA doses of 25 μg/ml and higher significantly reduced the motility of juveniles (Figure 2D).

DISCUSSION

Our experiments demonstrating the sensitivity of *C. elegans* to GA initially appear to contradict recent *in vitro* and *in vivo* evidence for GA resistance of *C. elegans* Hsp90 (David et al., 2003). In that study, nematodes were contin-

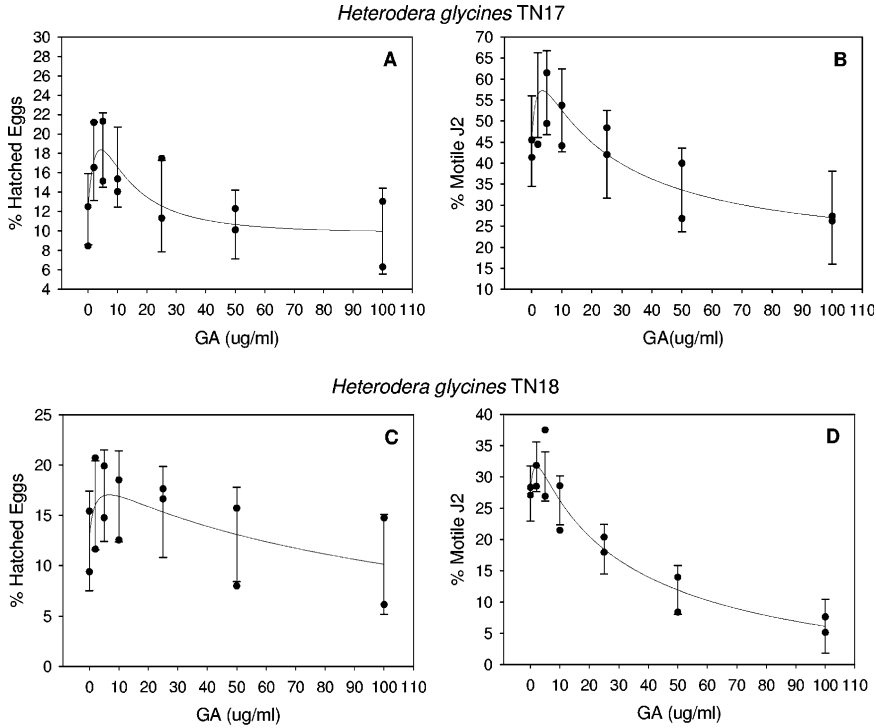

FIG. 2. Effect of geldanamycin (GA) on hatching and motility in *Heterodera glycines* inbred lines. Duplicate trials were used to fit a nonlinear dose-response regression to % hatched eggs and % motile juvenile values vs. GA concentration. Closed circles at each dose represent data means for the separate trials. Error bars were based upon the variation between duplicate trials pooled across the observed GA concentrations ($N = 14$) and indicate 95% confidence intervals for individual estimates of % hatched or % motile. (A) Percentage of *H. glycines* TN17 eggs that hatched after incubation for 4 d in various concentrations of GA or DMSO control. (B) Percentage of *H. glycines* TN17 hatched J2 that were motile after 4 d in GA or DMSO control. (C) TN18, egg hatch vs. GA concentration. (D) TN18, juvenile motility vs. GA concentration.

uously exposed to a single dose of GA, equivalent to our highest level (178 μM = 100 μg/ml GA), but no defects in viability, dauer formation, fertility, or life span were detected over several generations. This study followed the common procedure for testing compounds on nematodes, in which a high concentration is initially used to overcome the relative impermeability of the *C. elegans* cuticle (Rand and Johnson, 1995). In contrast, our multiple dose bioassays allowed us to uncover a complex response pattern that was not detectable with a single GA dose. The reversals of hatch and motility that we observed in *C. elegans* exposed

TABLE 2. EFFECT OF GELDANAMYCIN ON EGG HATCH AND MOTILITY OF THE PLANT PARASITIC NEMATODE *Heterodera glycines*, STRAINS TN17 AND TN18

	TN-17		TN-18	
GA conc. (μg/ml)	% Hatched eggs	% Motile J2	% Hatched eggs	% Motile J2
0	10.5 ± 0.7c	43.1 ± 6.5 cd	12.2 ± 2.7 b	27.5 ± 3.7 a
2	18.8 ± 0.9 a	61.6 ± 6.0 a	16.0 ± 3.4 a	30.0 ± 3.7 a
5	18.1 ± 0.9 a	55.6 ± 6.3 ab	17.0 ± 3.6 a	32.3 ± 3.8 a
10	14.7 ± 0.8 b	48.9 ± 6.5 bc	15.3 ± 3.3 a	25.0 ± 3.4 ab
25	14.3 ± 0.8 b	44.5 ± 6.4 c	16.4 ± 3.5 a	19.2 ± 3.0 b
50	11.3 ± 0.8 c	33.3 ± 6.0 de	11.9 ± 2.7 b	11.3 ± 2.3 c
100	9.7 ± 0.7 c	27.6 ± 5.5 e	10.3 ± 2.4 b	6.4 ± 1.7 c
Total no. eggs or J2	14,926	2330	13,260	2416

Means followed by different letters are different ($\alpha = 0.05$) by pairwise comparisons conducted on logit-transformed binomial response data in a logistic regression ($N = 14$; duplicate trials at each concentration).
Reported means ± standard errors were back-transformed to percentages from logit-scale estimates.

to high GA concentrations are, therefore, congruent with the prior study's inability to detect a phenotype at 100 μg/ml GA. Also, because the prior study administered GA to the surface of agar plates, it is not possible to discern whether uptake of GA by the nematodes was comparable in both studies. Comparison of low dose effects in both plate and liquid assay systems would be necessary to answer this question. These issues underscore the sensitivity of *C. elegans* to experimental conditions when testing responses to pharmacologic agents, and stress the importance of testing a range of doses and assay systems.

It is well established that Hsp90 is the definitive molecular target of geldanamycin binding (Prodromou et al., 1997; Stebbins et al., 1997). This fact, combined with information about the role of Hsp90 (*daf-21*) in *C. elegans*, provides clues about the possible pathways affected by geldanamycin. The different hatch responses to GA that we observed in *C. elegans* and *H. glycines* reflect differences in the way eggs hatch in each species. In *C. elegans,* all eggs are equally competent to hatch without any external stimulation (Albert and Riddle, 1988). The sensitivity of *C. elegans* egg hatch to GA exposure suggests the involvement of Hsp90 in this process, which is consistent with evidence for the presence of Hsp90/DAF-21 within the germline (Inoue et al., 2003). In contrast to *C. elegans*, the hatching process in cyst nematodes is complex, asynchronous, and influenced by environmental and host-derived factors (reviewed in Perry, 1997). The *H. glycines* TN17 and TN18 hatch responses to GA were similar to each other but very different from *C. elegans*. Because the hatching phenotypes of these inbred lines are temporal, exposure of eggs to one GA dose over time might reveal differences between these strains.

With respect to motility, in *C. elegans* the Hsp90 mutation *daf-21(p673)* is known to cause lethargy and defective chemotaxis to volatile odorants (Vowels and Thomas, 1994; Birnby et al., 2000). Also, the *C. elegans* protein UNC-45 functions as a cochaperone with Hsp90 for the folding and assembly of myosin, an integral component of muscle (Barral et al., 2002). Together, these associations predict a possible role for Hsp90 in movement or muscle development pathways in *C. elegans*, and GA inhibition of *C. elegans* juvenile motility is consistent with this interpretation. Overall, the differences in GA responses observed in *C. elegans* and *H. glycines* reflect the likely influence of genetic background upon the Hsp90-dependent pathways underlying hatching and motility. Because the gene for Hsp90 from *H. glycines* has recently been described (Skantar and Carta, 2004), further details of the Hsp90–GA interaction may be elucidated.

The complex nonlinear hatch and motility profiles in both nematode species display characteristics of hormesis, a model that describes the nonlinear responses of an organism to a wide range of compounds, including peptides, metals, and toxins (Stebbing, 2000; Calabrese and Baldwin, 2003). Whereas the external agent and the measured biological endpoint may vary, hormesis is commonly characterized by U- or inverse U-shaped dose–response curves. Prior studies pointed to the existence of hormetic effects in *C. elegans*. It has been established that different concentrations of pharmacological agents can cause contradictory effects in nematodes, and may indicate that the compound acts upon multiple targets (Rand and Johnson, 1995). Also, a hormetic link between thermotolerance and longevity has been described in *C. elegans* (Butov et al., 2001; Cypser and Johnson, 2002). The fact that *C. elegans daf-21*/Hsp90 is known to be involved in thermotolerance and upregulated by DAF-16 (McElwee et al., 2003) further supports involvement of Hsp90 in a hormetic biological switch involved in stress responses. Hsp90 could provide rheostatic control of client protein activities (e.g., protein kinases or hormone receptors), in response to cellular indicators of nutrient, thermal, or oxidative stress (Morano and Thiele, 1999; Knowlton and Sun, 2001). The reversal of egg hatching and J2 motility effects observed at GA doses may be such a manifestation of Hsp90-mediated hormesis. By profiling the GA doses that show observable effects, these studies should open the door to further investigations of Hsp90 and hormesis in nematodes.

Recent studies have shown that GA-producing strains of *Streptomyces* are effective against a variety of plant pathogens. The GA-producing strain *S. violaceusniger* YCED-9 was shown to antagonize the growth of seven fungal pathogens of turfgrass *in vitro* (Trejo-Estrada et al., 1998b). In greenhouse studies, this strain partially controlled grass seedling disease caused by *Rhizoctonia solani* and crown-foliar disease caused by *Sclerotinia homeocarpa* (Trejo-Estrada et al., 1998a). *S. hygroscopicus* spp. *geldanus* strain EF-76, was

shown to inhibit the causal agent of potato common scab, *Streptomyces scabies*, under field conditions, but did not affect potato yield (Beauséjour et al., 2003). In light of our GA results, these strains may have promise as biocontrol agents for nematodes as well.

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RAPID COMMUNICATION

SELECTIVE AND pH-DEPENDENT BINDING OF A MOTH PHEROMONE TO A PHEROMONE-BINDING PROTEIN

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Abstract—Fluorescence and circular dichroism (CD) data suggest that the major pheromone-binding protein (PBP) from the wild silkmoth, *Antheraea polyphemus*, ApolPBP1, undergoes a pH-dependent conformational change similar to that previously observed for the PBP from the silkworm moth, *Bombyx mori*, BmorPBP. All three constituents of the sex pheromone, E6,Z11-16Ac, E6,Z11-16Ald, and E4,Z9-14Ac, bound to ApolPBP1 with apparent high affinity at high pH, but reduced binding at low pH when tested individually in a "cold binding assay." In competitive assays, however, ApolPBP1 showed considerable preference for the major constituent of the sex pheromone, E6,Z11-16Ac. These data suggest that specificity of PBPs contributes at least in part to the remarkable selectivity of moth's olfactory system.

Key Words—pheromone-binding protein, *Antheraea polyphemus*, conformational change, circular dichroism, fluorescence, binding assay

INTRODUCTION

Pheromone-binding proteins (PBPs) assist in the transport of volatile, hydrophobic pheromones through the aqueous milieu separating the external environment from the odorant receptors in insect antennae (reviewed in Leal, 2005). Recent experimental evidence supports the hypothesis that PBPs are essential for the rapid uptake of pheromones from the external environment and

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delivery to the odorant receptors (Leal et al., 2005), but the literature regarding their role in pheromone discrimination is dichotomous (Wojtasek and Leal, 1999; Maida et al., 2000, 2003; Campanacci et al., 2001). For example, it has been found by fluorescence assays (Campanacci et al., 2001) that the three components of *A. polyphemus* sex pheromone, E6,Z11-16Ac, E6,Z11-16Ald, and E4,Z9-14Ac (Kochansky et al., 1975; Meng et al., 1989), were bound by the moth's major PBP, ApolPBP1 (Maida et al., 2000), with similar affinity (Campanacci et al., 2001). On the other hand, ApolPBP1 showed preferential binding to E6,Z11-16Ac over the other constituents of the sex pheromone in two different assays with radiolabeled pheromones (Maida et al., 2003). Ligand binding activity by *Bombyx mori* PBP, BmorPBP, was reduced at low pH because of a pH-dependent conformational change (Wojtasek and Leal, 1999), which is postulated to release the pheromone to the odorant receptor (Leal, 2005). By contrast, no pH influence was found for E6,Z11-16Ac binding to another PBP from the wild silkmoth, ApolPBP3 (Maida et al., 2003). These inconsistencies could be derived in part from different binding assays and recombinant proteins, and/or the unavailability of radiolabeled pheromones, which prevents employing the same standards by different laboratories. The present study was designed to address this controversy by using a recently developed "cold binding assay," which is based on the separation of bound and free ligands by a centrifugal device and does not require radiolabeled compounds (Leal et al., 2005). Here, we report that ApolPBP1 selectively binds the major pheromone constituent in competitive assays and undergoes a pH-dependent conformational change associated with a reduction of ligand binding at low pH.

METHODS AND MATERIALS

Protein Preparation. Recombinant ApolPBP1 was expressed in periplasmic space in *Escherichia coli* using a previously described pET-22b(+) expression system (EMD Biosciences; Wojtasek and Leal, 1999). The protein was extracted from the harvested cells with Tris–HCl 10 mM, pH 8, after three cycles of freeze (liquid nitrogen, 3 min) and thaw (room temperature), centrifuged at 16,000 $\times$ *g* to remove debris, and purified by multiple steps of ion exchange and gel filtration chromatography. Highly purified fractions [>99% monitored by liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI/MS)] were lyophilized and stored at -80°C until use. LC-ESI/MS was performed with a LCMS-2010 (Shimadzu) upgraded with a stainless-steel capillary probe. Chromatographic separations were done on a ZorbaxCB C8 column (150 $\times$ 2.1 mm; 5 μm; Agilent Technologies) with a gradient of water and acetonitrile containing a modifier (2% acetic acid).

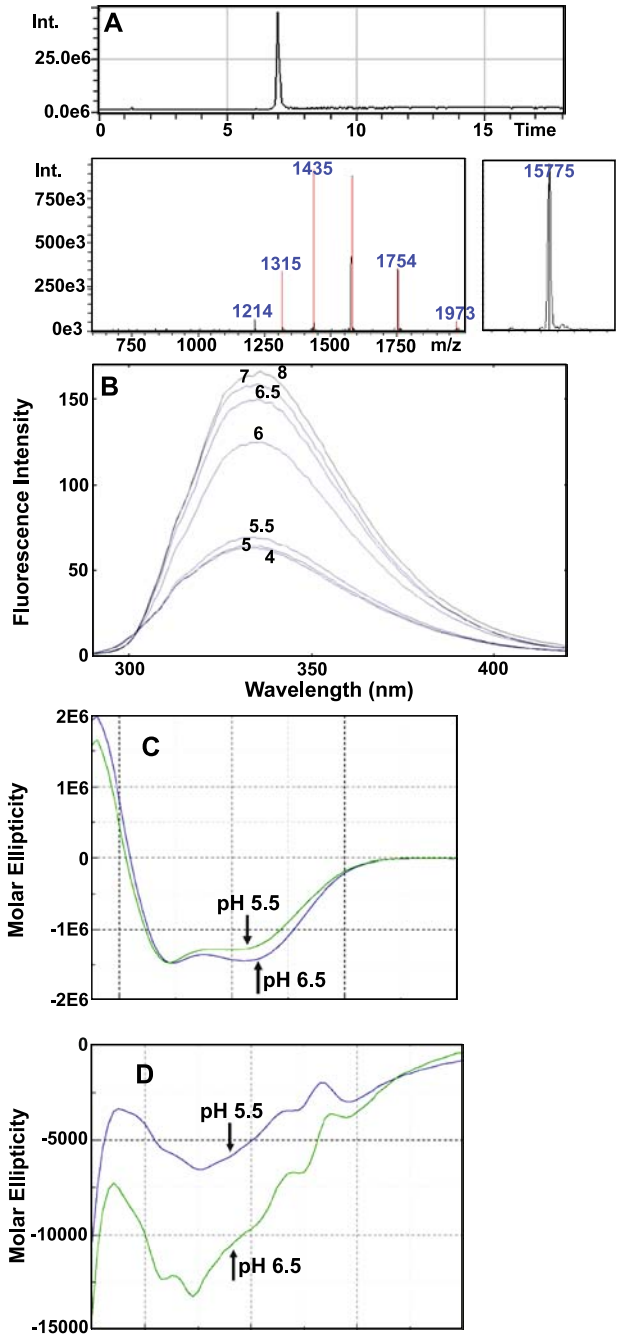
Fluorescence and CD Spectroscopy. Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorometer with 20 μg/ml of ApolPBP1 in 20 mM of one of the following buffers: NaOAc, pH 4–5; NH_4OAc, pH 5.5–7; Tris–HCl, pH 8. The protein solution was excited at 280 nm, and the emission spectra were recorded between 285 and 420 nm. Emission and excitation slit widths were set at 3 nm. Circular dichroism (CD) spectra were recorded by using a J-810 spectropolarimeter (Jasco) with 1 mg/ml or 0.3 mg/l of protein in 20 mM NH_4OAc buffer for near- and far-UV CD, respectively.

Cold Binding Assay. Binding tests were performed by the previously described "cold binding assay" (Leal et al., 2005). E6,Z11-16Ac, E6,Z11-16Ald, and E4,Z9-14Ac were purchased from Plant Research International (The Netherlands) and stocked at −80°C until use. Ethanol solutions (3.2 mM) were prepared after purity was confirmed by GC and GC-MS to be >98% for the acetates and >95% for the aldehyde. For competitive assays, we prepared one ethanol sample with 3.2 mM of each E6,Z11-16Ac, E6,Z11-16Ac, and E4,Z9-14 (Kochansky et al., 1975; Meng et al., 1989). One microliter of the test ligand was added to 50 μl of ApolPBP1 solution (6.2 μM) to make a final ligand-to-protein molar ratio of 10. GC and GC-MS analyses were performed on a 6890 Series GC system and a 5973 Network Mass Selective Detector, respectively. Both instruments were equipped with the same type of capillary column (HP-5MS, 25 m × 0.25 mm; 0.25 μm), which was operated at 100°C for 1 min, increased to 250°C at a rate of 10°C/min, and held at the final temperature for 10 min.

RESULTS AND DISCUSSION

We prepared a sample of recombinant ApolPBP1 by periplasmic expression, an expression system known to generate properly folded, functional PBPs (Wojtasek and Leal, 1999). Purity of rApolPBP1 was higher than 99%, as demonstrated not only by single bands in gel electrophoresis analysis (10 and 15% native PAGE; 15% SDS-PAGE) but also by LC-ESI/MS (Figure 1A). Both high-performance liquid chromatography trace and MS profile indicated a single protein peak with a molecular weight of 15,775 Da. This is in agreement

FIG. 1. (A) Mass spectral data for purified recombinant ApolPBP1. HPLC separation, mass spectrum of the major LC peak, and its deconvolution data (molecular mass of 15,775 Da). (B) Intrinsic fluorescence data indicating a great conformational transition between pH 6.5 and 5.5. (C) Far-UV CD and (D) near-UV CD spectra at pH 6.5 and 5.5 indicating minimal difference in helical content and a major change in the 3D conformation of the protein, respectively.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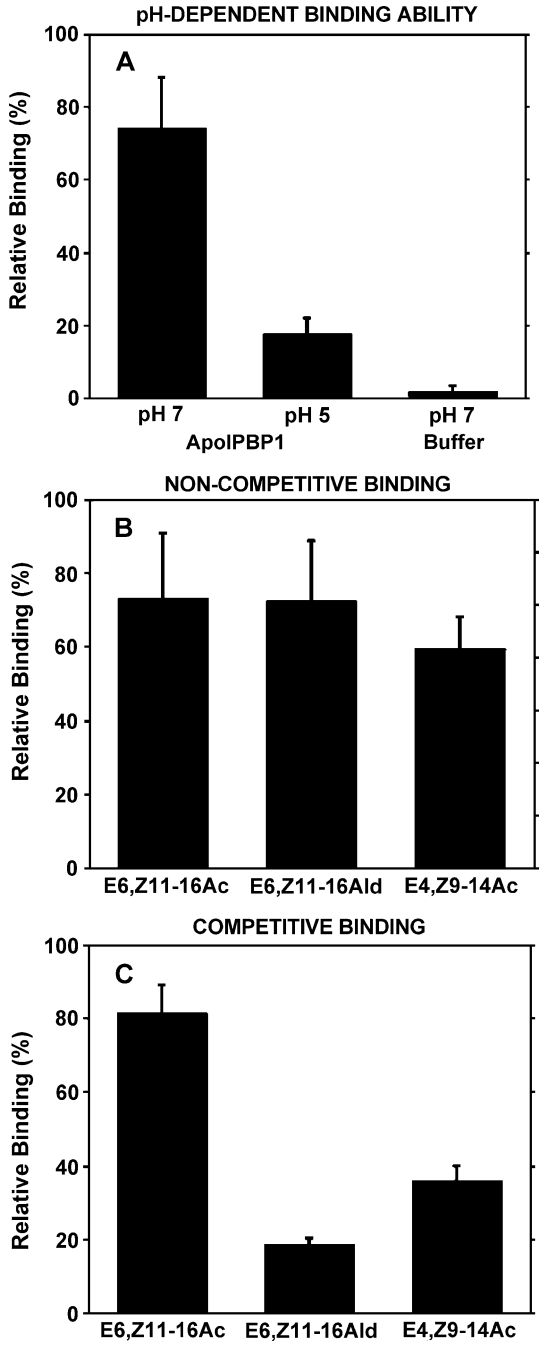
with the calculated molecular mass of ApolPBP1 (15,776 Da), considering the formation of three disulfide bridges.

Change in intrinsic fluorescence suggests a conformational transition between pH 6.5 and 5.5, with pronounced decrease of fluorescence signal between pH 6 and 5.5 (Figure 1B). Clearly, the large transition takes place above the p*I* (calcd., 4.74), whereas no significant change was observed when the pH was decreased below the p*I* (Figure 1B). Next, we obtained the far- and near-UV circular dichroism (CD) spectra of ApolPBP1. The far-UV CD spectrum at pH 6.5 (Figure 1C) indicated that ApolPBP1 is a helix-rich protein, in agreement with the reported NMR structure (Mohanty et al., 2004). Similarly to what has been found in BmorPBP (Wojtasek and Leal, 1999), the far-UV CD spectrum at low pH differed from the spectrum at high pH only in the minimal at ca. 228 nm (Figure 1C). Change in near-UV CD spectra at pH 6.5 and 5.5 (Figure 1D) is also strikingly similar to what has been observed in BmorPBP (Wojtasek and Leal, 1999). These findings are not consistent with the hypothesis that ApolPBP1 undergoes an overall conformational change involving most residues (Mohanty et al., 2004). The helical contents, nearly unchanged at low and high pH (Figure 1C), suggest that ApolPBP1 undergoes a pH-mediated helix formation compensated by some unwinding such as the formation of a C-terminus α-helix and unwinding of the N-terminus helix, as it has been documented for BmorPBP (Horst et al., 2001).

The pH-dependent conformational change in ApolPBP1 is reflected in binding affinity. ApolPBP1 binds E6,Z11-16Ac with apparent higher affinity at high pH and lower affinity at low pH (Figure 2A), as opposed to what has been observed for the association of the same ligand with ApolPBP3 (Maida et al., 2003). Next, we tested the specificity of this protein toward the three components of the sex pheromone system. When one ligand was tested at a time, ApolPBP1 bound to E6,Z11-16Ac, E6,Z11-16Ald, and E4,Z9-14Ac with the same apparent affinity (Figure 2B), but reduced binding at low pH (data not shown). However, in competitive assays in which the protein was incubated with equal amounts of the three compounds, ApolPBP1 selectively bound the major pheromone component, showed lower affinity for the shorter acetate, and had almost no affinity for the aldehyde (Figure 2C).

In summary, ApolPBP1 undergoes a pH-mediated conformational change, probably structurally related to what has been unveiled in BmorPBP. Whereas the basic form of ApolPBP1 bound the major pheromone constituent at the

FIG. 2. (A) Binding of ApolPBP1 at pH 7 and reduced binding at low pH. (B) When tested individually, ApolPBP1 bound all three constituents of the sex pheromone. (C) When subjected to an equal mixture of all three ligands at the same time in competitive assays, ApolPBP1 showed preferential binding to the major constituent of the sex pheromone, E6,Z11-16Ac.


pH-DEPENDENT BINDING ABILITY
A
Relative Binding (%)
100
80
60
40
20
0
pH 7
pH 5
pH 7
ApoIPBP1
Buffer
NON-COMPETITIVE BINDING
B
Relative Binding (%)
E6,Z11-16Ac
E6,Z11-16Ald
E4,Z9-14Ac
COMPETITIVE BINDING
C
Relative Binding (%)
E6,Z11-16Ac
E6,Z11-16Ald
E4,Z9-14Ac

sensillar lymph pH, binding is prevented or reduced in the acidic form. Assay methods testing one protein and one ligand at a time may conceal binding specificity. Under physiological conditions, PBPs encounter complex mixtures of compounds bombarding the sensilla. If they filter out some of these potential ligands, as the competitive assay suggests, the remarkable selectivity of the insect olfactory system does not have to rely entirely on the odorant receptors.

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CALIFORNIA GROUND SQUIRREL (*Spermophilus beecheyi*) DEFENSES AGAINST RATTLESNAKE VENOM DIGESTIVE AND HEMOSTATIC TOXINS

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Abstract—Previous studies have shown that some mammals are able to neutralize venom from snake predators. California ground squirrels (*Spermophilus beecheyi*) show variation among populations in their ability to bind venom and minimize damage from northern Pacific rattlesnakes (*Crotalus oreganus*), but the venom toxins targeted by resistance have not been investigated. Four California ground squirrel populations, selected for differences in local density or type of rattlesnake predators, were assayed for their ability to neutralize digestive and hemostatic effects of venom from three rattlesnake species. In Douglas ground squirrels (*S. b. douglasii*), we found that animals from a location where snakes are common showed greater inhibition of venom metalloprotease and hemolytic activity than animals from a location where snakes are rare. Effects on general proteolysis were not different. Douglas ground squirrels also reduced the metalloprotease activity of venom from sympatric northern Pacific rattlesnakes (*Crotalus oreganus oreganus*) more than the activity of venom from allopatric western diamondback rattlesnakes (*C. atrox*), but enhanced fibrinolysis of sympatric venom almost 1.8 times above baseline levels. Two Beechey ground squirrel (*S. b. beecheyi*) populations had similar inhibition of venoms from northern and southern Pacific rattlesnakes (*C. o. helleri*), despite differences between the populations in the locally prevalent predator. However, the venom toxins inhibited by Beechey squirrels did vary among venom from Pacific rattlesnake subspecies, and between these venoms and venom from allopatric western diamondback rattlesnakes. Blood plasma from Beechey squirrels showed highest inhibition of metalloprotease activity of northern Pacific rattlesnake venom, general proteolytic activity and hemolysis of southern Pacific

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rattlesnake venom, and hemolysis by allopatric western diamondback venom. These results reveal previously cryptic variation in venom activity against resistant prey that suggests reciprocal adaptation at the molecular level.

Key Words—*Spermophilus beecheyi*, *Crotalus oreganus*, *Crotalus atrox*, venom, natural resistance, coevolution, predator–prey.

INTRODUCTION

Some species of mammals are known to be resistant to the venom of the viperid snakes that hunt them (Thwin and Gopalakrishnakone, 1998; Pérez and Sánchez, 1999). Viper venoms contain toxins with a variety of effects, including metalloproteases that break down the lining of blood vessels and extracellular matrix (Gutiérrez and Rucavado, 2000). Other venom components disrupt the normal processes of coagulation and induce hemolysis (Markland, 1998; Braud et al., 2000). In susceptible prey (and humans), venoms can lead to extensive hemorrhage, edema, tissue necrosis, and death (Russel, 1980; Ownby, 1982). Even if prey successfully evade ingestion by a viperid predator, venom-induced shock and the digestive effects of toxins pose a serious threat. To maintain the ability to survive and reproduce in an environment that holds challenges from predators, competitors, and conspecifics, mammals must minimize hemorrhage, tissue destruction, and disruptions to hemostasis that follow envenomation.

The interactions of California ground squirrels (*Spermophilus beecheyi*) and northern Pacific rattlesnakes (*Crotalus oreganus oreganus*) may be the most intensively studied example of the relationship between rattlesnakes and preferred prey. Ground squirrels are an important food resource for rattlesnakes throughout California (Linsdale, 1946; Fitch, 1948). Fitch (1949) estimated that *S. beecheyi* make up to 69% (by weight) of the diet for adult northern Pacific rattlesnakes (*C. o. oreganus*) at one site in the foothills of the Sierra Nevada. Field observations reveal that northern Pacific rattlesnakes are common near burrow systems during the reproductive season and periods of pup emergence, and are responsible for approximately 40% of pup and juvenile mortality in ground squirrel colonies (Linsdale, 1946; Fitch, 1948; personal observation). Sympatry between California ground squirrels and Pacific rattlesnakes is indicated in late Pleistocene fossil assemblages (Miller, 1912; Stock, 1918; Brattstrom, 1953). Elsewhere in the United States, middle Miocene fossil deposits indicate longer sympatry between rattlesnakes and the *Otospermophilus* ancestor of modern sciurids (Black, 1963; Holman, 1979). Therefore, the intensity of this predator–prey relationship is probably not a recent phenomenon, and represents an important biotic interaction for both ground squirrels and rattlesnakes.

It is not surprising, therefore, that *S. beecheyi* exhibit a variety of strategies to deter rattlesnake predation. Beyond a primary defense of vigilance, these squirrels will aggressively confront snake predators detected near their burrows. Prolonged staring, conspicuous tail flagging, substrate throwing, and biting are used to discourage hunting snakes, and provoke defensive displays such as hissing or rattling that aid in assessment of the danger posed by snake predators (Owings and Coss, 1977; Coss and Owings, 1989; Rowe and Owings, 1990, 1996; Swaisgood et al., 1999). However, active harassment requiring close approach to rattlesnakes exposes squirrels to the risk of envenomation. To protect against this risk, California ground squirrels possess innate blood plasma factors that allow them to survive envenomation by northern Pacific rattlesnakes (Poran et al., 1987; Poran and Coss, 1990). Serum–venom binding in radioimmunoassay (RIA) has been used extensively to characterize variation in resistance among *S. beecheyi* populations (Poran et al., 1987; Coss et al., 1993). Unfortunately, RIA binding levels provide only indirect information about functional differences in venom neutralization among *S. beecheyi* populations. Although the RIA binding of pooled sera from squirrel populations correlates highly with the same sera used to protect envenomated mice in approximate LD_{50} tests (Poran et al., 1987), this method does not reveal the underlying biochemical dynamics of toxicity and resistance. Enzyme–inhibitor binding, such as that described for the resistance of opossum to rattlesnake venom (Catanese and Kress, 1993), is indistinguishable from a variety of other protein–protein interactions resulting in high binding levels (Mishell and Shiigi, 1980).

Experimental Questions. Adult northern Pacific rattlesnakes (*C. o. oreganus*) possess venoms with multiple proteolytic and hemolytic venom toxins that ground squirrel prey must defend against (Mackessy, 1988, 1993, 1996). Biardi et al. (2000) showed that plasma from California ground squirrels can reduce the activity of venom proteases on hide powder azure and gelatin. However, this study did not specifically measure the activity of venom metalloproteases or toxins affecting clotting and hemolysis. A more detailed understanding of the biochemical basis of resistance is essential when attempting to clarify the relationship between RIA binding levels and functional resistance to venom. For example, previous research has revealed conflicting evidence about resistance in two populations of Douglas ground squirrels (*S. b. douglasii*) that vary in local rattlesnake density. One location (Winters, Yolo County, CA) is in oak woodland habitat in the foothills on the west side of the Sacramento Valley where rattlesnakes are common; this site also holds a winter hibernacula for rattlesnakes (W. Hamilton, personal communication). As expected, squirrels from this location show high RIA binding levels (Coss et al., 1993). A nearby site (Davis, Solano Co.) is on the floor of the valley less than 20 km to the east where rattlesnakes are extremely rare (Poran et al., 1987). RIA binding levels in

Davis squirrels are 59% lower, which Coss et al. (1993) interpreted as a decline in resistance correlated with colonization of this rattlesnake-rare habitat when the populations diverged about 8.6 ± 5.9 kyr. However, Biardi et al. (2000) showed that plasma from these two populations are indistinguishable in their capacity to inhibit proteolytic activity of northern Pacific rattlesnake venom. One interpretation of this discrepancy is that the correlation between RIA and approximate LD_{50} dose, determined using squirrels from sites where snakes are common (Poran et al., 1987), may not be an informative measure of resistance in squirrel populations where encounters with rattlesnakes are rare. An alternative interpretation is that reduced inhibition of other venom toxins is responsible for lower binding in the Davis population. One goal of this study is to use three additional measures of venom activity (metalloprotease activity, fibrinolysis, and hemolysis) in order to determine whether decreased RIA binding reflects venom resistance or nonfunctional binding interactions.

Additional measures of venom biochemical activity can also provide more detailed information on how ground squirrel plasma defenses deal with venoms from different rattlesnake lineages. Other mammal species seem to be broadly resistant to toxins from a variety of venom. For example, Soto et al. (1988) showed that sera from opossum (*Didelphis virginiana*) and woodrats (*Neotoma micropus*) were effective at preventing hemorrhage by all 47 species of snakes they examined; Huang and Pérez (1980) measured resistance in cotton rats (*Sigmodon hispidus*) to nine viperid venoms; and Tomihara et al. (1990) showed that mongoose (*Herpestes edwardsii*) are resistant to hemorrhagic effects of 28 snake venoms. However, northern Pacific rattlesnakes (*C. o. oreganus*) are the only pit vipers sympatric with ground squirrels in northern and central California (Klauber, 1972). Folsom Lake in the foothills of the Sierra Nevada east of Sacramento lies within the core of this range, and has a high density of northern Pacific rattlesnakes (Poran et al., 1987). Ground squirrels (*S. b. beecheyi*) from this site show high resistance when measured by experimental envenomation, *in vivo* protection of venom-challenged mice (Poran et al., 1987), and RIA binding levels (Poran and Coss, 1990). Squirrels from the Santa Ynez Valley inhabit snake-rich location southwest of the Central Valley within the zone of intergradation between northern (*C. o. oreganus*) and southern Pacific (*C. o. helleri*) rattlesnakes (Klauber, 1972). Squirrels from these two regions show differences in allozyme frequencies (Goldthwaite, 1989), reflecting divergence during the late Pleistocene (Coss and Goldthwaite, 1995; Coss, 1999). They have also been subject to predation from simultaneously diverging lineages of Pacific rattlesnake predators (Pook et al., 2000; Ashton and de Queiroz, 2001).

Mackessy (1988) found no significant differences between venoms from northern and southern Pacific rattlesnake venoms collected from Santa Barbara, San Luis Obisbo, and Ventura Counties across several assays of enzymatic

activity and toxicity (to lizards). Similarly, Bee et al. (2001) detected only minor differences in metalloprotease activity between these two venoms. However, genetic divergence and geographic separation in squirrel populations suggests the opportunity for coevolutionary change (Thompson, 1994, 1997; Nuismer et al., 1999; Gomulkiewicz et al., 2000) that may affect the ability of plasma defenses to deal with venom from these two subspecies. In other words, we expect plasma defenses in squirrels from Folsom Lake to be more effective against northern Pacific rattlesnake venom. Conversely, plasma defenses in squirrels from the Santa Ynez valley should be more effective against southern Pacific rattlesnake venom. Finally, venom from western diamondback rattlesnakes (*C. atrox*) constitutes a novel challenge to plasma defenses of squirrels in both of these locations. This venom also shows proteolytic, hemorrhagic, and hemolytic activity (Soto et al., 1988), but western diamondback rattlesnakes are a lower Sonoran species restricted to the deserts of the southeastern part of California (Klauber, 1972). We know that California ground squirrel plasma inhibits *C. o. oreganus* proteolytic activity more than *C. atrox* activity (Biardi et al., 2000), and we expect a similar result when measuring effects on other venom toxins. Therefore, a second goal of this study was to assess the specificity of plasma defenses by challenging them with venom from sympatric and allopatric predators.

METHODS AND MATERIALS

Blood Collection and Storage. We collected blood samples from ground squirrels in four locations that varied in the density or identity of rattlesnake predators. The method of determining local snake density (~predation intensity) is described by Poran et al. (1987), supplemented by information on rattlesnake distributions prior to extensive human disturbance of the Central Valley (Grinnell and Camp, 1917; Nussbaum et al., 1983).

Two populations of Douglas squirrels (*S. b. douglasii*) were sampled: Winters (Yolo Co.) is in the Coast Range foothills and has a high density of northern Pacific rattlesnake predators (*C. o. oreganus*); the Davis site (Yolo Co.) is on the floor of the Sacramento valley where rattlesnakes are extremely rare (only two *C. o. oreganus* reported during the last 25 years). Two populations of Beechey squirrels (*S. b. beecheyi*) were sampled: Folsom Lake State Recreation Area (El Dorado Co.) has a high density of northern Pacific rattlesnakes (*C. o. oreganus*); Sedgwick Natural History Reserve (Santa Barbara Co.) in the Santa Ynez valley also has a high density of rattlesnakes, and lies within the range of southern Pacific rattlesnakes (*C. o. helleri*). The number of individuals collected at each location were as follows: Davis = 11; Winters = 16; Folsom Lake = 10; Sedgwick Reserve = 9.

Squirrels were live-trapped and anesthetized with ketamine–HCl (50 mg/kg, i.m.). Approximately 3 ml of whole blood were collected from individual adults via cardiac puncture and stored in additive-free Vacutainer blood collection vials (Becton Dickinson, Irvine, CA, USA). Samples were maintained at 4°C overnight, then clots were removed and discarded. The supernatant was centrifuged at 3000 *g* for 10 min at 4°C to separate out any remaining erythrocytes, then stored at −70°C until used.

Venom Source and Preparation. Lyophilized venoms were purchased from Sigma (St. Louis, MO, USA). Although venoms were not collected from the same sites as squirrel plasma samples, venoms from Pacific rattlesnakes were obtained from regions within the geographic range of California ground squirrels (Hall, 1981). According to the supplier, northern Pacific rattlesnake (*C. o. oreganus*), venom was obtained from multiple snakes originating in northern California and Oregon. Venom from southern Pacific rattlesnakes (*C. o. helleri*) was obtained from snakes originating in southern California. Information on the site(s) of origin for western diamondback (*C. atrox*) venom was unavailable.

Venoms were reconstituted at a concentration of 10 mg/ml in a buffer containing 20 mM Tris–HCl + 1 mM $CaCl_2$, pH 8.0, and stored at −20°C until use.

Assays of Venom Toxicity. Four assays examined the effects of ground squirrel plasma in inhibiting the proteolytic, collagen hydrolysis, hemolytic, and fibrinolytic activity of rattlesnake venom. Agarose–gelatin plates were prepared according to the method of Palmer (1993) and used as a measure of total venom proteolytic activity. A template was used to punch equally spaced 3-mm-diam wells in the surface of the gel, and wells were loaded with 20 μl of sample. The gel was then incubated at 37°C for 24 hr in a humidified chamber. Following incubation, unhydrolyzed gelatin was precipitated in a saturated ammonium sulfate solution at 70°C for 10 min, and the area of hydrolysis was calculated from the product of two perpendicular diameters across the clear lysis zone.

Snake venom metalloprotease activity was quantified using Azocoll (Sigma) as a collagen substrate to model the effects on mammalian connective tissue and extracellular matrix. Aliquots (1 ml) of 1% Azocoll (w/v) solution in 20 mM Tris–HCl, pH 8.0 were prepared in a microcentrifuge tube. Sample (20 μl) was added, and the reaction mix was incubated at 37°C for 2 hr with occasional agitation. Following incubation, the tubes were centrifuged for 2 min at 16,000 *g* to pellet unreacted substrate. The absorbance of the supernatant was determined at 520 nm using a Shimadzu UV160 spectrophotometer.

BBL Stacker plates (Carolina Biological Supply) containing 5% defibrinated sheep's blood in Columbia agar were used to assay for hemolytic activity. Wells (3 mm diam) were punched in the substrate in a grid pattern. Samples (20 μl) were loaded into each well, then the plate was sealed with parafilm and incubated at 37°C for 24 hr. The area of hemolysis, defined as the transparent

zone around the well cleared of red blood cells, was calculated from two perpendicular measurements of well diameter.

Fibrin–agarose plates prepared by a modification of the method of Astrup and Müllertz (1952) were used to measure venom fibrinolytic activity. Bovine fibrinogen was dissolved in Tris–HCl, pH 8.0, to a concentration of 1 mg/ml using a sonicator bath. Ten milliliters of fibrinogen solution was placed in a 100 × 15 ml Petri dish and clotted by the addition of 100 μl of thrombin (100 units/ml). Clot formation was completed within 30 min, as determined by the opacity of the fibrinogen solution. Sample treatments (20 μl) were applied to the surface of the clot, and the plate was incubated at 37°C for 24 hr in a humidified chamber. Following incubation, the area of hydrolysis was calculated from the product of two perpendicular diameters across the liquefied lysis zone.

Relative Activity Scores and Statistical Analyses. Plasma samples from individual squirrels were screened against relevant venoms, and individual scores were recorded for each of the four assays as the mean of at least three replicates. Samples for each assay consisted of a venom-only treatment (10 μl venom + 10 μl buffer) and a venom + plasma treatment (10 μl venom + 5 μl plasma + 5 μl buffer), as well as negative controls (buffer only and plasma only). The difference in activity between venom-only and venom + plasma treatments was expressed as a ratio:

$$\text{Relative activity} = \frac{\text{activity of [venom + plasma]}}{\text{activity of [venom only]}}$$

This allows for testing qualitative differences in the outcome of interactions between venom toxins and plasma defensive factors across the four assays. It simultaneously adjusts for differences in scaling due to differences among venoms in baseline activity on these assays (Soto et al., 1988).

Because relative activity scores are proportions, they were subject to arcsine transformation prior to statistical analysis to yield measures that followed an approximately normal distribution (Zar, 1984). Data were analyzed using a one-factor between-subjects (populations) and two-factor within-subjects (assay type, venom type) repeated measures ANOVA. Specific comparisons of population and venom effects were tested with planned contrasts of the relevant group means.

RESULTS

Plasma Defenses in Douglas Ground Squirrels (S. b. douglasii). Effects of Douglas ground squirrel plasma on venom activity are shown in Figure 1. The

two populations differed significantly in their ability to inhibit venom from the northern Pacific rattlesnake ($F_{3,18} = 18.96$, $P < 0.0001$). This was primarily due to significantly higher inhibition by animals from Winters (where snakes are common) in the collagenase assay ($F_{1,20} = 56.97$, $P < 0.001$). Winters animals were also slightly more effective at reducing venom hemolytic activity ($F_{1,20} = 7.25$, $P = 0.014$), although the mean difference was small—a change in relative activity of only 2.4% of baseline venom activity. There were no significant

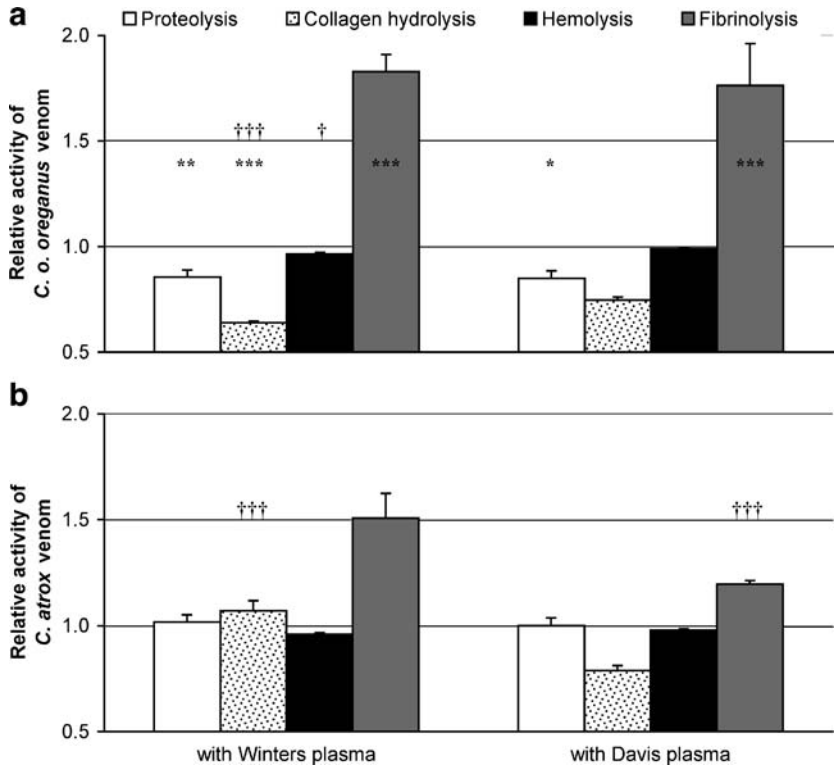

FIG 1. Activity (mean ± SE) of rattlesnake venoms incubated with plasma samples from two populations of Douglas ground squirrels (*S. b. douglasii*) that vary in local density of northern Pacific rattlesnakes (*C. o. oreganus*). Winters is a location where rattlesnakes are common, whereas Davis is a site where snakes are extremely rare. Western diamondback rattlesnakes (*C. atrox*) are allopatric with both populations of squirrels. Activity is expressed relative to venom-only controls as described in Methods and materials. Daggers indicate significant differences between the two squirrel populations (†$P < 0.05$; †††$P < 0.001$). Asterisks indicate significant differences between the two venoms (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

differences between plasma samples from Davis and Winters squirrels in their effects on general proteolytic activity of northern Pacific rattlesnake venom. The two populations also did not differ in their effect on fibrinolysis; in this case, relative activity increased to almost 1.8 times that of venom-only treatments (Figure 1a, b).

There were also significant differences in the effects of plasma samples on sympatric and allopatric venoms. Winters animals reduced proteolysis ($F_{1,25}$ = 17.21, P = 0.003) and collagen hydrolysis ($F_{1,20}$ = 115.45, $P < 0.001$) of northern Pacific rattlesnake venom more than western diamondback venom. Plasma samples from Davis animals were also more effective at reducing proteolytic activity of *C. o. oreganus* venom ($F_{1,25}$ = 6.44, P = 0.018), but showed no difference in their effect on collagen hydrolysis by the two venoms ($F_{1,26}$ = 0.67, $P > 0.05$). Plasma was less effective against *C. o. oreganus* venom than against *C. atrox* venom in the fibrinolytic assay ($F_{1,20}$ = 16.12, $P < 0.001$). There was no significant difference between venoms in the hemolytic assay ($F_{1,25}$ = 1.24, $P > 0.05$).

Plasma Defenses in Beechey Ground Squirrels (S. b. beecheyi). There were no differences between plasma from Folsom Lake and Sedgwick in their effects on venom proteolysis, collagen hydrolysis, or hemolysis (all tests: $P >$ 0.05) (Figure 2). Although plasma from Sedgwick squirrels generally induced higher relative activity in the fibrinolysis assays than Folsom plasma samples, this difference also was not statistically significant ($F_{1,10}$ = 1.43, $P > 0.05$). Consequently, data from the two Beechey populations were combined for analysis of the effects of plasma defenses across different venoms.

Beechey squirrel plasma varied in effectiveness against general proteolytic activity of the three venoms ($F_{2,32}$ = 19.8, $P < 0.001$)—Southern Pacific rattlesnake (*C. o. helleri*) venom was inhibited more than northern Pacific rattlesnake venom ($F_{1,16}$ = 11.09, P = 0.004), which in turn was inhibited more than western diamondback venom ($F_{1,16}$ = 18.24, $P < 0.001$). In contrast, Beechey plasma samples reduced northern Pacific rattlesnake metalloprotease activity more than southern Pacific ($F_{1,17}$ = 5.843, P = 0.032) or western diamondback ($F_{1,17}$ = 5.536, P = 0.031) venom. Hemolytic activity of western diamondback venom was reduced more than that of the two Pacific rattlesnake venoms ($F_{2,10}$ = 8.03, P = 0.008). There were no significant differences in the way Beechey plasma samples affected fibrinolysis by the three venoms.

DISCUSSION

Although California ground squirrel sera-to-venom binding level in RIAs correlates highly with approximate LD_{50} in mice, this method does not directly

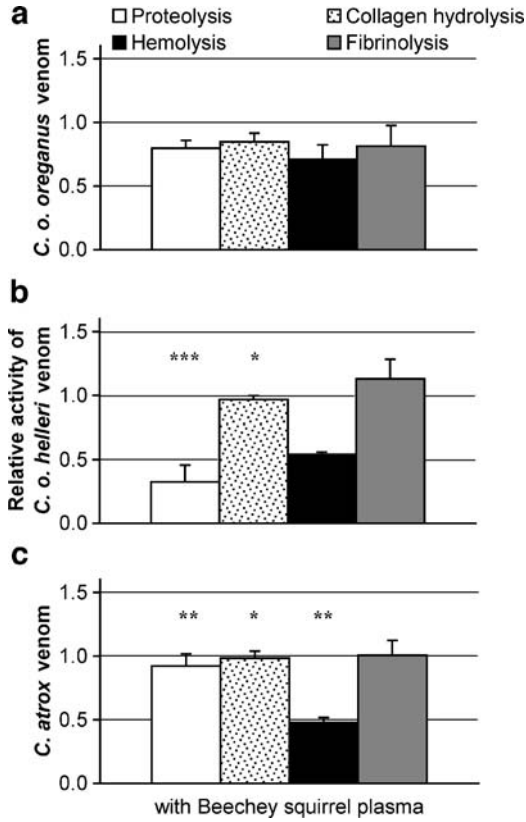

FIG 2. Activity (mean ± SE) of rattlesnake venoms incubated with plasma samples from two populations of Beechey ground squirrels (*S. b. beecheyi*) that are subject to predation by Pacific rattlesnakes. Folsom Lake is a site where northern Pacific rattlesnakes (*C. o. oreganus*) are common, whereas the Sedgwick Reserve is a site within the zone of intergradation between northern and southern Pacific (*C. o. helleri*) rattlesnakes. Western diamondback rattlesnakes (*C. atrox*) are allopatric with both populations of squirrels. Activity is expressed relative to venom-only controls as described in Methods and materials. Asterisks indicate significant differences from the effect on *C. o. oreganus* venom (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

measure the ability of prey sera to inhibit specific venom toxins. We observed distinct differences in the capacity of plasma proteins from California ground squirrels to affect the proteolytic and hemolytic activities of rattlesnake venoms. The protease, metalloprotease, and hemolytic activity of all three venoms were reduced in the presence of prey plasma from one or more ground squirrel

populations. However, Douglas squirrels are unable to decrease venom fibrinolysis. In fact, fibrinolytic activity of northern Pacific rattlesnake venom is almost twice as great when incubated with plasma from Douglas squirrels (Figure 1a) than when assayed alone (relative activity = 1.0) or against Beechey squirrel plasma (Figure 2a). Fibrinolysis may result from plasmin-like activity of venom toxins or from activation of endogenous serum components (Hofmann and Bon, 1987; Braud et al., 2000). Increased activity of venom when incubated with serum suggests that venom not only maintains effectiveness in the face of prey defenses, but can also capitalize on the biochemical properties of prey tissues. RIA binding levels, therefore, may represent a multitude of interactions between venom and prey tissues that simultaneously reduce and enhance the toxic activities of venom. Radioimmunoassays have been valuable in screening for population differences in venom resistance in California ground squirrels (Poran et al., 1987; Poran and Coss, 1990; Coss et al., 1993). However, assays of venom + plasma interactions clarify the functional basis of differences in binding levels, and will be more valuable in understanding the consequences of variation in prey defenses.

In contrast to enhanced fibrinolytic activity in the presence of Douglas blood plasma, samples from all four populations significantly reduced the metalloprotease activity of northern Pacific rattlesnake (*C. o. oreganus*) venom (Figures 1a and 2a). This is consistent with our understanding of antihemorrhagic factors identified in other mammalian prey of viperid snakes, which have been identified as snake venom metalloprotease inhibitors (SVMPI) (Pérez and Sánchez, 1999). The ability to inhibit hemorrhagic toxins appears to be a convergent response to envenomation, perhaps because the major threat of viper venoms, hemorrhage *in rhexis*, is thought to result from hydrolysis of collagen-rich connective tissue lining the capillary endothelium (Baramova et al., 1989; Tu, 1991). Minimizing the ability of venom toxins to rupture blood vessels can have a cascade of multiple subsequent protective benefits—including reduced access of other venom toxins to target tissues, reduced edema, and continued circulation of blood to regions near the bite—thereby enhancing tissue repair and minimizing necrosis (Gutiérrez and Rucavado, 2000; Anai et al., 2002). However, it is surprising that the metalloprotease activity of southern Pacific and western diamondback venoms was relatively unaffected, because defenses specialized to one or a few venoms seems to be rare. For example, Soto et al. (1988) showed that sera from opossum (*D. virginiana*) and woodrats (*N. micropus*) was effective at preventing hemorrhage by all 47 species of snakes they examined. California ground squirrels encounter only a single rattlesnake species (*C. o. oreganus*) from central California northward to British Columbia; we suspect that coevolution may have favored plasma defenses in populations from the Central Valley that are especially effective against venom from this species.

Generalized resistance to venom metalloproteases is similar to the pattern observed in animals from the Davis site, where rattlesnakes are extremely rare (cf. Figure 1a, b). Compared to the nearby Winters population, plasma factors were not as effective against the local rattlesnake predator (*C. o. oreganus*), but maintained some effectiveness against western diamondback (*C. atrox*) venom. Antihemorrhagic proteins isolated from other mammals show homology to proteins in the immunoglobin supergene family (Perales and Domont, 2002), which includes plasma serpins and other protease and metalloprotease inhibitors. Catanese and Kress (1993) identified differences in the amino acid sequence of oprin (the antihemorrhagic factor from *D. virginiana*) that distinguish it from other mammalian α_1-proteinase inhibitors. Presumably because of these differences, oprin can bind and inhibit venom metalloproteases while these toxins rapidly hydrolyze human α_1 proteinase. We suspect that consistent selection from northern Pacific rattlesnake toxins in the Winters, Folsom Lake, and Sedgwick Reserve animals has favored sequence changes in ground squirrel SVMPI that maximize effectiveness against this venom. As venom metalloproteases are also subject to selection favoring escape from inhibition by prey defenses, reciprocal changes may have led to SVMP and SVMPI variants unique to these species. Reactive center sequences in plasma serpins are hypervariable and affect protease recognition and inhibition effectiveness (Hill and Hastie, 1987; Goodwin et al., 1996). It will be interesting to see whether Davis dwellers, and squirrels from other sites where snakes are rare or absent, possess plasma factors with a reactive site sequence similar to the precursor of SVMPIs in this species. Such sequences may confer some level of protection against xenologous metalloproteases, including those from a variety of snake venoms, but may not represent a form optimized to inhibit SVMP from the lone sympatric species, northern Pacific rattlesnakes.

The contrast between squirrel blood plasma effects on metalloprotease and fibrinolytic activity may reflect alternative solutions to venom toxicity by rattlesnakes and ground squirrels. Although ground squirrel plasma factors can reduce the activity of venom metalloproteases, rattlesnakes may still benefit by immobilizing prey as they disrupt hemostasis. Elevated fibrinolytic activity of *C. o. oreganus* venom may aid snakes in subduing prey by the induction of hypotensive shock if internal bleeding after envenomation is prolonged (Mackessy, 1993; Mackessy et al., 2003). This would increase the likelihood of obtaining a valuable meal, as long as sufficient SVMP activity is maintained to facilitate the rupture of prey viscera after ingestion (Thomas and Pough, 1979). From the squirrel's perspective, avoidance of tissue damage from digestive venom toxins may be the crucial task of plasma defenses because behavioral defenses may buffer against the risk posed by venom-induced shock. Recruitment of other colony members to mob rattlesnakes (Owings and Coss, 1977; Hersek and Owings, 1993) may minimize the opportunity for snakes to

ingest envenomated squirrels, even if they are temporarily immobilized. In this case, the digestive effects of metalloproteases may pose a greater threat to fitness because of the potential for lasting damage to tissues. Studies of inhibition by plasma from rock squirrels, a closely related species that also shows aggressive confrontation of rattlesnake predators (Owings et al., 2001), likewise indicates that metalloprotease inhibition is of primary importance (Biardi, 2000).

Our four measures of venom activity also revealed significant differences between Pacific rattlesnake venoms when preying on the Beechey subspecies of California ground squirrels (*S. b. beecheyi*). We expected to find differences between squirrels from the Folsom and Sedgwick sites, but were unable to distinguish between them using any of the four assays of resistance. Instead, we observed striking differences between venoms. Northern Pacific rattlesnake venom maintained greater activity in the general proteolytic and hemolytic assays, whereas southern Pacific rattlesnake venom maintained greater effectiveness in the metalloprotease and fibrinolysis assays (cf. Figure 2a, b). This was surprising because previous studies have not identified clear differences in activity between these venoms (Mackessy, 1988; Bee et al., 2001). However, our results show that these venoms pose distinctly different threats to ground squirrels inhabiting a site (such as the Sedgwick Reserve) where they are likely to encounter both snake species.

The role of Douglas squirrel plasma in enhancing fibrinolytic activity, inhibition of venom metalloproteases by ground squirrel SVMPI, and the detection of differences in northern and southern Pacific rattlesnake venoms in the presence of Beechey squirrel plasma all highlight the adaptive context of venoms and resistance. Our assays of venom activity in the presence of resistance factors revealed variation that is not detected in assays of venoms alone, and reinforce the idea that the outcome of envenomation will depend on both predator and prey, and in turn will affect the evolution of both venom toxicity and resistance. Detection of previously cryptic differences between *C. o. oreganus* and *C. o. helleri* venoms may reflect an arms race that has generated variation within and between predators and prey. Relationships among species over evolutionary time can foster reciprocal adaptation of traits crucial to their interaction (Futuyma and Slatkin, 1983). Because populations of California ground squirrels vary in their ability to neutralize venom toxins (Biardi, 2000; Biardi et al., 2000; this study), and because this variation has important implications for the fitness of both predators and prey, both theoretical (e.g., Thompson, 1994, 1997) and modeling (e.g., Gomulkiewicz et al., 2000) approaches to coevolution predict corresponding local variation in venom from northern Pacific rattlesnakes. There are no published studies describing population differences in venom for this species; unfortunately, our use of pooled venoms also does not allow us to detect differences among

rattlesnakes at the population level. However, population variation in venoms related to diet is not unknown. For example, Daltry et al. (1996a,b) demonstrated that population variation in venom composition of the Malayan pit viper (*Calloselasma rhodostoma*) is strongly influenced by diet. However, this study related variation to the relative representation of different prey categories (amphibians, reptiles, and "endotherms") in the diet, making it unclear whether the effect is attributable to basic structural and physiological differences among prey categories, or more specific defenses to venoms such as we observe in ground squirrels. The subsequent debate between Sasa (1999a,b) and Wüster et al. (1999) centering on neutral vs. adaptive explanations for variability in snake venoms highlighted the importance of studying venoms in their natural context. To date, however, studies designed to test the coevolution of venomous snakes and their prey remain rare (e.g., Heatwole and Powell, 1998; Jorge da Silva and Aird, 2001).

Research on snake venom chemistry has an extensive history, driven primarily by interest in the clinical treatment of snakebite and the characterization of toxins in a therapeutic and research context. However, approaches to understanding venoms in a natural context (Mackessy, 1988; Daltry et al., 1996a,b; Kordis and Gubensik, 2000; Creer et al., 2003; Mackessy et al., 2003), especially considering venom resistance (Poran et al., 1987; Poran and Coss, 1990; Coss et al., 1993; Heatwole and Poran, 1995; Chiszar et al., 1999; Biardi et al., 2000; Heatwole et al., 1999), are becoming increasingly common. Future studies that simultaneously evaluate local populations of snakes and prey are likely to provide additional surprising insights into toxicity and resistance arising from the coevolutionary arms race at the molecular level.

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CHEMICAL DEFENSE IN HARVESTMEN (ARACHNIDA, OPILIONES): DO BENZOQUINONE SECRETIONS DETER INVERTEBRATE AND VERTEBRATE PREDATORS?

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Abstract—Two alkylated 1,4-benzoquinones were identified from the defensive secretion produced by the neotropical harvestman *Goniosoma longipes* (Gonyleptidae). They were characterized as 2,3-dimethyl-1,4-benzoquinone and 2-ethyl-3-methyl-1,4-benzoquinone. We tested the effectiveness of these benzoquinone secretions against several predator types, including invertebrates and vertebrates. Different predators were exposed to the harvestmen's gland secretion or to distilled water in laboratory bioassays. Our results indicate that secretions containing the 1,4-benzoquinones released by *G. longipes* can be an effective defense against predation, and that the effectiveness of the secretion is dependent on the predator type. The scent gland secretion repelled seven ant species, two species of large wandering spiders, and one frog species, but was not an effective defense against an opossum. Our study also demonstrates that the scent gland secretion of *G. longipes* can work as a chemical shield preventing the approach of three large predatory ants for at least 10 min. The chemical shield may protect the harvestman against successive attacks of the same ant worker and also allow the harvestman to flee before massive ant recruitment. Our data support the suggestion that chemical defenses may increase survival with some but not all potential predators. This variation in defense effectiveness may result from many interacting factors, including the attack strategy, size, learning ability, and physiology of the predators, as well as the chemical nature of the defensive compounds, type of emission, and amount of effluent released by the prey.

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Key Words—Allomone, antipredator defense, chemical defense, chemical shield, Formicidae, *Philander*, predation, *Proceratophrys*, 1,4-benzoquinones.

INTRODUCTION

The species of the order Opiliones are characterized by the presence of a pair of exocrine glands located at the anterior margins of the cephalothorax near the base of the second pair of legs (Shultz, 1990). These glands produce a variety of volatile secretions that are released under the threat of predation (Eisner et al., 1978). To date, nearly 30 species have had their secretions chemically characterized, and a phylogenetic pattern may be observed. Species belonging to the suborder Eupnoi produce a variety of acyclic compounds, such as short-chain ketones and alcohols among the sclerosomatids and naphthoquinones among the phalangiids (references in Roach et al., 1980). In the suborder Laniatores, species of the superfamily Travunioidea produce mainly terpenoids, whereas among the Gonyleptoidea, the secretion is composed of phenols and alkylated 1,4-benzoquinones (references in Eisner et al., 1978; Acosta et al., 1993; Gnaspini and Cavalheiro, 1998). Quinones are widely distributed in the scent gland secretions of arthropods such as insects and millipedes (Blum, 1981), but the particular alkylated quinones produced by harvestmen seem to be of more restricted occurrence (Eisner et al., 1978).

Although much is known about the chemical nature of the compounds produced by harvestmen, knowledge about their biological role is still scarce. Several authors suggest that the scent gland secretions in harvestmen could be used for intraspecific communication, including trail marking, sexual recognition, mutual attraction, and alarm (see Holmberg, 1986; Machado et al., 2002). However, the main function attributed to the scent secretion is defense against predators (review in Holmberg, 1986). Laboratory observations have shown that ants are repelled by the secretion released by the cosmetid *Vonones sayi* (Eisner et al., 1971), the leiobunine *Leiobunum formosum* (Blum and Edgar, 1981), and the gonyleptid *Acanthopachylus aculeatus* (Eisner et al., 2004). Anecdotal observations indicate that some spiders may be also deterred by harvestman secretions (e.g., Cloudsley-Thompson, 1958; Juberthie, 1976), but others are not (e.g., Bristowe, 1949; Immel, 1954). Recently, Eisner et al. (2004) demonstrated that the scent gland secretion of the harvestman *A. aculeatus* was unable to deter the attack of a lycosid spider. The effectiveness of the scent gland secretion against vertebrates is also controversial because some species of frog seem to present a strong aversive response (e.g., Edgar, 1971), whereas some mammals feast on harvestmen (e.g., Pelegatti-Franco and Gnaspini, 1996). Despite these

field and laboratory observations, the defensive role of the secretion against invertebrate and especially vertebrate predators remains to be experimentally tested through a rigorous protocol.

In this study, we chemically characterized the scent secretion of *Goniosoma longipes* (Roewer, 1913; Gonyleptidae) and conducted a series of bioassays to test the effectiveness of the 1,4-benzoquinone secretions produced by this neotropical harvestman against several predator types. This is a comprehensive study on the defensive role of the fluids released by harvestmen and the first to assess experimentally that the specific alkylated benzoquinone secretions released by harvestmen can deter vertebrates. Because after releasing the odoriferous secretion, residual effluents remain on the harvestmen's body (Eisner et al., 1971), we also tested if the secretion can also work as a "chemical shield".

METHODS AND MATERIALS

Studied Species. The harvestman *G. longipes* is conspicuous (Figure 1A, B) and widespread in forested areas of southeastern Brazil. This species was chosen as the source of secretion because it is abundant in our study site and because individuals produce a great amount of secretion when disturbed (see Machado et al., 2000). Moreover, the ecology and behavior of the species were previously studied (see Machado and Oliveira, 1998; Machado et al., 2000), aiding the selection of more realistic potential predators. The external morphology of the ozopore of *G. longipes* has been described (see Figures 19 and 20 in Hara and Gnaspini, 2003), and the mode of emission occurs as follows: (a) first, a large transparent droplet of enteric fluid composed exclusively of water is regurgitated, slips along the lateral area of the dorsal scute, runs by capillarity in tegumentary grooves, and accumulates in the posterior portion of the body (Figure 1A); (B) if the harvestman is persistently disturbed, a second fluid may be secreted by the odoriferous glands, adding to the previously colorless fluid a yellowish coloration and a characteristic sour smell (Figure 1B). Sometimes, the secretion may also be sprayed as a jet directly toward the aggressor without mixing with enteric fluid (Machado et al., 2000; Hara and Gnaspini, 2003).

All individuals of *G. longipes* used in this study were collected inside caves at the Parque Florestal do Itapetinga (23°15′S, 46°45′W, 900- to 1300-m altitude, 1600 mm rain/year), Atibaia, São Paulo state, southeastern Brazil (for details on the study site, see Machado and Oliveira, 1998 and Machado et al., 2000). In the laboratory, adult *G. longipes* were maintained in a communal terrarium (60 × 40 cm base, 35 cm high) containing cotton wetted with water to maintain the humidity and fed with dead insects twice a week.

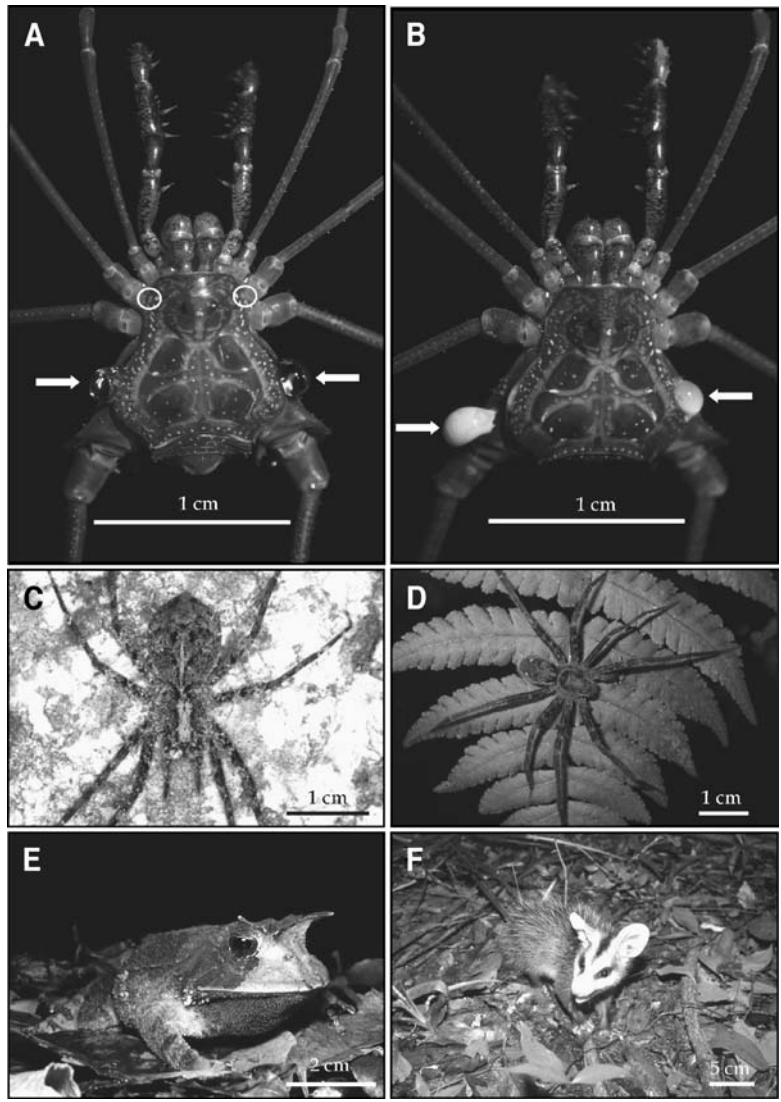

FIG. 1. Adult male of the harvestman *G. longipes* (A) with two droplets (arrows) of aqueous enteric fluid and (B) with a droplet of secretion (arrows) formed by the mixture of enteric fluid and defensive secretion (photos: B.A. Buzatto). The circles indicate the external opening of the scent gland. (C–F) Model predators used in the bioassays: (C) the hunting spiders *E. cyclothorax* (photo: R.J. Sawaya) and (D) *T. biocellata* (photo: G. Machado); (E) the small horned frog *Pr. boiei* (photo: I. Sazima) and (F) the white-eared opossum *D. albiventris* (photo: E.G. Martins).

Analyses of Defensive Secretions. Chemical secretions from *G. longipes* (about 8 mg/individual) were collected by pressing cotton wools (50–70 mg) onto the gland openings. The exudates were then either extracted with ethyl acetate (about 5 ml) and analyzed by gas chromatography–mass spectrometry (GC-MS) or with $CDCl_3$/tetramethylsilane (TMS) (600 μl) for the NMR experiments. GC-MS analyses were carried out using an HP 6890/5973 system equipped with an HP5 fused silica capillary column (30 m × 0.25 mm × 0.25 μm). Oven temperatures were programmed from 50 to 200°C at 10°C min^{-1}, and subsequently from 200 to 290°C at 16°C min^{-1}. The injector temperature was 250°C. Helium was used as carrier gas at a flow rate of 1 ml min^{-1}. The electron impact mass spectrum (EIMS) were taken at 70 eV. Scanning speed was 3.54 scans s^{-1} from *m/z* 40 to 450. High-resolution mass spectrum was obtained from VG AutoSpec EI (70 eV) equipment. Nuclear magnetic resonance (NMR) spectral data were acquired from Varian Inova spectrometer, operating at 499.88 MHz for ^{1}H-NMR and 125.71 MHz for ^{13}C-NMR. $CDCl_3$ was used as solvent and TMS as an internal reference (δ = 0.0 ppm). Chemical shifts δ were recorded in ppm and coupling constants *J* in Hz. Thin-layer chromatography analyses were performed on Merck F_{254} Aluminum Sheets, and the spots were visualized by UV light (254 nm). All solvents were of high analytical grade, bidistilled before using. Cotton wools were successively extracted with ethyl acetate, and the solvent was evaporated under vacuum.

Bioassays. The experiments were conducted from October 2002 to May 2003 and were performed between 19:00 and 23:00 hr under dim light conditions. Immediately before each trial, an adult individual of *G. longipes* was milked of secretion by seizing it by hand. Males and nonovigerous females were milked because the total amount of secretion released by them is not different (8.3 ± 4.4 mg). The exudate was collected with capillary tubes and diluted into a syringe with 100 μl of distilled water. The secretion was mixed with water because many harvestman species, including *G. longipes*, dilute their own gland secretion in aqueous enteric fluid (Eisner et al., 1971, 1978; Acosta et al., 1993; Gnaspini and Cavalheiro, 1998; Hara and Gnaspini, 2003; see also Figure 1A). Different syringes were used for each experimental group. A different unmilked individual was used in each trial because repeated milking of the same individual could reduce the concentration and quantities of the secretion (Eisner et al., 1971). Different model predators were exposed to the harvestmen's gland secretion (treatment) or to distilled water (control).

Most laniatorean harvestmen, including *G. longipes*, have spines covering the body and legs and present a variety of defensive behaviors that may be divided in evasive responses (fleeing and dropping from the substrate) and aggressive responses (Machado, 2002). The latter includes several mechanisms that threaten, harm, or deter aggressors, such as attacking with the

pedipalps, biting with the chelicerae, pinching with the sharp projections of the femora and coxae IV, and releasing scent gland secretions (Machado, 2002; Hara and Gnaspini, 2003). To test the potential defensive role of the glandular secretion only, and prevent other possible defensive mechanisms, individuals of *G. longipes* were not offered to the predators. Rather, the common cricket *Gryllus gryllus* (nearly 1 cm of body length) was used as model of prey in the experiments with spiders, frogs, and marsupials (see details below).

Tests with Ants. Two bioassays were conducted with ants: one in the field to test the potential of the scent gland secretion as a repellent and another in the laboratory to test the effectiveness as a "chemical shield". The field experiment consisted of the presentation of filter paper baits imbibed with honey solution, placed on plastic dishes (5-cm diam). These baits were randomly distributed on the forest floor 5 m from each other. In the treatment group, when ants had come to feed at the margin of the bait, the glandular secretion of one individual of *G. longipes* diluted in 100 μl of water was discharged in the center of the filter paper with a syringe. A similar procedure was used in the control group, but only 100 μl of water were discharged in the bait. In both experimental groups, the number of ants in contact with the bait was counted before and 5 sec after presentation of either secretion or water. Five ant species attended the baits in the field and were used in this experiment: (1) Formicinae—*Camponotus lespesi* (four baits; two controls and two treatments); (2) Myrmicinae—*Pheidole* sp. (eight baits; four controls and four treatments); (3) Ponerinae—*Gnamptogenys* sp. (eight baits; four controls and four treatments), *Odontomachus chelifer* (six baits; three controls and three treatments), and *Pachycondyla striata* (six baits; three controls and three treatments). After square-root transformations of the data, a repeated-measures ANOVA was performed on the number of workers of the five ant species in contact with the baits before and after the treatment in both experimental groups (Pagano, 1994).

Three ant species were used in the laboratory experiments: *Pachycondyla villosa* (one colony), *Odontomachus hastatus*, and *Camponotus crassus* (three colonies each). Inside the tray of each colony, we presented a glass coverslip (1 × 5 cm) divided in two sides randomly designated by the flip of a coin as treatment or control. The treatment consisted of a filter paper (1 cm^2) wetted with 200 μl of a saturated sugar solution mixed with the glandular secretion of one individual of *G. longipes*. The control side contained only a filter paper (1 cm^2) wetted with 200 μl of a saturated sugar solution. The ant's response was evaluated by counting the total number of workers feeding at the baits at 1-min intervals during 10 min after the first contact.

Tests with Spiders. Enoploctenus cyclothorax (Ctenidae) is a large (3- to 5-cm body length) wandering spider that is commonly found near cave

entrances (Figure 1C) and occasionally preys on adult *G. longipes* (Machado et al., 2000; Machado, personal observations), as well as other gonyleptid harvestmen (Willemart and Kaneto, 2004). *Trechalea biocellata* (Trechaleidae) is also a large (2- to 3-cm body length) wandering spider, abundant in the study site (Figure 1D), which is generally found near river margins or in other moist habitats, such as caves. An adult male of this spider was once observed preying on a harvestman, probably a *Discocyrtus* (Gonyleptidae), at night (Machado, personal observations). Individuals of both spiders were collected at the Parque Florestal do Itapetinga, from October 2002 to May 2003. They were maintained in individual cages (20 × 10 cm base, 15 cm high) containing cotton wetted with water to maintain the humidity and starved for 5–6 d before the experiments. Only subadults and adults of both sexes of *T. biocellata* (N = 40) and *E. cyclothorax* (N = 20) were used in the experiments. Individuals were offered a cricket, which they promptly took as prey. To ensure that the crickets were unable to promote injuries on the spiders, their hind legs (armed with several spines) were removed just before the experiment. After the spiders had started feeding, each individual was stimulated with either secretion of one harvestman diluted in 100 μl of water (treatment) or the same volume of distilled water (control), applied with a syringe directly to the base of the chelicerae. Individuals that extricated the chelicerae and abandoned the prey within 5 min were scored as respondents (cf. Eisner et al., 1997). The number of spiders that released the prey was compared between the two experimental groups using a Fisher exact test (Pagano, 1994). The time between the presentation of secretion and the response of the spiders was compared between species using a Mann–Whitney *U*-test.

Tests with Frogs. The effectiveness of the scent secretion against frogs was tested using adults (16 males and 4 females) of the small horned frog, *Proceratophrys boiei* (Leptodactylidae). The species is a leaf-litter dweller whose adults may reach 5–7.5 cm of body length (Figure 1E). Because of its large mouth, the diet of this species includes large ground arthropods, such as spiders, cockroaches, beetles, and crickets (Giaretta et al., 1998; Teixeira and Coutinho, 2002). Although there is no record of harvestmen among the food items of *Pr. boiei*, other species of the genus, such as *Pr. apendiculata*, include harvestmen in their diet (Machado, personal observations). Small horned frogs (N = 20) were collected at the Parque Florestal do Itapetinga during the breeding season from November 2002 to January 2003. They were maintained in a communal terrarium (60 × 40 cm base, 35 cm high) and starved 3 d before the experiments. They were offered a cricket, which they promptly took as prey. As soon as a frog swallowed the cricket, we caught it by hand, forced its mouth open, and used a syringe to inject either secretion of one harvestman diluted in 100 μl of water (treatment) or the same volume of distilled water (control). Anurans generally catch their prey by a quick flip of

their sticky tongue and may swallow chemically protected arthropods, such as millipedes or beetles, before the secretion of the prey is discharged (Eisner and Meinwald, 1966). Hence, the secretion might be released outside the mouth, inside the mouth, or even inside the stomach. Our experimental protocol simulated the mouth scenario. Individuals that regurgitated the prey within 5 min were scored as respondents. The number of frogs that regurgitated the prey was compared between the two experimental groups using a Fisher exact test (Pagano, 1994).

Tests with Opossums. The white-eared opossum *Didelphis albiventris* (Didelphidae) is a medium-sized mammal (0.5–2.0 kg; Figure 1F), whose omnivorous diet includes harvestmen (Cáceres and Monteiro-Filho, 2001; Cáceres, 2002). Although *D. albiventris* occurs at the Parque Florestal do Itapetinga, experimental individuals were collected at the Clube Náutico Araraquara (21°43′S, 48°01′W, 653-m altitude, 1300 mm rain/year), a forested area near Américo Brasiliense, São Paulo state. In this locality, the species is abundant, and juvenile individuals (150–450 g) can be trapped using Sherman live traps. The opossums ($N = 8$) were maintained in individual cages for 12 hr after collection, and 30 min before the experiment, they were transferred to a dark closed box (60 × 40 cm base, 35 cm high) and fed with a piece of banana to bring all of them to a similar hunger level.

The presentation of the prey was made by using a tube inserted in the box's wall that placed the cricket in the center of the cage. The first cricket introduced in the cage had a 4 × 8 mm piece of filter paper carefully placed under its wings (no glue was used), wetted with the glandular secretion of one individual of *G. longipes* diluted in 100 μl of water (treatment). Two minutes after the introduction of the first cricket, a second cricket with a piece of filter paper wetted with 100 μl of distilled water (control) under its wings was placed in the cage. Prey were offered in this sequence because if the first cricket was rejected and not the second, it would be possible to identify a rejection response. This second cricket was also a control of the hunger level of the opossums. We recorded the time elapsed between the presentation of the crickets and the attack of the opossums, as well as the behavioral response of the animals to each type of prey. Because species of the genus *Goniosoma* are capable of ejecting their secretion forcibly as a spray (Machado, 2002; Hara and Gnaspini, 2003), one adult individual of *G. longipes* was also introduced in each cage after the presentation of the crickets to investigate the response of the opossums to this special type of emission.

Vouchers. One of the trials with frogs and all trials with the marsupials were video recorded, digitalized, and are available from the authors upon request. Voucher specimens of all studied species (except *D. albiventris*) were deposited at the Museu de História Natural da Universidade Estadual de Campinas and Museu de Zoologia da Universidade de São Paulo, Brazil.

RESULTS

Chemistry. Two alkylated 1,4-benzoquinones were identified from the defensive secretion of the harvestman *G. longipes* (Figures 2 and 3):

2,3-Dimethyl-1,4-benzoquinone (compound 1). GC-MS *m*/*z* 136 (100), 108 (57), 107 (56), 82 (44), 79 (52), 65 (5), 54 (50); HRMS (EI, 70 eV): M^+ 136.05392 ($C_8H_8O_2$; calc. 136.05244); ^{1}H-NMR ($CDCl_3$, TMS, 499.88 MHz) δ (ppm) = 2.04 (s, H-7 and H-8, 6H), 6.72 (s, H-5 and H-6, 2H); ^{13}C-NMR δ (ppm) = 187.39 (C-1, C-4), 140.99 (C-2, C-3), 136.24 (C-5, C-6), 12.18 (C-7, C-8). Other spectroscopic methods as ^{13}C-NMR DEPT-135 and DEPT-90, ^{1}H–^{1}H gCOSY, ^{1}H–^{13}C ^1J HSQC, and ^{1}H–^{13}C ^nJ gHMBC were also employed (data not shown).

2-Ethyl-3-methyl-1,4-benzoquinone (compound 2). GC-MS *m*/*z* 150 (100), 135 (10), 122 (37), 121 (18), 107 (79), 82 (21), 79 (38), 67 (12), 54 (22); HRMS: 150.06824 ($C_9H_{10}O_2$; calc. 150.06810). For further spectroscopic data as ^{1}H-NMR ($CDCl_3$, TMS, 499.88 MHz), ^{13}C-NMR, DEPT-135 and DEPT-90, ^{1}H–^{1}H gCOSY, ^{1}H–^{13}C ^1J HSQC, and ^{1}H–^{13}C ^1J gHMBC, see Table 1.

The fragmentation pattern of compound 1 under EIMS was compared with literature (Budzikiewicz et al., 1967), and it was identical to the spectrum reported for 2,3-dimethyl-1,4-benzoquinone. Because of molecular symmetry, compound 1 displayed two singlets in ^{1}H-NMR spectrum: at δ = 2.04 ppm for six methyl protons and at δ = 6.72 ppm for the other two aromatic protons. This spectral simplicity allowed the structural identification of the other component in the mixture.

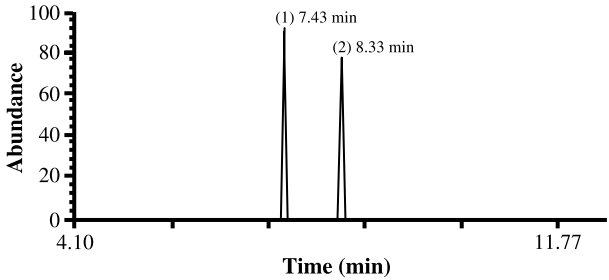

FIG. 2. GC-MS analysis of male *G. longipes* defensive secretion. Peaks corresponding to compounds 2,3-dimethyl-1,4-benzoquinone and 2-ethyl-3-methyl-1,4-benzoquinone are indicated as (1) and (2), respectively.

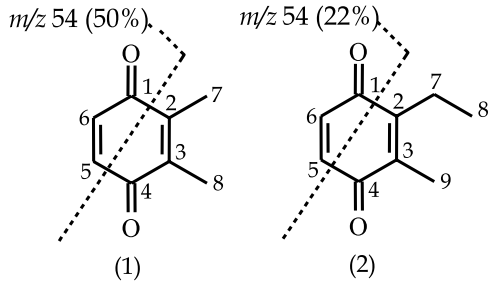

FIG. 3. Chemical defense compounds identified from the scent secretion of the harvestman *G. longipes*. The origin of the fragment *m/z* 54 in the EIMS spectra is also shown.

Compound 2 fragmentation pattern under EIMS did not match any mass spectral data available for other 1,4-benzoquinones derivatives identified in arthropods. The use of one and bidimensional NMR techniques allowed almost complete chemical shift assignments for this compound, which was identified as 2-ethyl-3-methyl-1,4-benzoquinone. The molecular formula $C_9H_{10}O_2$ was confirmed by high-resolution mass spectrometry (M^+ at *m/z* 150.06824; calc.

TABLE 1. ^1H AND ^{13}C NMR ($CDCl_3$) CHEMICAL SHIFT ASSIGNMENTS FOR 2-ETHYL-3-METHYL-1,4-BENZOQUINONE (COMPOUND 2)

C	δ_H	gCOSY ^1H–^1H	δ_C	HSQC, ^1H,^{13}C (^1J)	gHMBC, ^1H,^{13}C (^nJ)	DEPT[c]
1	–	–	187.10	–	–	–
2	–	–	146.15	–	–	–
3	–	–	140.37	–	–	–
4	–	–	187.90	–	–	–
5	6.71(broad s, 1H)	–	136.19[a]	136.19 (C-5)[b]	187.90 (C-4)	CH
6	6.71(broad s, 1H)	–	136.37[a]	136.37 (C-6)[b]	187.10 (C-1)	CH
7	2.51 (q, *J* 7.6 Hz, 2H)	1.06	19.73	19.73 (C-7)	12.84 (C-8); 140.37 (C-3); 146.15 (C-2); 187.10 (C-1)	CH_2
8	1.06 (t, *J* 7.6 Hz, 3H)	2.51	12.84	12.84 (C-8)	19.73 (C-7); 146.15 (C-2)	CH_3
9	2.05 (s, 3H)	–	11.63	11.63 (C-9)	140.37 (C-3); 146.15 (C-2); 187.90 (C-4)	CH_3

The letters *a* and *b* indicate interchangeable assignments, and *c* indicates results from DEPT-90 and DEPT-135 experiments. Splitting pattern are as follows: s = singlet; t = triplet; q = quartet.

150.06810). A CH_2CH_3 substituent was easily characterized by the A_3X_2 spin system (δ = 2.51 ppm for 2H and δ = 1.06 ppm for 3H) in the ^{1}H-NMR spectrum, which was further confirmed by ^{1}H, ^{1}H homonuclear correlations spectrum (gCOSY). The substitution pattern of compound 2 was first suggested by the presence of the intense fragment at *m*/*z* 54 in the low-resolution mass spectrum, confirmed by HRMS (observed *m*/*z* 54.01036; calc. for C_3H_2O *m*/*z* 54.01056; Figure 3). This was corroborated by the H-7/C-3, H-7/C-1, H9/C-2, and H-9/C-4 ^3J correlations observed by ^{1}H, ^{13}C heteronuclear long-range correlations spectrum (gHMBC). Further spectroscopic methods as gHSQC (^{1}H,^{13}C ^1J), DEPT-135, and DEPT-90 were also employed in chemical shift assignments for compound 2 (Table 1).

The chemical composition of defensive secretions from male and female individuals was the same in *G. longipes*, but the proportion of the two components is slightly different. Compound 2 always displayed smaller relative abundance in GC-MS analyses. This feature was also explored in the interpretation of the NMR experiments, in which compound 2 signals were always less intense than those from compound 1.

Tests with Ants. The presentation of scent gland secretion in the treatment group induced a marked reduction in the number of ants tending the sugar baits in the field (Figure 4). This reduction was not observed in the control baits (Figure 4). The repellent effect was observed for recruiting species such as

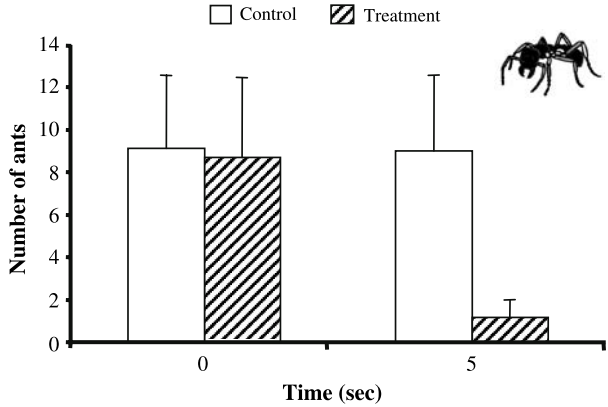

FIG. 4. Number of ants in the field tending sugar baits before (time = 0 sec) and after (time = 5 sec) stimulation with harvestman secretion (treatment) or distilled water (control). Data combined from 32 trials (16 controls and 16 treatments) on five species of ants. There was a significant effect of time (repeated-measures ANOVA, $F = 13.77$, $P < 0.001$) and experimental group (repeated-measures ANOVA, $F = 39.61$, $P < 0.001$).

Pheidole sp., *C. lespesi*, and *Gnamptogenys* sp. and also for solitary hunters such as *O. chelifer* and *P. villosa*. After leaving the treatment baits (but not the control baits), workers of all tested species typically cleaned their antennae and front legs.

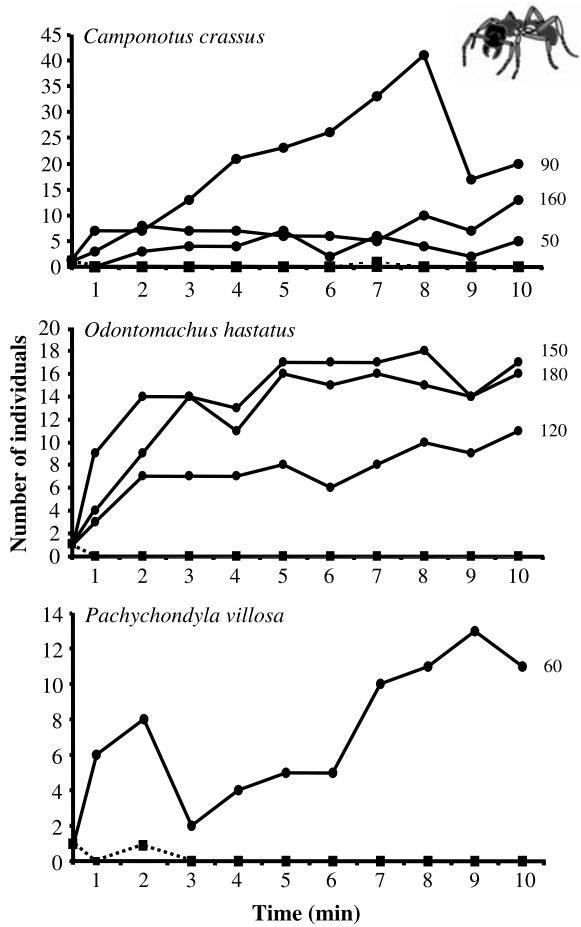

FIG. 5. Number of visits over time of three ant species tending control baits of sugar solution (circular dots, full line) and treatment baits of sugar solution mixed with harvestman secretion (square dots, dotted line) in the laboratory. The numbers at the right lateral of the graphics indicate the size of the ant colonies (for *C. crassus* and *Odontomachus*, three colonies each, and for *P. villosa*, one colony). Because the number of ants in contact with treatment baits was generally zero, it is not possible to distinguish the three dotted lines.

In the laboratory, the number of ants tending control baits generally increased during the 10-min period, whereas few or no ant contacted treatment baits (Figure 5). The workers that touched the filter papers of the treatment baits presented a behavioral response similar to that observed in the field experiment,

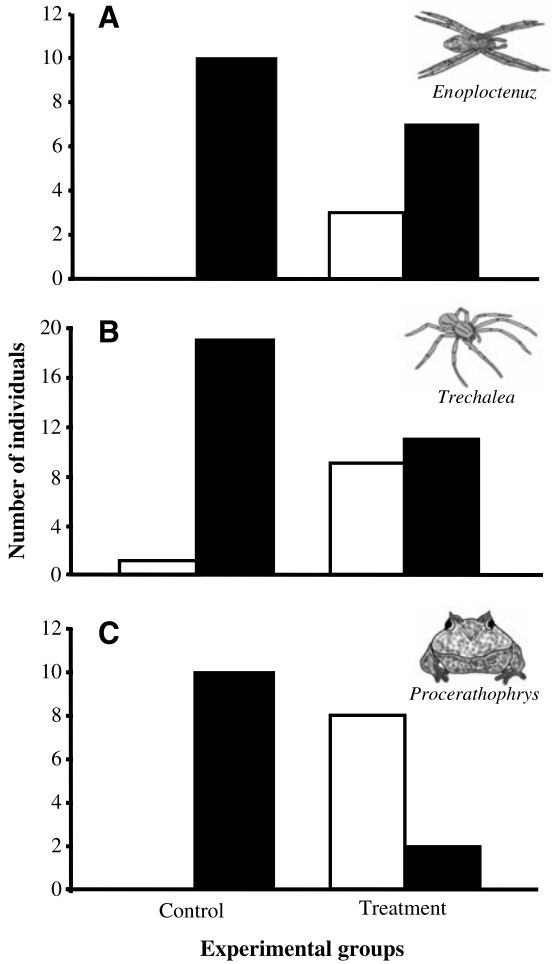

FIG. 6. Number of individuals of the spiders (A) *E. cyclothorax* and (B) *T. biocellata* that released (white bars) or not (black bars) the prey after stimulation with harvestman secretion (treatment) or distilled water (control). (C) Number of individuals of the small horned frog *Pr. boiei* that regurgitated (white bars) or not (black bars) the prey after stimulation with harvestman secretion (treatment) or distilled water (control).

i.e., they immediately cleaned their antennae and front legs and did not return to the bait.

Tests with Spiders. The frequency of individuals of *E. cyclothorax* (Fisher exact test, $P = 0.003$) and *T. biocellata* (Fisher exact test, $P = 0.008$) that released the prey in the treatment group was significantly higher than in the control group (Figure 6A, B). There was no difference between species in the frequency of individuals that released the prey in the treatment group (Fisher exact test, $P = 0.43$). Respondent individuals of both species extricated their fangs, dropped the prey, and rubbed their mouthparts against the substrate. The time elapsed between the presentation of the secretion and the release of the prey also did not differ between species ($U = 23.0$; $P = 0.37$) and ranged from 1 to 30 sec in *E. cyclothorax* and from 1 to 50 sec in *T. biocellata*.

Tests with Frogs. The frequency of individuals of *Pr. boiei* that regurgitated the prey in the treatment group was significantly higher than of those in the control group (Fisher exact test, $P = 0.004$; Figure 6C). Frogs in the treat-

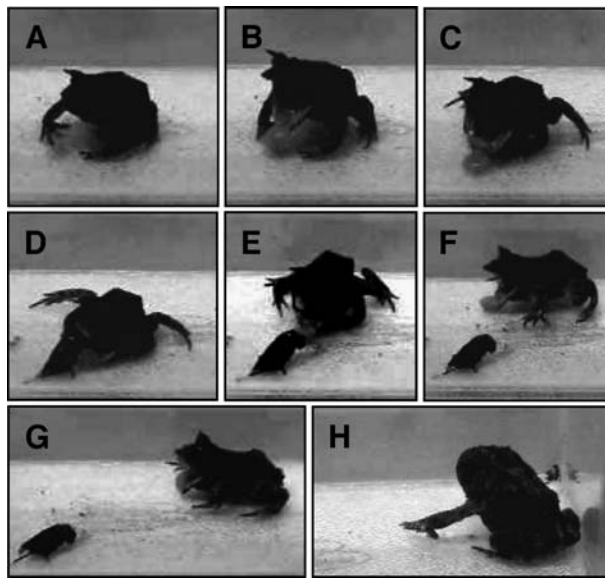

FIG. 7. (A–H) Sequence extracted from videotape showing the reaction of a small horned frog, *Pr. boiei*, to the scent gland secretion of the harvestman *G. longipes*. (A) The individual immediately before the test. (B) After the harvestman secretion was presented inside its mouth, the individual had abdominal contractions, and (C–E) regurgitated the cricket. (F–G) The frog scratched the mouth on the substrate and moved backward. (H) Nearly 30 sec after regurgitation, the individual still showed clear signs of aversion, maintaining the eyes shut and presenting strong abdominal contractions.

ment group presented clear aversion reactions such as scratching the tongue on the substrate, shutting the eyes, and strong abdominal contractions (Figure 7). These behavioral reactions occurred both before and after prey regurgitation. Interestingly, for all respondent individuals, the prey was regurgitated alive.

Tests with Opossums. All individuals of *D. albiventris* consumed the crickets of both experimental groups. There was no clear sign of aversion when the opossums ingested the crickets wetted with secretion. All opossums also ingested adults of the harvestman *G. longipes*. Four of five subadult individuals subdued the harvestmen using the front legs to step on it, which probably promoted the releasing of secretion. After that, the harvestmen were grasped with the front legs and consumed. One subadult and three juveniles subdued the harvestmen using the snout to bite the prey. The biting elicited the release of a jet of secretion that probably reached the nose and the eyes of the opossums. In these cases, the opossums responded to the discharge by releasing the prey and wiping their eyes and snout with their front legs for nearly 2 min. After this period, the opossums approached the dead body of the prey and ate it. In all cases, only the body of the harvestmen was consumed and the legs were discarded. During actual consumption, the opossum showed no clear signs of aversion, such as those presented by the small horned frogs.

DISCUSSION

Like most representatives of the superfamily Gonyleptoidea, the defensive secretion of the harvestman *G. longipes* is a mixture of compounds. The occurrence of alkylated 1,4-benzoquinones in *G. longipes* is chemotaxonomically in agreement with the constitution of the defensive secretions previously reported for other Gonyleptidae (Estable et al., 1955; Fieser and Ardao, 1956; Roach et al., 1980; Acosta et al., 1993; Eisner et al., 2004), including congeneric species, such as *Goniosoma spelaeum* (Gnaspini and Cavalheiro, 1998). The derivative 2,3-dimethyl-1,4-benzoquinone has been already identified in other harvestman species belonging to the families Cosmetidae, Gonyleptidae, and Manaosbiidae (Estable et al., 1955; Fieser and Ardao, 1956; Eisner et al., 1977, 2004; Roach et al., 1980; Acosta et al., 1993), but this is the first report of 2-ethyl-3-methyl-1,4-benzoquinone as a natural product. Moreover, the use of bidimensional nuclear magnetic resonance spectroscopy and high-resolution mass spectrometry is reported for the first time for chemical characterization of substances from harvestmen, providing additional protocols for studies of scent secretions in the group.

Alkylated benzoquinones are produced by harvestmen of the suborder Laniatores, millipedes of the orders Spirobolida, Spirostreptida, and Julida, and

insects of the orders Coleoptera, Dermaptera, Blattodea, Isoptera, Orthoptera, and Hemiptera (Eisner and Meinwald, 1966; Blum, 1981). The caustic, irritating, and odoriferous properties of these substances may cause invertebrate predators to desist from the attack and to perform vigorous cleaning activities (Eisner and Meinwald, 1966). Recently, Eisner et al. (2004) showed that the secretion of the gonyleptid harvestman *A. aculeatus* (Pachylinae) was an effective deterrent against the ant *Formica exsectoides*. Using a different protocol, we here demonstrate that secretions containing the 1,4-benzoquinones produced by the gonyleptid *G. longipes* (Goniosomatinae) effectively repel five species of ants belonging to three subfamilies. This result is expected because 1,4-benzoquinones from the secretion of several arthropods, including cockroaches, carabid, and tenebrionid beetles, have been shown to be highly repellent to ants (Eisner, 1958a,b; Peschke and Eisner, 1987).

As long as their defensive stores last, chemically defended arthropods can repel potential predators, but when exudates are depleted, they may quickly fall prey (Whitman et al., 1994). Many harvestmen, including representatives of the families Biantidae, Cosmetidae, Gonyleptidae, Manaosbiidae, Phalangodidae, and Sclerosomatidae, have mechanisms of emission that increase the evaporating surface of the defensive secretion (reviewed in Acosta et al., 1993; Hara and Gnaspini, 2003). These mechanisms are divided in two main types: (1) emission of secretion as fine spray that moistens the dorsum of the harvestman and (2) displacement of the secretion along the lateral area of the dorsal scutum. Both types of emission are supposed to create a "chemical shield" around the body of the harvestman protecting the animal after the emission of secretion (Acosta et al., 1993; Hara and Gnaspini, 2003). Our study demonstrates that secretions containing the 1,4-benzoquinones released by *G. longipes* in fact work as a "chemical shield" preventing the approach of three large predatory ants for at least 10 min. The "chemical shield" may protect the harvestman against the successive attacks of the same ant worker and also allow it to flee before massive ant recruitment. Because the families that have mechanisms to increase the evaporating surface of the defensive secretion are not closely related, it is possible that the "chemical shields" have evolved independently many times in the order Opiliones, and it may be related to the constant exposure to swarming insects, mainly ants.

The efficiency of the defensive secretion of harvestmen seems to vary among different spider families and genera. The ctenid *Ctenus fasciatus* is an important predator of gonyleptids in the wild (Gnaspini, 1996; Machado et al., 2000), and, therefore, it seems that the 1,4-benzoquinone secretions released by their prey have no effect on this spider. In captivity, individuals of the wolf spider *Lycosa ceratiola* (Lycosidae) were minimally affected by the effluent of the harvestman *A. aculeatus*, showing little response when the effluent was applied to their mouthparts as they fed on mealworms (Eisner et al., 2004).

Using a similar protocol, we demonstrate here that secretions produced by *G. longipes*, whose composition is almost the same of *A. aculeatus*, are able to repel two large species of wandering spiders, *E. cyclothorax* (Ctenidae) and *T. biocellata* (Trechaleidae).

Contrary to what would be expected, Eisner et al. (2004) reported that live individuals of *A. aculeatus* were consistently rejected by spiders immediately after contact, before they were even prompted to release the defensive secretion. Three previous studies have reported that individuals of *E. cyclothorax* attack gonyleptid harvestmen but immediately retreat, avoiding biting the prey (Sabino and Gnaspini, 1999; Machado et al., 2000; Willemart and Kaneto, 2004). Eisner et al. (2004) discuss that *A. aculeatus* may contain additional chemical factors on the tegument that are repellent to spiders. It is also possible that such a repellent property derives from some residual action of the secretion, which may function as a "long persisting chemical shield", and/or that harvestmen actually release small amounts of secretion, not able to be detected visually. Experimental specimens that are seized or pressed normally produce excessive amounts of fluids because of a highly stressful situation, but minimal amounts of fluids may be secreted in "slightly unpleasant" situations before an extremely shocking situation appears (Whitman, personal communication).

As far as we know, our study is the first to evaluate the defensive role of 1,4-benzoquinone secretions produced by a harvestman species against vertebrate predators. Given that the great majority of the neotropical Laniatores are nocturnal (Machado, personal observations), we focused on two groups of vertebrates that are active at night: frogs and marsupials. Both vertebrate predators used here occur syntopically with *G. longipes* and may include harvestmen in their diets, thus being appropriate model organisms for the bioassays. The defensive effluent of *G. longipes* stimulated a strong aversive reaction on the frogs, causing them to regurgitate the prey, which left the frogs' stomach still alive. Similar results were previously obtained in experiments using millipedes and beetles that have quinone-producing glands (see Eisner and Meinwald, 1966). As occurs with spiders, the effectiveness of the harvestmen secretion in anurans may vary among different species or genera. Especially in the genera *Bufo* (Bufonidae) and *Rana* (Ranidae), which are mainly constituted by large-bodied species, there are many records of predation on harvestmen (Bristowe, 1949; Smith and Bragg, 1949; Jenssen and Klimstra, 1966; Berry, 1970; Clarke, 1974; Acosta et al., 1995). Therefore, it is possible that the size of the frog influences the resistance to the secretion, so that smaller species are more likely to be affected than the larger ones. However, this suggestion remains to be tested.

Marsupials seem to be highly resistant to the chemical defenses of arthropods (Eisner, 1965, but see Whitman et al., 1994). Moreover, field data on the diet of some Brazilian didelphids has revealed that gonyleptid harvestmen

are important food items for at least three species: *Philander opossum*, which enter caves to feed on resting individuals of *G. spelaeum* (Pelegatti-Franco and Gnaspini, 1996), *D. albiventris*, and *Didelphis aurita*, in which harvestmen are the most frequent prey (Cáceres and Monteiro-Filho, 2001; Cáceres, 2002). Indeed, the tested white eared-opossum *D. albiventris* consumed all crickets in both experimental groups, as well as the adult harvestmen offered in our experiment. However, individuals that bit the prey were clearly affected by the spray of secretion released by *G. longipes*. This spray reached the eyes and the snout of the opossums, making them immediately release the dead prey and spend nearly 2 min grooming their face. Similar results were obtained by Eisner (1965) who offered the walkingstick *Anisomorpha* (Phasmida) to the mouse opossum *Micoureus demearare*. Individuals of *Anisomorpha* also sprayed their defensive secretion on the face of the marsupial, and, despite the "obvious discomfort" caused, the opossum attacked and consumed all walkingsticks that were introduced into the cage.

Many gonyleptids show gregarious habits, and among the representatives of the subfamily Goniosomatinae, this behavior is especially common (Machado, 2002). Recently, it was demonstrated that the defensive secretion of *Goniosoma* aff. *proximum* elicits alarming behavior among aggregated individuals and that the reaction response to the signal is negatively correlated with the size of the group (Machado et al., 2002). The time elapsed between the presentation of the secretion and the dispersion of the individuals ranged from 2 to 52 sec. If after the first strike toward a harvestmen aggregation, a naive marsupial takes up to 120 sec to recover from the exposition to the secretion, there is enough time for all aggregated individuals to disperse. Because the aggregated individuals are not close relatives, there is no apparent advantage to the attacked harvestman, which dies after the first strike of the marsupial. However, the emission of defensive secretion by one individual may retard the attack of the marsupial and enhance the escape capability of conspecifics in an aggregation of harvestmen (see Discussion in Machado, 2002). Gregariousness, therefore, may be a defensive mechanism especially important against mammal predators because harvestman secretions seem not to be so effective.

The results of our study indicate that the 1,4-benzoquinones released by *G. longipes* are an effective defense against predation, and that the effectiveness of the secretion depends on the predator type. The emission of scent gland secretions repelled seven species of ants, two species of wandering spiders, and one species of frog, but it was not an effective defense against an opossum. Our data support the suggestion that chemical defenses may increase survival with some but not all potential predators (e.g., Krall et al., 1999; Staples et al., 2002; Eisner et al., 2004). This variation in defense effectiveness may result from many interacting factors, including on one hand the attack strategy, size, learning ability, and physiology of the predators and, on the other hand the

chemical nature of the defensive compounds, type of emission, and amount of effluent released by the prey (Whitman et al., 1994). In this way, harvestmen are good model organisms because they release a great variety of compounds and are attacked by numerous potential and a few actual predators.

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PROCESSING OF A SESQUITERPENE LACTONE BY *Papilio glaucus* CATERPILLARS

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Abstract—*Papilio glaucus* caterpillars encounter a diverse array of sesquiterpene lactones, including parthenolide, in the leaves of host plants *Liriodendron tulipifera* and *Magnolia virginiana*. These compounds are toxic to unadapted herbivores, and the development of *P. glaucus* caterpillars likely depends on their ability to excrete or detoxify them efficiently. A new metabolite of parthenolide, 2-α-hydroxydihydroparthenolide, identified by crystal structure determination and nuclear magnetic resonance, was present in the waste of the caterpillars. The parent compound was modified by the reduction of an α-methylene group, rendering the compound less reactive, and the addition of a hydroxyl group, which increases the polarity and prepares it for the conjugation reactions of phase II metabolism. Unmetabolized parthenolide was also present in large amounts in waste. *P. glaucus* larvae are apparently capable of excreting intact sesquiterpene lactones and sesquiterpene lactone metabolites during consumption of foliage rich in these compounds.

Key Words—*Papilio glaucus*, sesquiterpene lactone, parthenolide, 2-α-hydroxydihydroparthenolide, detoxification, Magnoliaceae, reductase, cytochrome P-450.

INTRODUCTION

Tiger swallowtail butterfly caterpillars, *Papilio glaucus* L. (Lepidoptera, Papilionidae), are polyphagous and can utilize hosts from several families. Their preferred hosts include tulip tree (*Liriodendron tulipifera* L.) and sweet

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bay (*Magnolia virginiana* L.), both in the family Magnoliaceae (Scriber, 1986; Scriber et al., 1991). The leaves of these trees contain a variety of toxic constituents, including sesquiterpene lactones (Doskotch et al., 1975; Song et al., 1998).

Sesquiterpene lactones, feeding deterrents to both vertebrates (Peters and van Amerongen, 1998) and invertebrates (Doskotch et al., 1981; Passreiter and Isman, 1997), are also toxic to a wide variety of organisms due to a relatively nonspecific mechanism of action. The toxic activity is attributed to the presence of various electrophilic groups, such as the α-methylene-γ-lactone, that react with electron-rich atoms such as sulfur and oxygen in proteins and nucleic acids (see Figure 1). The presence of multiple alkylating sites enhances activity and

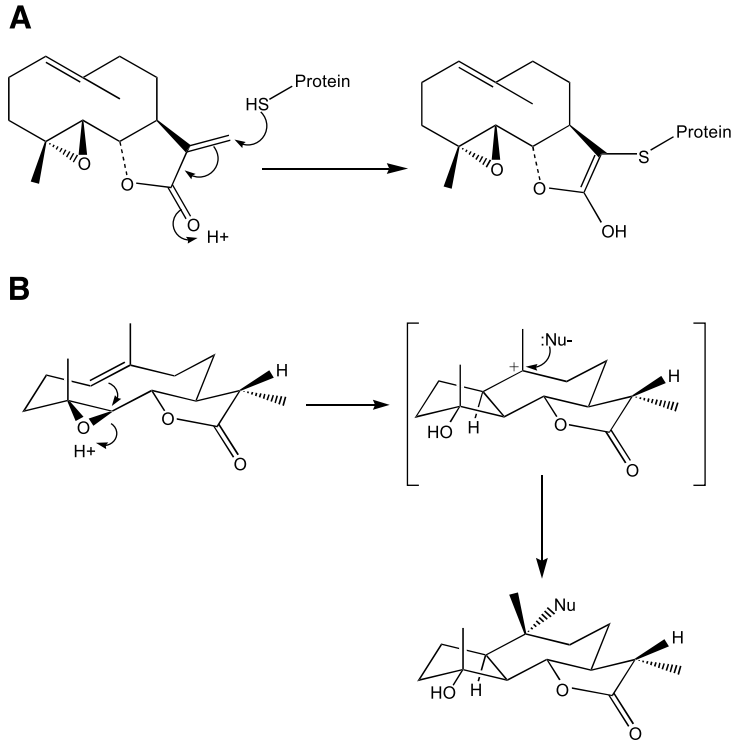

FIG. 1. Mechanisms of nucleophilic addition to parthenolide. (A) Michael addition of the sulfur group of a protein to the exocyclic α-methylene group of parthenolide. (B) Addition of a nucleophile (:Nu-) facilitated by transannular attraction of the double bond electrons for the electropositive carbon adjacent to the epoxide. (Adapted from Fischer et al., 1998). (Modified from Fischer et al. 1998 with permission from Elsevier).

may result in the cross-linking of DNA (Lee et al., 1971; Fischer et al., 1998; Schmidt, 1999a,b).

In addition to disrupting the function of critical macromolecules, parthenolide causes oxidative stress in cells, leading to apoptosis and the formation of gut lesions (Wen et al., 2002; Barbehenn, 2003). Parthenolide also causes the depletion of the tripeptide glutathione, an antioxidant found in all cells (Schmidt, 1999a), as well as the insect gut lumen (Barbehenn et al., 2001; Barbehenn, 2003). Glutathione scavenges free radicals, acts as cofactor for several antioxidant enzymes (Barbehenn, 2003), and forms conjugates with other electrophilic allelochemicals to facilitate their excretion. Therefore, its depletion may augment the phytochemically induced oxidative assault on insect cells.

Sesquiterpene lactones exert their cytotoxic effects at low concentrations, on the order of micrograms per milliliter (Lee et al., 1971; Wen et al., 2002). Fresh *M. virginiana* leaves can contain up to 0.5% parthenolide (W. Schühly, personal observation). Therefore, caterpillars feeding on sweet bay leaves may encounter substantial amounts, often consuming several milligrams per leaf. The successful utilization of parthenolide-containing plant tissues for growth and development demands the ability to tolerate large doses, expel it without absorption, or metabolize it to a less reactive and ultimately excretable form (Brattsten, 1986). In this paper, we analyzed the waste of *P. glaucus* caterpillars for parthenolide and its metabolites in order to examine the postingestion fate, which may suggest mechanisms responsible for the ability to use the toxic foliage as a food source.

METHODS AND MATERIALS

Isolation of Metabolite from Caterpillar Frass. Adult female *P. glaucus* butterflies were captured in Highlands and Levy counties in Florida. The adult females were set up for oviposition at the Archbold Biological Station in Lake Placid, FL, USA, in clear plastic containers with locally growing *M. virginiana* leaves (Scriber, 1993). Each day eggs were collected, and females were fed a 20% honey solution. Hatchling caterpillars were reared together in plastic containers on *M. virginiana* leaves. To retain leaf moisture, petioles of the leaves were placed in water-filled aquapiks. As the majority of the caterpillars reached the fifth instar, frass (waste) was collected in plastic Ziploc bags and stored in the freezer until it was transported on ice to the University of Mississippi. Frozen frass (325 g) was ground in a blender with 100% methanol and extracted exhaustively to yield 42 g of extract. Two 20 g aliquots of the crude extract were partitioned between chloroform and water (3×300 ml). The chloroform layer (31 g) was concentrated by vacuum, and aliquots were

subjected to vacuum liquid chromatography (VLC) (silica gel, 40–63 μm, EM Science, Gibbstown, NJ, USA; 100% hexane with increasing amounts of ethyl acetate, then increasing methanol). Thin-layer chromatography (TLC) and nuclear magnetic resonance (NMR) were used to compare fractions. Fractions containing a major compound by TLC (R_f = 0.28, 30:70 hexane/ethyl acetate, blue spot after spraying with vanillin/H_2SO_4 and heating) were combined (0.3 g) and rechromatographed by VLC (80:20 hexane/ethyl acetate, with increasing ethyl acetate and methanol). Compound-containing fractions were again pooled (0.16 g) and resubjected to VLC (60:40 hexane/ethyl acetate, 100% ethyl acetate, 100% methanol). One of these fractions yielded crystals suitable for analysis by X-ray crystallography [crystal data: $C_{15}H_{22}O_4$, orthorhombic space group $P2_12_12_1$, a = 7.6930(10), b = 11.446(2), c = 15.637(3) Å, V = 1,376.9(4) Å^3, Z = 4, T = 100 K, 18,811 data with MoKα radiation by Nonius Kappa CCD, R = 0.033 for 2,937 observed (of 3,101 unique) data having <33.7° and 181 refined parameters, Cambridge Database deposition number CCDC 266646]. Reverse-phase high-performance liquid chromatography (HPLC) was used to purify the compound from additional fractions with an isocratic 70% methanol solvent system (Phenomenex ultracarb 5 ODS 30 5 μ 250 × 10.00 mm, Torrance, CA, USA), and 1-D and 2-D NMR spectra were also used to elucidate the structure of the compound (Figures 2 and 3).

Isolation of Pure Parthenolide. *M. grandiflora* leaves were collected from the University of Mississippi campus, air-dried, ground (1,000 g), and extracted three times with hexane–ethyl acetate (1:1) to yield 65 g of crude extract. Ten grams of this extract were subjected to VLC (120 g Silica 60, 40–63 μm,

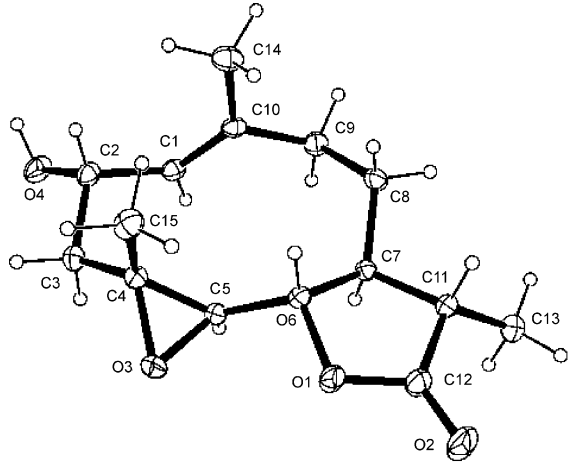

FIG. 2. Crystal structure of 2-α-hydroxydihydroparthenolide.

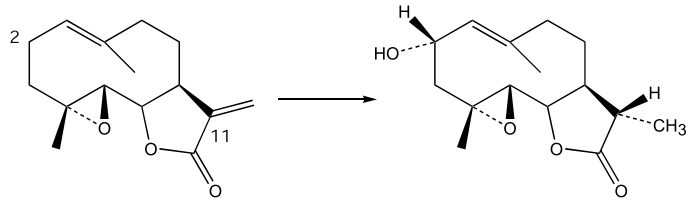

FIG. 3. Conversion of parthenolide to 2-α-hydroxydihydroparthenolide in the caterpillar gut. The hydroxylation at C2 and reduction at C11 are proposed to be catalyzed by a cytochrome P-450 monooxygenase and an NADPH reductase, respectively.

Sorbent Technologies, Atlanta, GA, USA) with hexane and increasing amounts of ethyl acetate. TLC revealed that the fraction eluting with approximately 7:3 hexane/ethyl acetate contained mainly parthenolide. The crude parthenolide (250 mg) was subjected to solid-phase extraction on an RP-18 SepPak cartridge (5,000 mg) using a gradient of water with increasing amounts of methanol to remove chlorophyll and other impurities. Parthenolide-containing fractions were combined, and parthenolide crystallized as clear prisms from the methanol/water. The ^{1}H and ^{13}C NMR spectra of the pure compound matched the values given in the literature (Fischer et al., 1992).

Parthenolide Feeding Study. *P. glaucus* females were collected in Oglethorpe County, GA, USA and sent to the University of Mississippi. Hatchling caterpillars from each mother were randomly assigned to either the parthenolide or control group, and were reared individually in 5.5-in.-diam plastic Petri dishes. For the first three instars, all caterpillars were reared on black cherry (*P. serotina*) leaves, which do not contain sesquiterpene lactones. The petioles of the leaves were inserted into water-filled floral piks to prevent desiccation. During the last two instars, the parthenolide group received black cherry leaves coated with parthenolide, in an amount equivalent to 0.2% of the weight of the cherry leaf. Parthenolide was dissolved in methanol to make a stock solution, and the correct volume was applied evenly to the top surface of each preweighed leaf by syringe. The control group received leaves treated similarly with methanol only. The solvent was allowed to evaporate before the leaves were offered to the caterpillars. Leaves were replaced as needed, which amounted to every other day for early fourth instars and every day for fifth instars. The leaves were made up the day that they were needed, and frass from each group was collected daily and stored at −20°C until its extraction. Once the caterpillars voided their guts and entered the prepupal state, they were removed from the experiment.

Extraction of Frass from Parthenolide Feeding Study. Frass from the parthenolide (106 g) and control (52.2 g) treatments was freeze-dried and

extracted sequentially with hexane, ethyl acetate, and methanol (3 × 700 ml each) and concentrated in vacuo. TLC revealed the presence of a spot in the methanol extract of the treated frass that was not present in the control frass, and this compound was chosen for purification. An aliquot (2.6 g out of 8.4 g total) of the methanol extract of the parthenolide treatment was dissolved in 100% deionized H_2O and filtered. The resulting filtrate was freeze-dried to yield 1.5 g, and 1 g of this was vacuum chromatographed (silica gel, 32–63 μm, Sorbent Technologies, GA, USA) with a gradient of chloroform and methanol (98:2 chloroform/methanol to 100% methanol). Fractions 20–21 were combined based on TLC and eluted through a SepPak with 60:40 hexane/2-propanol before normal-phase HPLC (Phenomenex Luna 10 μ silica 100 Å, 250 × 10 mm, gradient of 90:10 hexane/2-propanol to 70:30 in 35 min). Like fractions from multiple runs were combined and rechromatographed on the same column in isocratic 85:15 hexane/2-propanol. The pure compound was subjected to NMR, confirming its identity as the previously isolated metabolite.

RESULTS AND DISCUSSION

The compound isolated from the frass of *Magnolia*- and parthenolide-fed caterpillars was determined to be 2-α-hydroxydihydroparthenolide by X-ray crystallography as well as 1-D and 2-D NMR (Figures 2 and 3). ^{1}H NMR (MeOD) δ 5.29 (1H, d, J = 10.3 Hz, H-1), 4.63 (1H, ddd, J = 10.5, 10.4, 5.8 Hz, H-2), 3.99 (1H, dd, J = 9.1, 9.1 Hz, H-6), 2.91 (1H, d, J = 9.1 Hz, H-5), 2.45 (2H, m, H-3, H-11), 2.33 (1H, dd, J = 12.9, 6.4 Hz, H-9), 2.15 (1H, dd, J = 12.4, 12.4 Hz, H-9), 2.03 (1H, m, H-7), 1.96 (1H, dd, J = 15.1, 6.3, H-8), 1.80 (3H, s, H-14), 1.78 (1H, m, H-8), 1.31 (3H, s, H-15), 1.26 (3H, d, J = 7.0 Hz, H-13), 1.16 (1H, dd, J = 11.3, 11.3 Hz, H-3); ^{13}C NMR (MeOD) δ 179.2 (C-12), 136.1 (C-10), 129.1 (C-1), 82.5 (C-6), 66.8 (C-5), 66.0 (C-2), 61.2 (C-4), 51.8 (C-7), 45.6 (C-3), 42.4 (C-11), 41.2 (C-9), 29.2 (C-8), 17.6 (C-15), 16.7 (C-14), 12.3 (C-13).

Subsequent feeding experiments confirmed that the compound is a metabolite of parthenolide, which has been modified in the gut by the reduction of the exocyclic methylene group and the addition of a hydroxy group to the 2 position (Figure 3). These chemical modifications likely occur in two steps mediated by different enzymes. The addition of an oxygen atom to an allylic position is a typical cytochrome-P-450-catalyzed reaction (Wolf, 1982). The reduction of the double bond of sesquiterpene lactones is commonly seen in microbial systems (Clark and Hufford, 1979; Galal et al., 1999; Kumari et al., 2003), and is likely carried out by an nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reductase, such as an α,β-ketoalkene double bond reductase (Takeda et al., 1997). The 2-α-hydroxydihydroparthenolide may be a

by-product of microbial metabolism or larval detoxification enzymes present in the midgut or Malpighian tubules. In the latter case, the hydroxy group increases the polarity of the compound and could promote subsequent conjugation to sugars or glutathione to facilitate efficient excretion or removal from tissues by ATP-dependent glutathione transport pumps (Ishikawa, 1992). The role of reductases in the detoxification of natural products may be common; the ketone group of the cardenolide uscharidin is metabolized to two isomeric alcohol products by homogenates of monarch caterpillar guts in an NADPH-dependent reaction. This reaction is unaffected by monooxygenase inhibitors but is blocked by boiling or the addition of denaturing solvents, suggesting an enzyme-mediated but cytochrome-P-450-independent event (Marty and Krieger, 1984).

Although not quantified, a substantial amount of unmetabolized partheno-lide was also present in the frass. The excretion of unaltered secondary metabolites has been reported for several moth and butterfly larvae of varying diet breadth (Bowers and Puttick, 1986; Boros et al., 1991; Fiedler et al., 1993), and may reflect incomplete digestion of plant material. However, specialized species with highly efficient and inducible enzyme systems tend to metabolize toxic compounds more efficiently than their unadapted counterparts (Cohen et al., 1992). For example, the black swallowtail butterfly, *P. polyxenes*, which feeds exclusively on furanocoumarin-containing host plants, metabolizes 73% of the xanthotoxin consumed to two major metabolites in 4 h compared to 9% metabolized by the fall armyworm, *Spodoptera frugiperda*, a species that does not normally encounter this compound. Consequently, the armyworms contained much higher levels of xanthotoxin in their body tissues than the black swallowtail caterpillars (Ivie et al., 1983). In contrast to the specialist *P. polyxenes*, the lycaenid butterfly larvae *Callophrys rubi* feed on plants from several unrelated angiosperm families including the alkaloid-containing flowers of *Lupinus polyphyllus* and *Genista tinctoria*, and the HPLC alkaloid profile of the waste mirrors that of the flowers (Fiedler et al., 1993).

The presence of unaltered diet-derived compounds in the waste of polyphagous species may either reflect barriers to the absorption of allelochem-icals through the gut or highly efficient absorption and excretion through the Malpighian tubules. Maddrell and Gardiner (1976) demonstrated that the Malpighian tubules dissected from *Manduca sexta*, *Pieris brassicae*, and *Rhodnius prolixus* all efficiently excreted nicotine from the fluid bathing them. However, mechanisms for the removal of this potentially charged and polar molecule may not extend to more lipophilic compounds. In addition, absorption, excretion, and even sequestration necessitate the buildup of higher levels of toxic allelochemicals in the hemolymph of the insect that may not be tolerated in the absence of efficient detoxification enzymes or target site insensitivity in the gut, body fat, or other tissues (Berenbaum, 1986). Whereas fifth instar *M. sexta*, a solanaceae specialist, survived injection with 500 μg of nicotine and

continued feeding, *P. brassicae* larvae were readily killed by devouring young cabbage leaves that had taken up nicotine through the roots, despite the high rate of nicotine excretion in the Malpighian tubules (Maddrell and Gardiner, 1976 and references therein). Similarly, Isman (1985) found that although *Melanoplus sanguinipes* tolerated sesquiterpene lactones such as parthenin when ingested or applied topically, they proved toxic when injected into the hemocoel of the grasshoppers, suggesting that the alimentary canal and integument provide effective barriers to sesquiterpene lactones. Although the exact mechanism is unknown, the larvae of the tortoise beetle, *Physonota arizonae* (Cassidinae) uses egestion to its advantage by covering itself with diet-derived, sesquiterpene lactone-rich fecal deposits as protection from predation (Aregullín and Rodríguez, 2003).

Cell-based assays with parthenolide and related compounds indicate that the cell membrane does not pose a barrier to their entry into the cell (Lee et al., 1971; Fischer et al., 1998; Schmidt, 1999b), so other barriers may prevent their penetration through the gut wall to the hemolymph. Insects have a peritrophic membrane, consisting of a mixture of proteins, glycoproteins, and proteoglycans attached to a chitin microfibrillar mesh that lines the gut and is shed periodically. Reactive sesquiterpene lactones may bind to the nucleophilic amino acid side-chain groups present in the peritrophic membrane (Barbehenn, 2001) preventing their absorption, or lipophilic aggregates of allelochemicals may be size excluded from absorption through the peritrophic membrane.

Many other species in the genus *Papilio* feed on plants in the Lauraceae, Rutaceae, and Apiaceae that produce either sesquiterpene lactones or furanocoumarins (also containing an α,β unsaturated lactone group), or both types of compounds (Scriber et al., 1991; Berenbaum, 1995). Therefore, the long, historic association of this genus with these reactive constituents may have led to the evolution of mechanisms to minimize their toxicity, either through highly efficient mechanisms of excretion, the deployment of detoxification enzymes, or barriers to their absorption through the gut.

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SIMILARITY OF CUTICULAR LIPIDS BETWEEN A CATERPILLAR AND ITS HOST PLANT: A WAY TO MAKE PREY UNDETECTABLE FOR PREDATORY ANTS?

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Abstract—Ithomiine butterflies (Nymphalidae) have long-lived, aposematic, chemically protected adults. However, little is known about the defense mechanisms in larvae and other juvenile stages. We showed that larvae *Mechanitis polymnia* are defended from ants by a chemical similarity between their cuticular lipids and those of the host plant, *Solanum tabacifolium* (Solanaceae). This is a novel defense mechanism in phytophagous insects. A field survey during one season showed that larval survivorship was up to 80%, which is high when compared with other juvenile stages. In a laboratory bioassay, live larvae on their host plant were not attacked by the predatory ant *Camponotus crassus* (Formicidae). Two experiments showed that the similarity between the cuticular lipids of *M. polymnia* and *S. tabacifolium* protected the larvae from *C. crassus:* (a) when the caterpillar was switched from a host plant to a non-host plant, the predation rate increased, and (b) when a palatable larva (*Spodoptera frugiperda*, Noctuidae) was coated with the cuticular lipids of *M. polymnia* and placed on *S. tabacifolium* leaves, it no longer experienced a high predation rate. This defensive mechanism can be defined as chemical camouflage, and may have a double adaptive advantage, namely, protection against predation and a reduction in the cost of sequestering toxic compounds from the host plant.

Key Words—*Camponotus crassus*, cuticular hydrocarbons, chemical camouflage, chemical defense, Formicidae, Ithomiinae, *Mechanitis polymnia*, chemical mimicry, Solanaceae, *Solanum tabacifolium*.

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INTRODUCTION

Insects use chemicals to defend themselves in unique and varied ways (Evans and Schmidt, 1990, and references therein). Defense through chemical similarity *via* cuticular components has been suggested for insects and may involve crypsis or mimicry (e.g., Espelie et al., 1991; Buckner, 1993). Indeed, many inquilines of social insects are likely to use this mechanism (Howard, 1993; Lenoir et al., 2001; Howard and Blomquist, 2005, and references therein). However, phytophagous insects have not been shown to avoid predation by using cuticular chemical profiles similar to those of their host plant. In this paper, we provide evidence that this phenomenon occurs in the larvae of the ithomiine butterfly *Mechanitis polymnia*. Ithomiinae butterflies are long-lived, aposematic butterflies that are well-protected against predation through the sequestration of pyrrolizidine alkaloids from adult food sources, such as nectar and withered leaves of *Heliotropium* (Boraginaceae) and *Eupatorium* (Asteraceae) (Brown, 1984, 1985, 1987; Masters, 1990, 1992; Trigo et al., 1996). In more primitive genera (*Tellervo*, *Tithorea*, *Aeria*, and *Elzunia*), the larvae feed on Echitoideae vines (Apocynaceae) (Brown, 1985; Drummond and Brown, 1987; Brown and Freitas, 1994) from which they sequester pyrrolizidine alkaloids (Trigo and Brown, 1990; Orr et al., 1996; Trigo et al., 1996). The other ithomiine genera feed on Solanaceae, but the sequestration of chemical compounds from their host plants is poorly understood; *Placidula euryanasa* larvae sequester tropane alkaloids from their host plant *Brugmansia suaveolens* (Freitas et al., 1996), and calystegines found in adults of *M. polymnia* are probably sequestered from their host plants (Nash et al., 1993). There are no reports on the defensive mechanisms of larvae in this subfamily.

Mechanitis polymnia is a common butterfly in southeastern Brazil, and the adults are well protected against the orb weaving spider *Nephila clavipes* by pyrrolizidine alkaloids (Brown, 1984, 1985). Female *M. polymnia* lay their eggs on the upper surface of the leaves of several *Solanum* species, *Lycopersicum esculentum*, *Cyphomandra velutina*, and *Cyphomandra fragrans* (Brown, 1984; Drummond and Brown, 1987; Brown and Freitas, 1994). In a study of the natural history of immature *M. polymnia*, Portugal (2001) found that the rate of predation on larvae (about 20%) was lower than in the other stages. Only two species of unidentified Vespoideae, *Crematogaster* ants, and the reduviid *Montina confusa* were found to prey upon *M. polymnia* larvae (Trigo pers. obs.). Comparison of the high survivorship in all instars of *M. polymnia* (data from Portugal, 2001) with that of other insects suggested that a specific defensive mechanism was responsible for this difference, since a high survivorship is not usual in immature stages of insects but depends of the organism's life history (for a review, see Cornell and Hawkins, 1995). The ant *Camponotus crassus* patrols the host plants of *M. polymnia* and sometimes

attends homopterans, but never has been observed preying upon immature *Mechanitis*.

These observations led us to investigate whether cuticular chemistry was involved in the defense of the larva. We designed experiments to examine the hypothesis that the larvae were camouflaged by the similarity of their cuticular chemistry to that of their host plants and were, therefore, less likely to be preyed upon by chemically-oriented predators such as ants.

METHODS AND MATERIALS

Study Sites and Organisms. Eggs and larvae of *M. polymnia casabranca* Haensch on *S. tabacifolium* Dunnal were collected from Fazenda Santa Elisa, which belongs to the Instituto Agronômico de Campinas, Campinas (22°49′S, 47°17′W), and on the campus of the Universidade Estadual de Campinas (UNICAMP), Campinas (22°49′S, 47°05′W). Plant specimens were deposited in the herbarium of the Departamento de Botânica, Instituto de Biologia, UNICAMP (UEC). Larvae were reared on mature leaves collected daily from field individuals at UNICAMP and kept in plastic pots until they reached the 5th instar (at 25°C, 12 hr L/D cycle, uncontrolled relative humidity). The pots were cleaned, and leaves were change daily. In 5th instar, the larvae were killed by freezing and maintained at -15°C until the bioassays or chemical extraction.

Predation Bioassays. Since ants are important predators of immature butterflies (Smiley, 1985; Freitas and Oliveira, 1996), we used the neotropical ant *C. crassus* Mayr (Formicidae, Formicinae) as a generalist predator to test our hypothesis. This species nests in live trees and in dead and decaying logs (Kusnezov, 1951), and is frequently seen patrolling *M. polymnia* host plants and tending homopterans.

The colonies of *C. crassus* were collected from the Reserva Biológica e Estação Experimental de Mogi-Guaçu, Instituto de Botânica, Mogi-Guaçu (22°18′S, 47°10′W), and immediately transferred to test tubes (20 cm long, 2 cm diam) covered with red plastic film. The colonies were reproductive (immatures were also observed), and had a similar number of ants (about 50–70), as judged by visual inspection. Test tubes were placed in a plastic container (20 × 20 × 5 cm) the walls of which were coated with Fluon© to prevent the ants from escaping. This container was connected to a foraging arena (16 cm diam, 5 cm high) where the experiments were done. By doing the experiments in a foraging arena we avoided any defensive behavior by the ants that could result because of proximity to the nesting chambers. The colonies were kept at 25°C on a 12 hr L/D cycle and uncontrolled relative humidity, and were fed daily with a honey-water solution and weekly with 5–6th instar larvae of the palatable noctuid moth *Spodoptera frugiperda* L. The nests were fed 5–6th instar *S. frugiperda* larvae

48 hr before the bioassays. During the bioassays, five 5th instar *M. polymnia* larvae were offered on leaves of their respective host plant in order to simulate gregariousness. The mature leaves of host plant, collected immediately from field individuals at UNICAMP, were temporarily stored in a vial with water to prevent drying. Larval mortality was recorded after 24 hr.

Alteration of the Chemical Similarity by Changing the Host Plant. We predicted that the predation rate would be higher in the predation bioassay if we reduced the chemical similarity of *M. polymnia* larvae by changing their host plant. To simulate this scenario, we used freeze-dried 5th instar larvae of *M. polymnia* on mature leaves of their own host plant (*S. tabacifolium*) and on *Brugmansia suaveolens* mature leaves, a solanaceous plant not used by *M. polymnia* (Brown, 1987; Drummond and Brown, 1987), in a predation bioassay similar to the predation with live larvae. If, after 24 hr, the *M. polymnia* larva was undamaged by the ants, we offered a palatable freeze-dried 5th instar larva of *S. frugiperda*. We thus validated the bioassay if this second larva was damaged by the ants within 24 hr.

Alteration of the Chemical Similarity by Changing the Lipid Profile of the Larvae. We also predicted that the ants would not recognize larvae as prey that had the same lipid profile as the plant on which the larvae were offered. To test this we first ran preliminary bioassays using a cuticular lipid extract from *M. polymnia* larvae since the amount of extract was limited because of the low number of *M. polymnia* larvae captured in the field. Five 5th instars were dipped in 5 ml of hexane (SupraSolv, Merck, Germany) for 5 min (modified from Haverty et al., 1996, and Young and Schal, 1997) and then removed. The hexane was subsequently treated with anhydrous Na_2SO_4, filtered, and dried gently in a stream of N_2.

The bioassay was done using freeze-dried 2nd instars of *S. frugiperda* coated with the cuticular lipids of *M. polymnia* larvae. We removed the cuticular lipids from *S. frugiperda* by dipping the larvae in 5 ml of hexane for 10 min. Young and Schal (1997) demonstrated that this time is sufficient to extract all cuticular lipids from nymphs of *Blattella germanica* (Blattellidae). One *S. frugieperda* larva was treated with 50 μl of hexane extract from 10 equivalent larvae of *M. polymnia* (treated larvae, TC). The larval equivalent was calculated based on the ratio of the dry weight of *S. frugiperda* to the dry weight of *M. polymnia* larvae. We used ten times the equivalent to compensate for the loss of hexane extract since part of it could penetrate the body of the larvae instead of remaining on the external cuticle. The second larva was treated only with 50 μl of hexane (non-treated larvae, NTC). After the treatment the hexane was gently dried on a N_2 flux. Ten min after, both were placed on the upper surface of a leaf of *S. tabacifolium* and a recruitment index was calculated as the percentage of ants in contact with the first larva relative to the total number of ants recruited to both larvae during a 30 min period. After either larva was found for the first time by the ants, the number of ants in contact with each larva

was recorded at the end of each minute. At the end of the experiment, there were 30 records for each of the larvae. With the sum of the 30 records for each larva, the recruitment index (RI) was calculated as follows:

$$\mathrm{RI} = \left(\frac{\text{number of ants on TC}}{\text{number of ants on TC} + \text{number of ants on NTC}} \right) \times 100$$

A RI of 50 or close to it indicated no preference for the two larvae, whereas an index lower than 50 indicated a preference for the non-treated larva, and an index higher than 50 indicated a preference for the treated larva. Since the RI ($\bar{X} \pm$ SE) in preliminary bioassays was 2.66 ± 0.83 ($N = 5$), indicating that ants did not recruit to larvae treated with the hexane extract of *M. polymnia*, we ran double choice bioassays in a more elaborated design, using a hexane extract from *S. tabacifolium* leaves. We applied the chemicals to freeze-dried 5th instars of *S. frugiperda* treated as described above, with three experimental groups as follows:

1. High similarity condition: one larva was treated with 50 μl of hexane extract from *S. tabacifolium* (treated larvae, TC), using the same amount of hexane extract equivalent to ten 5th instar larvae of *M. polymnia*; a second larva was treated only with 50 μl of hexane (non-treated larvae, NTC). Both larvae were placed on the upper surface of a *S. tabacifolium* leaf. Recruitment index was subsequently calculated as described above.
2. Low similarity condition: one larva was treated with 50 μl of hexane extract from *S. tabacifolium* as above (TC) while a second larva was treated only with 50 μl of hexane (NTC). Both larvae were placed on the upper surface of a *B. suaveolens* leaf and the recruitment index was calculated as described above.
3. Control: one larva was treated with 50 μl of hexane (TC) while a second larva was not treated with hexane (NTC). Both larvae were placed on the upper surface of a *S. tabacifolium* leaf and the recruitment index was calculated as described above.

RESULTS

Of 125 individuals of *M. polymnia* bioassayed against ants in the laboratory (45 1st, 20 2nd, 20 3rd, 15 4th, and 25 5th instar), only five individuals of the 1st instar and four of the 2nd were attacked and killed, indicating a low predation rate (4%). *Camponotus* ants did not seem to recognize *Mechanitis* larvae as prey and walked over them without showing any aggressive behavior. This suggested that *M. polymnia* larvae had some kind of chemical protection in their cuticle that prevented them from being recognized as prey by *Camponotus* ants.

Freeze-dried 5th instar *M. polymnia* larvae placed on the non-host plant (*B. suaveolens*) were attacked significantly more (67%, $N = 15$) than those placed on the natural host plant (27%, $N = 15$) (binominal test for two proportions, $Z = 2.196$, $P = 0.028$).

The recruitment of ants, based on the RI, also varied with treatment. The RI was significantly lower than 50 for 2nd instar *S. frugiperda* larvae that had the chemical profile of *M. polymnia* and were placed on *S. tabacifolium* (RI = 2.66 ± 0.83, $t = 56.92$, $df = 4$, $P < 0.001$, $N = 5$). The same results were obtained when 5th instar *S. frugiperda* larvae were treated with the chemical profile of *S. tabacifolium* and then placed on *S. tabacifolium*. Larvae that had the chemical profile of *S. tabacifolium* and were placed on *B. suaveolens* and those treated only with hexane in the control experiment had a high RI (one way ANOVA followed by the Tukey multiple comparison test, $F_{2,27} = 24.02$, $P < 0.01$, Figure 1;

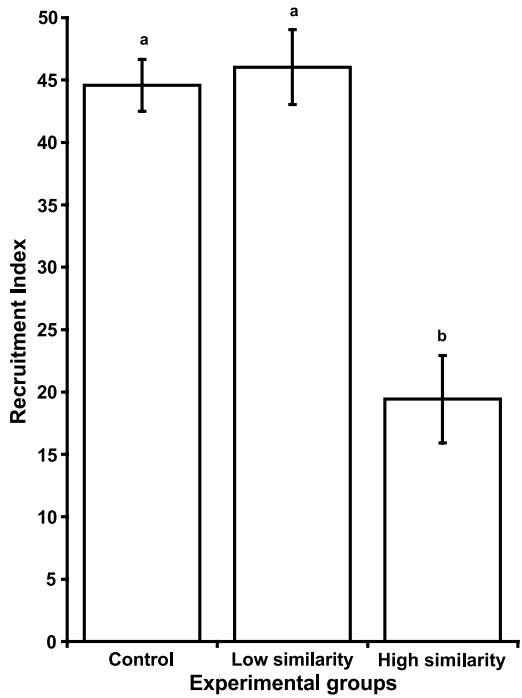

FIG. 1. Recruitment index ($\bar{X}$ ± SE) to *Solanum tabacifolium*-treated larvae of *Spodoptera frugiperda* placed on leaves of *Solanum tabacifolium* (high similarity) or *Brugmansia suaveolens* (low similarity) and to hexane-treated larvae (control). Ten replications were done for each experimental group. Different letters indicate a significant difference ($P < 0.05$).

FIG. 2. Bioassay with freeze-dried *Spodoptera frugiperda* larvae placed on a leaf of *Solanum tabacifolium* with ants (*Camponotus crassus*). The larva on the right was treated with the *Solanum tabacifolium* extract and the one on the left was treated with hexane only.

see also Figure 2 for a picture of the bioassay). We did not compare the RIs among the two bioassays since variations in larval size of *S. frugiperda* (2nd instar used for chemical profile of *M. polymnia* and 5th instar for chemical profile of *S. tabacifolium*) could have influenced the distribution of the chemicals applied to their cuticle and, consequently, ant recruitment.

DISCUSSION

The high similarity between the profile of cuticular lipids in insects and that of their biotic environment has been considered as a mechanism that enable insects, such as syrphid flies, eucharitid wasps, scarabeid and staphylinid beetles, and other ant species, to live with ants, by mimicking them. Generally, these interactions lead to parasitism and predation of the models by the mimic (see reviews in Dettner and Liepert, 1994; Stowe, 1988; Singer, 1998). Cuticular

lipids have also been shown to mediate other interactions among prey and predators. (Espelie et al., 1991) suggested a possible defense mechanism for phytophagous insects based on the similarity between their cuticular lipids and those of their host plants. Fishlyn and Phillips (1980), Hay et al., (1990), and Stachowicz and Hay (1999) described chemical defense mechanisms for marine herbivores based on chemical similarities between them and their host plants; the nature of these chemicals are unknown. However, as pointed out by Stachowicz and Hay (1999) there are few rigorous demonstrations of prey using such a mechanism to avoid predators. Our results in laboratory bioassays suggest that *M. polymnia* is not perceived by the predatory ant *C. crassus* due to the similarity of the cuticular lipids profile with its host plant *S. tabacifolium*. Although the lipids have not yet been chemically analyzed, the bioassays showed that *C. crassus* may perceive the contrast between larval lipids and plant lipids and recognize the larvae as prey. Among ants, the discrimination of nest mates and intruders is essentially based on the perception and similarity of cuticular substances (Hölldobler and Wilson, 1990; Vander Meer and Morel, 1998). In field and laboratory bioassays, predatory ants sometimes walk over larvae, which stay immobile; this thanatose-like behavior, together with a postulated chemical similarity may enhance the protection of *M. polymnia* larvae.

How similar the lipid profiles need to be to protect the larvae remains an open question. Besides chemical analyses, additional bioassays with different blends of lipids will be needed to verify if qualitative differences in the cuticular lipid profile between larvae and host plants are as important as quantitative ones for defense. Another question is the effectiveness of this defense mechanism against other chemically oriented predators (e.g., predatory ants, bugs). Whether larvae sequester lipids from plants or biosynthesize them *de novo* will be subject for future studies. Both mechanisms have been reported for insects. For example, a scarab beetle acquires cuticular hydrocarbons from its ant host (Vander Meer and Wojcik, 1982), and a staphylinid beetle biosynthesizes them, mimicrying their specific termite host (Howard et al., 1980). Feeding experiments with labeled lipid precursors (such as amino acids or acetate) could answer this question.

In respect to definition of this defense mechanism, the nomenclature distinguishing chemical mimicry from chemical camouflage is confusing (Dettner and Liepert, 1994). We adopt the definitions of Vane-Wright (1976), where mimicry involves an organism (the mimic) simulating the signal properties of a second living organism (the model), which are perceived as signals of interest by a third living organism (the operator) in such a way that the mimic enhances its fitness as a result of the operator identifying it as an example of the model. Camouflage or crypsis involves the simulation by organisms of background or uninteresting objects, or forms, i.e., the frame of reference in which

the operator searches for things of importance (but see Endler, 1981 for another definitions of mimicry and camouflage). *Mechanitis polymnia* has characteristics of a model (*S. tabacifolium* leaf chemistry) that is not of interest to the operator, *C. crassus*, thereby camouflaging itself against the ants.

Regardless of the open questions on the acquisition of cuticular lipids by larvae and the definitions used, to our knowledge, this report is the first to demonstrate that phytophagous insects can hide from predators by the similarity between their cuticular lipids and those of their host plants. Orivel and Dejean (2000) reported that the larvae of the hesperiid butterfly *Vettius tertianus* were not attacked by the ponerine ants *Pachycondyla goeldii*, although they do not display defensive devices. However, those authors did not find similarities when comparing the hesperid larval cuticular hydrocarbons with those of the host plant, *Aechmea mertensii*, or of the ants.

Chemical camouflage due to cuticular hydrocarbons may provide a double adaptive advantage to *M. polymnia* larvae. The first, and most obvious, is protection against predation, and the second is to free *M. polymnia* from the burden of using other defensive mechanisms against predation, such as restricted feeding periods or the sequestration of toxic compounds, which could involve an energetic cost (Bowers, 1993; Salazar and Whitman, 2001). Although this chemical hiding does not involve the modification of the foraging mode to avoid predators or the sequestration of toxic compounds, it implies the sequestration or biosynthesis of cuticular lipids by *Mechanitis*. The costs are not known yet.

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DIVERGENCE IN STRUCTURE AND ACTIVITY OF PHENOLIC DEFENSES IN YOUNG LEAVES OF TWO CO-OCCURRING *Inga* SPECIES

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Abstract—The leaves of tropical forest trees are most likely to suffer herbivore damage during the period of expansion. Herbivore selection on young leaves has given rise to a variety of leaf developmental strategies and age-specific chemical defense modes. We are studying correlations between leaf developmental types and chemical defenses in the Neotropical genus *Inga*. We have characterized defense metabolites in *Inga goldmanii* and *Inga umbellifera*, two species that co-occur in the lowland moist forest of Panama. These congeners have markedly different young-leaf developmental phenotypes but suffer approximately equal rates of herbivory. Bioassays of whole and fractionated leaf extracts using larvae of *Heliothis virescens* show that *I. goldmanii* chemical defenses are nearly three times more inhibitory than those of *I. umbellifera*. In both species, most of the inhibitory activity resides in complex mixtures of monomeric and polymeric flavan-3-ols. This group comprises >30% of young leaf dry weight in both *I. goldmanii* and *I. umbellifera*. The species' phenolic chemistry differs markedly, however, both in the structure of the monomeric units and in the distribution of polymer sizes. The differences in chemical structure have pronounced effects on their bioactivities, with *I. goldmanii* flavans being twice as inhibitory to *H. virescens* larvae as *I. umbellifera* flavans, and more than three times more efficient at protein binding. Given the extraordinarily high polyphenol concentrations that are found in the young leaves of these species, protein precipitation could be an important mechanism of growth inhibition. Nevertheless, our data show that another mode of phenolic action, possibly oxidative stress, occurs simultaneously.

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Key Words—*Inga goldmanii*, *Inga umbellifera*, *Heliothis virescens*, epicatechin-4β→8-catechin-4α→8-epicatechin, flavan-3-ol glycoside, procyanidin, condensed tannin, polyphenol, phenolic, protein precipitation, bioassay, bioactivity, chemical defense evolution.

INTRODUCTION

Young leaves of tropical forest trees suffer far higher rates of herbivory than mature leaves of the same species. Surveys of leaf herbivory across a range of tree species in a moist forest in Panama show that nearly 70% of lifetime herbivore damage occurs during the days to weeks of leaf expansion (Coley and Aide, 1991; Coley and Barone, 1996). This is a remarkable finding in that the leaves of tropical moist-forest trees typically last several years. High herbivory pressure on young leaves has led to a variety of developmental and defense chemical phenotypes. We are investigating correlations between developmental pattern, chemical defense, and herbivory in young leaves of the Neotropical genus *Inga* (Fabaceae). As a starting point, we have identified two co-occurring *Inga* species that have markedly different young-leaf development. *Inga goldmanii* is a "normally greening'' species in which leaves flush singly, expand slowly, and are photosynthetic. In contrast, *Inga umbellifera* is a "delayed-greening'' species: Leaves are deployed in large flushes, expansion is rapid, and full chloroplast development occurs only after leaves have reached full size. Populations of these two species growing in lowland moist forest on Barro Colorado Island, Panama, suffer approximately equal rates of young-leaf herbivory, approximately 22% (Coley 2 et al., in press).We are characterizing the full range of defense metab-olites synthesized in these two species to determine: (1) the relative contributions of chemistry to overall herbivory-avoidance strategies, (2) the structure/activity relationships of defense metabolites, and (3) whether chemical defenses, like developmental strategies, have diverged in these congeners.

We have developed an insect growth assay using larvae of the noctuid lepidopteran *Heliothis virescens*. We used the assay to test whole young-leaf extracts and to guide chemical fractionation. We found pronounced differences between *I. goldmanii* and *I. umbellifera* in the bioactivities of whole young-leaf extracts. Through assays of individual chemical fractions, we showed that, in both species, complex mixtures of phenolic compounds are accumulated at high concentrations and account for the majority of insect growth inhibition. In this paper, we describe the phenolic chemistry in these two species and show that structural divergence within this class of compounds has led to marked differences in bioactivities.

METHODS AND MATERIALS

Collections of Plant Material. Collections from *I. goldmanii* and *I. umbellifera* were made at Barro Colorado Nature Monument (BCNM, 9°10'N, 79°50'W), Republic of Panama. Young leaves, 5–80% of full expansion, were collected from shaded, understory trees during periods of leaf flush between January 2001 and December 2002. Collections were made randomly from trees across BCNM. Leaves were pooled during a given harvesting period, typically 3–4 mo. Tissues were processed shortly after collection by maceration, first in a Waring blender and then a Polytron 3100 (Brinkmann Instruments, Westbury, NY, USA) in 95% EtOH. Suspensions (leaf solids + dissolved extractables) of each species were shipped to the University of Utah on dry ice and then stored at −80°C until processing.

Fractionation of Plant Material, Isolation of Phenolic Components, and Determination of Phenolic Mass. Two sets of extractions were made, one to prepare a whole-leaf extract for bioassay, and one to separate phenolics from leaf solids and all other leaf extractables. The whole-leaf extract contained all soluble metabolites. To prepare this, a portion of leaf material (usually 5–10 g fresh weight) was filtered and extracted repeatedly with 80% EtOH/water, 70% acetone/water, dichloromethane (DCM), and 25 and 70°C water. All solutions were combined and reduced under vacuum to a concentration of 10–20 mg/ml. (Low molecular weight terpenes are not present in the shade phenotypes of these species.) The exact concentration was determined gravimetrically by vacuum-drying 5-ml subsamples.

A second extraction was performed on the remainder of the sample to (1) isolate phenolic components into purified fractions and (2) determine the mass percentage of phenolics of the total extractable metabolites. Leaf samples were submitted to a partitioning process (details in Index), which resulted in five extract fractions plus the marc. Two of these were shown by subsequent high-performance liquid chromatography (HPLC) and mass/nuclear magnetic resonance (NMR) spectral analyses to be composed nearly entirely of phenolic components, and were designated phenolics I and II. Except where noted, these were combined into a single "phenolics" fraction. The remaining three fractions, "lipids", "proteins", and "aqueous", were vacuum-dried along with the marc. The weight of each fraction was recorded to determine total leaf dry weight (DW) and the mass percentage of phenolics. The crude and phenolic fractions were submitted to bioassay (details below). The marc was further processed (see the following section).

Butanol–HCl Digestion of Marc. Samples of *I. goldmanii* and *I. umbellifera* marc were acid digested following the method of Porter et al. (1986). After digestion, the marc was washed with water to remove the acid and then extracted with BuOH until most of the cyanidin had been removed. The marc

was dried, lyophilized, and its mass recorded. Portions of extracted and unextracted marc were subsequently submitted to bioassay (details below).

Chromatographic Separations of Phenolics. Characterization of the phenolic content of *I. goldmanii* and *I. umbellifera* began with the isolation of individual components. Low molecular weight compounds were separated from EtOAc extracts of the phenolics fraction of *I. goldmanii* by flash chromatography on silica gel using dichloromethane/MeOH, 92:8 → 80:20. A similar separation of low molecular weight compounds from *I. umbellifera* is described elsewhere (Lokvam et al., 2004).

A higher molecular weight phenolic compound, a procyanidin trimer (compound **1**, Figure 1a), was isolated from *I. goldmanii* by HPLC. The compound was separated on a 10 × 200 mm polyhydroxyethyl aspartamide (PHA) semipreparative column (5 μm, PolyLC Corp., Columbia, MD, USA) and then purified using a 10 × 250-mm Microsorb ODS semipreparative column (5 μm, Varian Analytical Instruments, Walnut Creek, CA, USA) with a guard column (ODS, 8 μm, Varian). Compound structural characterization was based on one- and two-dimensional NMR spectra (described below) and on the high-resolution matrix-assisted laser desorption initiation time-of-flight (HR-MALDI-TOF) mass spectrum, obtained using a Voyager DE-STR spectrometer (MDS Sciex, Concord, ON, CA) with the sample dissolved in MeOH/CHCA. The separation and characterization of individual procyanidins from *I. umbellifera* are described elsewhere (Lokvam et al., 2004).

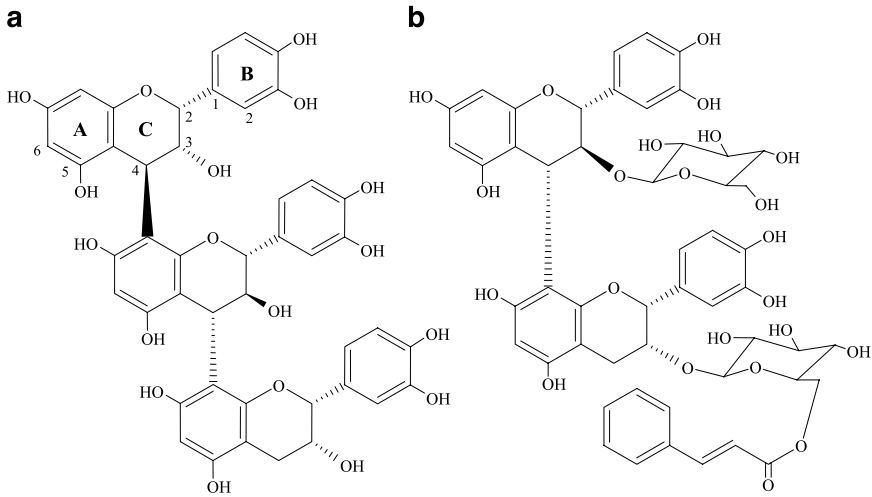

FIG. 1. (a) Trimeric procyanidin (compound **1**) from *I. goldmanii*. (b) Representative substituted procyanidin from *I. umbellifera*.

As a qualitative test of the purity of the phenolics extracts, phenolics from both *I. goldmanii* and *I. umbellifera* were analyzed by HPLC using a diode array detector (DAD) and an evaporative light-scattering detector (ELSD, SEDERE S.A., Alfortville, France). For both sample analyses, comparisons of the ELSD and DAD outputs were made to check for the presence of non-UV-absorbing compounds. *I. umbellifera* phenolics were further analyzed by liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS; details below).

NMR. ^{1}H and ^{13}C NMR spectra were acquired on a Unity iNOVA 500-MHz spectrometer (Varian). The *I. goldmanii* trimer was dissolved in CD_3OD. ^{1}H and ^{13}C spectra were referenced to the centerline of the solvent multiplets, 3.31 and 49.15 ppm from trimethylsilyl, respectively. Acquisition temperatures were $-39°C$ (for trimer ^{13}C spectrum) and $-20°C$ (for trimer ^{1}H spectrum), respectively. ^{1}H–^{1}H connectivities as well as short- and long-range ^{1}H–^{13}C correlations were observed with gradient-selected DQCOSY, HSQC, and HMBC pulse sequences.

Electro-Spray Ionization Mass Spectrometry. I. goldmanii phenolics were analyzed with a Finnigan LCQ (Thermo-Finnigan, Bremen, Germany) operated in the negative ion ESI mode. *I. umbellifera* phenolics were analyzed with a MicroMass Quattro II mass spectrometer (Waters, Milford, MA, USA) operated in the positive ion ESI mode. For both analyses, phenolic solutions in MeOH at a concentration of approximately 0.1 μg/μl were introduced into the instrument by direct infusion at a rate of 5 μl/min. *I. umbellifera* phenolics were further analyzed by LC/MS using a Waters 2690 HPLC system with a Waters 2487 UV Detector (recording at 280 nm) configured in tandem with the MicroMass spectrometer described above.

Separation of I. goldmanii Phenolics into Size Classes. Samples of *I. goldmanii* phenolics I (0.75 g) and phenolics II (0.3 g) dissolved in 50% MeOH were each applied to a water-equilibrated LH-20 column and eluted with 50% MeOH and 70% acetone. Solvents were removed under reduced pressure, and all fractions were vacuum-dried. This gave four fractions, designated phenolics I–MeOH, phenolics I–acetone, phenolics II–MeOH, and phenolics II–acetone. (*I. umbellifera* phenolics cannot be separated on LH-20 under the protocol described here.)

Estimation of Degree of Polymerization. The mean degree of polymerization (mDP) and catechin/epicatechin ratios of each of the *I. goldmanii* LH-20 fractions were determined by thiolysis, acid-catalyzed degradation of polymers in the presence of benzylmercaptan (Thompson et al., 1972). This reaction releases lower units as free monomers and extender units as their respective thioethers. Product mass percentages were estimated by comparison of their peak areas (280 nm) to calibration curves made with catechin or epicatechin standards (Sigma, St. Louis, MO, USA) or the purified thioethers. Following

conversion to moles, the mDP was calculated by the formula: (catechin + epicatechin + thioethers) / (catechin + epicatechin). HPLC analyses of derivatized and underivatized phenolics yields the relative masses of catechin and epicatechin as free monomers and as lower or extender units.

The mDP and the monomer/dimer mass percentages of *I. umbellifera* phenolics were estimated using a combination of mass and evaporative light scattering detection following the HPLC separations outlined above. The molecular ion masses of the major ELSD-detected peaks were determined using LC/ESI-MS. From these data, the number of flavanol + pyranose + cinnamate units in each of the major peaks was deduced. The overall monomer/dimer mass percentages were determined by summing the appropriate peak area percentages. Upper and lower bounds for the mDP of *I. umbellifera* phenolics were estimated by mass correction of each major polymer peak, so that area percent value reflected flavan-monomer number. The mDP was calculated from the weighted area percentages of the major polymer peaks.

Procyanidin–Protein Binding. A bovine serum albumin (BSA) precipitation assay (Hagerman and Butler, 1978) was used to assess protein binding by *I. goldmanii* and *I. umbellifera* procyanidins. This method measures the quantity of procyanidin (as absorbance of iron-phenolate at 510 nm) that precipitates with protein from a standard BSA solution over a range of total phenolic concentrations (0.125–3.0 mg/ml). Binding curves are thus obtained. The slopes of the linear portions, reported as absorbance units per milligram total phenolics per milliliter, are used as a relative measure of procyanidin–protein binding capacity. In both cases, r^2 values were >0.99.

Insect Feeding Trials. The bioactivities of whole-leaf extracts, all phenolic fractions, and the pre- and post-butanol-extracted marcs from both species were tested in growth trials using larvae of *H. virescens* (Lepidoptera: Noctuidae). Larvae were fed an artificial diet amended with plant products, and their growth relative to controls was measured. The diet was modified from Chan et al. (1978). Whole-leaf extracts (as the solutions that were described above) and phenolics (as powders) were added at prescribed mass percentages of the total diet DW. For the marc assays, marc was ground to a fine powder using a Retsch MM 200 Mixer Mill (Retsch GmbH, Haan, Germany) and was substituted at the same mass percentage (35% total diet DW) for the cellulose in the diet. Once prepared, the diet was divided into individual portion cups, typically 16 per treatment level. Controls were prepared in the same manner as treatments but without the addition of plant products. (We have verified that following high vacuum-drying there are no effects from solvents used in the whole-leaf extracts, so solvent controls were omitted.) One freshly hatched, unfed *H. virescens* larva was sealed into each cup with a vented lid and kept in a 75% RH controlled growth chamber on a 12–12 h L/D schedule for 8 d. At the end of this period, treatments and controls were weighed. Treatment weights were divided

by the control weight (GRC = growth relative to control) and fitted to the following dose–response function using the NLIN procedure in SAS:

$$\mathrm{GRC} = \frac{a_0 - a_0}{1 - (\log\ C/b_2)^{b_1}}$$

where a_0 is the response at low concentration, C is the dose in percent, b_1 is the slope, and b_2 is the logarithm of the concentration that inhibits growth by 50%. The antilog of b_2, the GI_{50}, was used to compare the relative inhibitory capacities of given plant chemical classes (Streibig et al., 1993). This statistic is based on the combined results of several assays, such that it can be assigned a 95% confidence interval (CI). If, for a given *I. goldmanii*/*I. umbellifera* assay class (whole-leaf extract or phenolics), there was no overlap in 95% CIs, the GI_{50} values were considered statistically different.

RESULTS

I. goldmanii Phenolics. I. goldmanii phenolics are composed almost entirely of monomeric catechin and epicatechin or polymers of these subunits.

TABLE 1. ^{1}H AND ^{13}C NMR SHIFTS FOR COMPOUND 1 IN CD_3OD AT −20°C (^{1}H) AND −39°C (^{13}C)[a]

	Upper		Middle		Lower	
C no.	δ ^{13}C	δ ^{1}H, mult.: Hz	δ ^{13}C	δ ^{1}H, mult.: Hz	δ ^{13}C	δ ^{1}H, mult.: Hz
A/C-2	75.5	5.18, br s	84.3	3.77, d: 10.0	79.6	5.09, br s
A/C-3	72.6	3.63, br s	74.2	4.58, m	65.9	4.38, o
A/C-4	36.9	4.44, br s	38.5	4.38, dd: 10.0, 7.5	23.5	2.55, br d: 16.0; 2.17, br d: 16.0
A/C-5	157.3		156.3		156.0	
A/C-6	95.5	5.22, o	97.2	6.12, s	94.6	6.02, s
A/C-7	157.3		155.4		154.7	
A/C-8	95.0	5.66, d: 2.0	110.5		108.6	
A/C-9	155.8		154.7		153.0	
A/C-10	103.9		107.8		100.4	
B-1	133.3		132.7		132.1	
B-2	115.7	6.90, br s	115.3	6.69, br s	112.6	6.35, br s
B-3	145.5		145.4		146.2	
B-4	144.7		146.2		145.3	
B-5	115.3	6.55, d: 8.0	115.3	6.62, o	116.2	6.42, o
B-6	118.9	5.47, o	121.3	6.41, o	118.6	5.46, o

[a]All values relative to the center line of the solvent multiplet (3.31 ppm for ^{1}H, 49.15 ppm for ^{13}C).

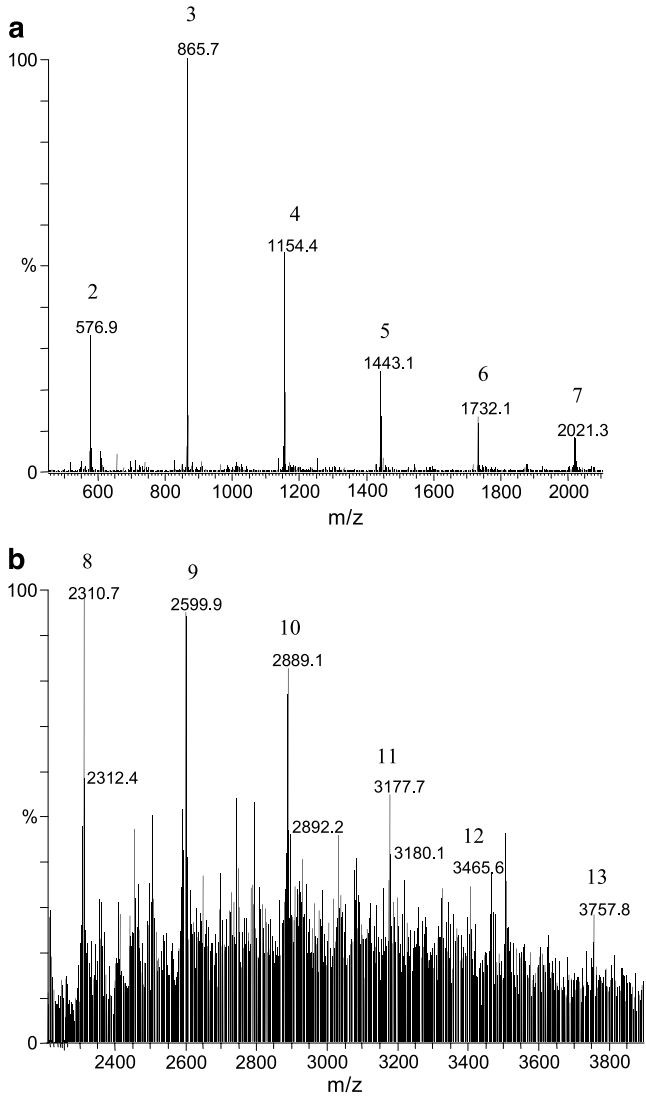

FIG. 2. ESI mass spectra of *I. goldmanii* phenolics. The low mass spectrum (a) shows masses from 500 to 2100 amu; the high mass spectrum (b) shows masses from 2200 to 3900 amu. Peaks are labeled by mass and polymer length.

This conclusion was based on NMR and mass spectral analyses and on thiolytic degradation followed by HPLC separation and diode array detection. Both catechin and epicatechin were isolated as monomers by flash chromatography on silica gel. They were identified by comparison of their ^{1}H NMR spectra to standards (Sigma). In addition, a procyanidin trimer, compound **1** (Figure 1a) was isolated by semipreparative HPLC. The structure of this compound, epicatechin-4β→8-catechin-4α→8-epicatechin, was deduced from 1D (Table 1) and 2D NMR spectra. HR-MALDI-TOF MS gave an $[M + Na]^+$ ion of *m/z* 889.1996, calculated for $C_{45}H_{38}O_{18}Na$ (5.1 ppm error) and consistent with a singly linked trimeric procyanidin. Compound **1** was first isolated but only minimally characterized by (Hsu et al., 1985).

The ESI mass spectrum of *I. goldmanii* phenolics showed only molecular masses consistent with monomeric catechin/epicatechin or singly linked polymers thereof (Figure 2). $[M - H]^-$ ions were observed ranging from 576.9 to 3757.8 amu, corresponding to degrees of polymerization 2 to 13. No evidence of multiply charged ions was detected. Thiolytic degradation of *I. goldmanii* phenolics followed by HPLC separation and diode array detection of the products indicated that the great majority of the product mass is catechin/epicatechin and their thioethers. This was corroborated using HPLC separation and detection by evaporative light scattering, a mass-sensitive technique, which showed no other major components.

TABLE 2. MASS PERCENTAGES, PHYSICAL PROPERTIES, AND BIOACTIVITIES OF SOME *I. goldmanii* AND *I. umbellifera* METABOLITE FRACTIONS

	% DW	mDP[a]	PP[b]	GI_{50}[c]	95% CI
I. goldmanii crude extract	48	–	–	1.1	0.87–1.42
I. umbellifera crude extract	61	–	–		2.11–3.64
I. goldmanii whole phenolics	29	7.2[d]	0.44	0.59	0.38–0.8
I. umbellifera whole phenolics	31	2.2–3.4	0.13	1.45	1.31–1.58
I. goldmanii LH-20 fractions					
Phenolics I–MeOH	6	2.0[e]	0.12	1.0	0.3–1.6
Phenolics I–acetone	14	6.1	0.56	1.2	0.7–1.9
Phenolics II–MeOH	2	nd	0.16	nd	–
Phenolics II–acetone	6	15.3	0.72	1.1	0.4–3.0

nd = Not determined.
[a] Mean degree of polymerization.
[b] Procyanidin–protein binding capacity: slope of linear binding curve (absorbance per milligram phenolic per milliliter).
[c] Concentration (% DW) of extract in insect diet that is required to reduce growth by 50% relative to controls.
[d] Weight average, based on LH-20 fractions.
[e] Free monomer content removed.

I. goldmanii phenolics I and II fractions were analyzed for percent monomer composition and degree of polymerization after separation on LH-20 (96 and 94% recovery, respectively). Monomeric catechin and epicatechin in the *I. goldmanii* phenolics were approximately 15.0 and 0.8%, respectively, of the total phenolic mass as estimated by HPLC analysis of underivatized phenolics I–MeOH, the only fraction that contained detectable quantities of free monomer. The mDP of the *I. goldmanii* phenolics was determined by HPLC analysis following thiolysis of the interflavanyl linkage in the presence of toluene-α-thiol (Table 2). Phenolics I–MeOH contained monomeric catechin/epicatechin and low molecular weight procyanidin with an mDP estimated to be 2.0. Phenolics II–MeOH was not amenable to analysis by these methods and gave only low concentrations of the normal thiolysis products, i.e., free monomers and thioethers. Based on its weak protein-precipitation ability, it appears to contain procyanidin. We suspect that this fraction consists of low molecular weight procyanidin/carbohydrate complexes whose chemistry prevents acid hydrolysis under the conditions we used. Phenolics I– and II–acetone contained higher molecular weight procyanidins with mDPs estimated to be 6.1 and 15.3, respectively. Based on the weighted averages of the individual fractions, the mDP of the entire phenolic population was estimated to be 7.2. Catechin–epicatechin ratios of the free monomers and for each of the *I. goldmanii* LH-20 fractions are listed in Table 3. Catechin is the predominant monomeric form and the most common procyanidin initiator unit, particularly in the higher molecular weight polymers. In contrast, epicatechin comprises more than half of the extender units in all *I. goldmanii* fractions.

I. umbellifera Phenolics. I. umbellifera phenolics, like *I. goldmanii* phenolics, are composed entirely of compounds based on catechin and epicatechin. In contrast to *I. goldmanii*, however, *I. umbellifera* flavanols are each substituted at the C3 position with either a simple hexose or a hexose that is mono- or disubstituted with a cinnamoyl ester (see Figure 1b). These conclusions are based on several lines of evidence. Purification and rigorous structural characterization of several *I. umbellifera* flavans (Lokvam et al., 2004) yielded the building blocks of these compounds: catechin/epicatechin, hexose (glucose in each case that was explicitly determined), and cinnamate. ESI mass spectra showed that all observed molecular masses in *I. umbellifera*

TABLE 3. CATECHIN–EPICATECHIN RATIOS OF *I. goldmanii* PHENOLICS

	Free monomer	Extender units	Lower units	Full fraction
Phenolics I–MeOH	18.7	0.9	1.9	1.3
Phenolics I acetone	–	0.7	4.5	1.0
Phenolics II–acetone	–	0.5	4.8	0.6

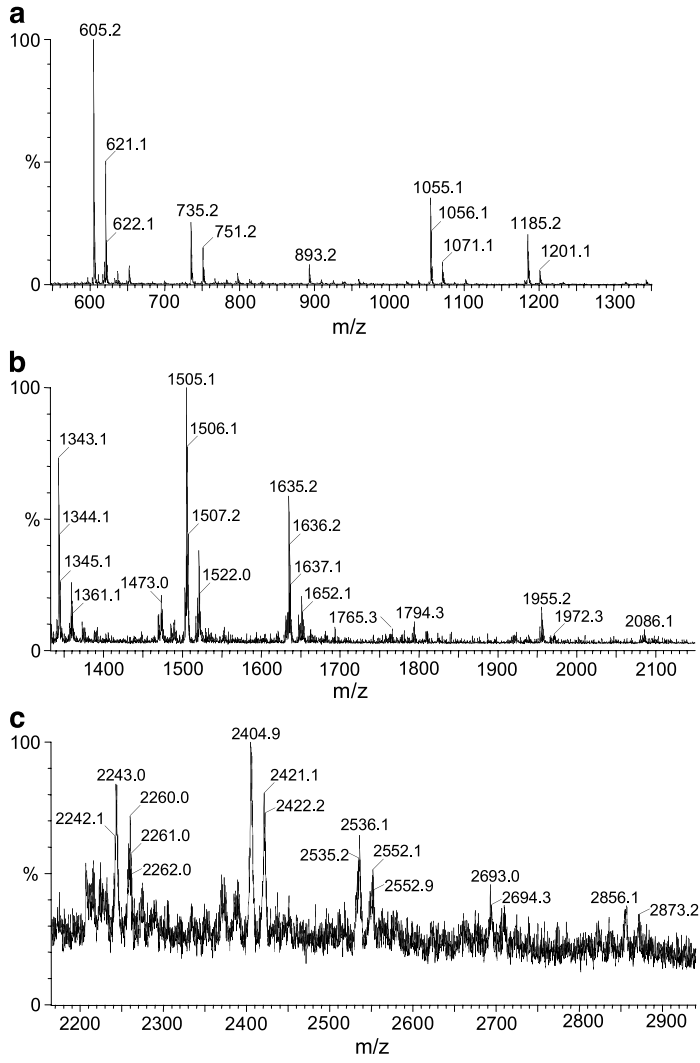

FIG. 3. ESI mass spectra of *I. umbellifera* phenolics. (a) Shows masses from 550 to 1350 amu, (b) shows masses 1350 to 2150, and (c) shows masses from 2150 to 2950 amu. In the positive ion mode, M + Na and M + K are the dominant molecular ions observed in spectra of *I. umbellifera* phenolic extracts. Peaks are labeled by mass (M + Na ions). For structural inferences, see Table 2.

TABLE 4. STRUCTURAL INFERENCES BASED ON MASS SPECTRA OF *I. umbellifera* PHENOLICS

$(M + Na)^+$	Flavan no.	Pyranose no.	Cinnamate no.
605.2	1	1	1
735.2	1	1	2
893.2	2	1	1
1,055.1	2	2	1
1,185.2	2	2	2
1,343.1	2	2	3
1,473.0	3	2	2
1,505.1	3	3	1
1,635.1	3	3	2
1,765.2	3	3	3
1,794.3	4	3	1
1,955.2	4	4	1
2,086.1	4	4	2
2,243.0	5	3	3
2,404.9	5	4	2
2,536.1	5	5	2
2,693.1	6	5	1
2,856.1	6	6	1

phenolics can be accounted for by combinations of the known monomeric subunits (Figure 3, Table 4, and the following section). *I. umbellifera* flavanols, unlike mixtures of unsubstituted *I. goldmanii* procyanidins, separate cleanly by ODS HPLC. DAD chromatograms of *I. umbellifera* phenolics showed over 40 (mostly minor) peaks having UV absorbance spectra nearly identical to the purified monomeric and dimeric compounds already structurally characterized. In addition, evaporative light scattering detection showed only minor contributions to the phenolics fraction mass from non-UV-absorbing components.

The ESI mass spectrum of *I. umbellifera* phenolics (Figure 3, Table 4) was recorded between 200 and 4000 amu. It showed monomer molecular masses of 605.2 and 735.2, and polymer masses between 893.2 and 2856.1 amu. These latter correspond to polymer sizes ranging from dimer (monoglucosyl, monocinnamoyl) to hexamer (hexaglucosyl, monocinnamoyl). As with *I. goldmanii*, no evidence of multiple charging was observed. The monomer and dimer concentrations and mDP of *I. umbellifera* phenolics were estimated following separation by HPLC and detection by complementary modes, mass and evaporative light scattering (Figure 4). Monomeric forms comprise approximately 30% of the total phenolic mass, and dimeric forms an additional 37%. Following mass correction of each peak to molar flavanol equivalents, the mDP of the *I. umbellifera* polymer population was estimated to be between 2.2 and 3.4.

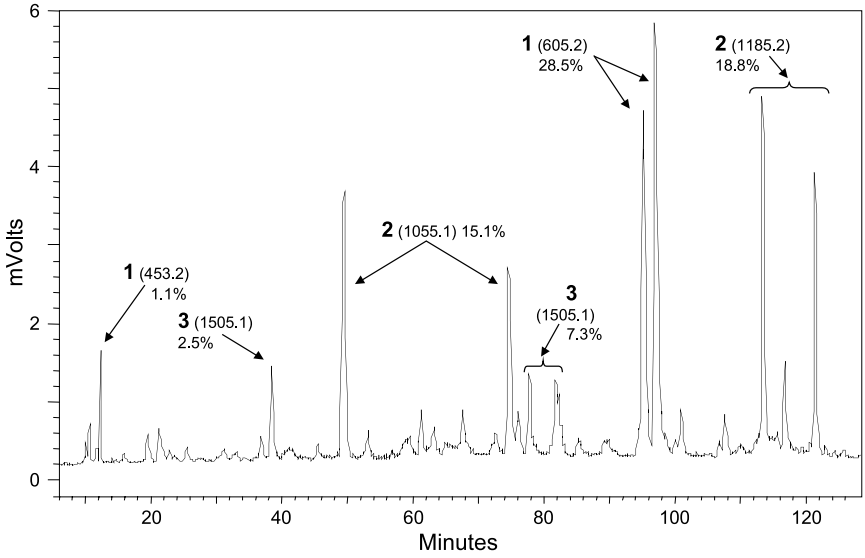

FIG. 4. HPLC chromatogram of *I. umbellifera* phenolics as detected by evaporative light scattering. Main peaks are labeled by flavanol number, mass (M + Na ions), and area percent of the full integrated chromatogram. For structural inferences, see Table 2.

Total Phenolics Mass. The extraction procedure used removed approximately 30% of young leaf DW as phenolics from both *I. goldmanii* and *I. umbellifera* (Table 2). In both species, approximately 70% of the extractable phenolics dissolved into aqueous EtOH. A further 30% of the extractable phenolics was removed using aqueous acetone. In addition to soluble leaf phenolics, butanol/HCl extraction of the marc produced a dense cyanidin solution, the result of acidolysis and oxidation of carbohydrate-bound procyanidins (Shen et al., 1986). The extraction removed approximately 32 and 52%, respectively, of the starting mass of the marcs of *I. goldmanii* and *I. umbellifera* (Table 5). The exact

TABLE 5. PRE- AND POST-ACID DIGESTED MASSES AND BIOACTIVITY OF *I. goldmanii* AND *I. umbellifera* MARC

	% Leaf DW	GRC[a]	*P*
I. goldmanii marc (+) phenolics	52	0.12	<0.05
I. goldmanii marc (−) phenolics	35	0.69	<0.01
I. umbellifera marc (+) phenolics	39	0.12	<0.05
I. umbellifera marc (−) phenolics	18	0.87	n.s.

[a] Growth relative to the control at a single concentration (35% of dry weight).

proportion of the extracted mass that was phenolic was not determined, but this proportion was likely considerable.

Procyanidin–Protein Binding. Procyanidin–protein binding curves were obtained for phenolics from both species. The linear portions were 0.125–1.0 mg phenolics/ml for *I. goldmanii*, and 0.125–2.5 mg phenolics /ml for *I. umbellifera*. In both cases, r^2 values were >0.99. The slopes of the linear portions of the binding curves were used to compare procyanidin–protein binding between the two species (Table 2). In comparison to *I. umbellifera* phenolics, approximately 3.5 times more *I. goldmanii* phenolics were involved in precipitating protein from a standard BSA solution. A similar analysis of *I. goldmanii* phenolics I–MeOH/acetone and phenolic II–acetone (Table 2) having mDPs of 2.0, 6.1, and 15.3, respectively, showed that protein-binding capacity increases sharply as a function of polymer length.

Bioactivities of I. goldmanii and I. umbellifera Young Leaf Metabolites. Whole-leaf extracts of the young leaves of *I. goldmanii* and *I. umbellifera* gave GI_{50} values (Table 2) of 1.1 and 2.8%, respectively, in the *H. virescens* larval growth assay. Assays of specific fractions (see "Methods and Materials'') showed that phenolics accounted for more than 50% of the growth-inhibiting activity observed in the whole-leaf extracts of each species. The *I. goldmanii* phenolics fraction, however, was more than twice as inhibitory as that from *I. umbellifera* (GI_{50} = 0.59 and 1.45%, respectively, 95% CIs nonoverlapping). The marcs from both species were assayed before and after digestion with BuOH/HCl to remove tissue-bound phenolics (Table 5). When substituted into the insect diet at the mass percentage of cellulose (35%), marc (+) phenolics reduced insect growth by more than 80% relative to controls in both species. In contrast, the growth reduction caused by marc (−) phenolics was not significant for *I. umbellifera* and was approximately 30% for *I. goldmanii*.

DISCUSSION

Feeding trials with larvae of the noctuid moth *H. virescens* show that whole-leaf extracts of young leaves from *I. goldmanii* and *I. umbellifera* have markedly different growth-inhibitory capacities. Overall, the suite of extractable metabolites synthesized by the normally greening species, *I. goldmanii*, is nearly three times more inhibitory than those synthesized by the delayed-greening species, *I. umbellifera*. Isolation and bioassay of the phenolic portion of the extractable metabolites shows that most of the observed activity is caused by metabolites in this fraction. Detailed chemical characterization indicates that, in both species, the phenolic fraction consists of a complex mixture of monomeric and polymeric flavan-3-ols.

In *I. goldmanii*, flavan-3-ols are synthesized as monomeric catechin/epicatechin and oligomeric to highly polymerized procyanidins. The polymeric forms have an average length of approximately 7.2. In *I. umbellifera*, like *I. goldmanii*, catechin and epicatechin are the only phenolics that were detected. In this species, they occur exclusively as their 3-*O*-glycosyl or 3-*O*-(cinnamoyl)-glycosyl derivatives. Moreover, in contrast to *I. goldmanii*, *I. umbellifera* flavan-3-ols are dominated by lower molecular weight monomeric and dimeric forms.

Flavan-3-ols are a primary line of chemical defense in *I. goldmanii* and *I. umbellifera*. This class of compounds is synthesized at extraordinarily high concentrations in the young leaves of these species. The phenolic extraction protocol used in this study was not exhaustive, yet approximately 30% of the dry weight of both species was recovered as soluble flavans. In addition, an acid–butanol digestion of the marc showed the presence of appreciable amounts of tissue-bound flavans such that flavanol content may approach 50% of the leaf dry weight. In the *H. virescens* assay, the GI_{50} values for the whole phenolics extracts were 0.6% for *I. goldmanii* and 1.3% for *I. umbellifera*, concentrations that are far below what *Inga* herbivores encounter in the wild. Assays of pre- and post-acid-digested marc indicate that tissue-bound phenolics also contribute substantially to the overall phenolic toxicity. Bioassays such as these, against a naive, generalist herbivore, demonstrate the potency.

Structural differences in the phenolic metabolites expressed by *I. goldmanii* and *I. umbellifera* produce differentiable effects on the growth of *H. virescens* larvae. *I. goldmanii* phenolics are composed of unsubstituted procyanidin polymers. On a mass basis, this set of compounds is more than twice as inhibitory as the substituted forms produced by *I. umbellifera*. Similar results were observed in bioassays conducted in Panama with *Phoebis philea*, a lepidopteran that specializes on mimosoid legume leaves (Lokvam and Kursar, unpublished data).

Phenolics are among the most widely distributed and abundant secondary metabolites in plants (Bate-Smith, 1962, 1968), but their mode of action and ecological function have proven difficult to define, particularly with respect to insects. Following Feeny's (1969) observation that polyphenols could interfere with herbivore digestion by precipitating enzymes and/or dietary proteins, considerable attention was focused on the defensive role of high molecular weight phenolics (Feeny, 1976; Rhoades and Cates, 1976; Swain, 1979; Zucker, 1983). Their mechanism of action, however, remains obscure. The "tannin–protein binding'' mode of action has been questioned since (1) the guts of many herbivorous lepidoptera, with their high pHs and detergent content, are well adapted to prevent formation of polyphenol/protein complexes (Martin and Martin, 1984; Blytt et al., 1988), and (2) this mechanism has never been demonstrated *in vivo*. Martin et al. (1987) showed that in *Manduca sexta* gut fluid hydrolyzable tannin–RUBISCO ratios must be at unity or greater for appreciable protein precipitation to occur. They suggest that such high

polyphenol/protein ratios would only rarely be encountered in nature, and that protein precipitation is, therefore, probably not an ecologically relevant process. The extraordinarily high procyanidin content that we observe in young *Inga* leaves, however, argues against this point. At concentrations of 25–30% leaf dry weight, procyanidins could inhibit digestion through protein binding.

Simple phenolics do not precipitate protein, yet have been shown to have strong negative effects on insect herbivores. For instance, functioning as pro-oxidants, the *o*-diphenols, caffeic and chlorogenic acid, were shown to alter gut chemistry in *Helicoverpa zea* (Summers and Felton, 1994), and are correlated with low larval growth rates in that species. These compounds function as toxins by degrading the gut epithelium. A distinction can be made then between the activity of *o*-diphenols (which include procyanidins) as outright toxins and high molecular weight phenolics as "digestibility reducers'' (Duffey and Stout, 1996). In a chemically complex group like the flavan-3-ols, the distinction must allow for considerable functional overlap (Butler et al., 1986; Blytt et al., 1988), but it is likely that there are multiple modes of activity that occur simultaneously.

Using LH-20, we separated *I. goldmanii* procyanidins into size classes having mean degrees of polymerization of 2.0, 6.1, and 15.3. The BSA precipitation assay showed that there is a strong positive correlation between protein-binding capacity and procyanidin molecular weight, a finding that is in agreement with earlier studies (Kumar and Horigome, 1986; Horigome et al., 1988). Bioassays showed, however, that both the low and high molecular weight fractions were equally inhibitory to *H. virescens* larvae. Similarly, comparisons of the BSA binding characteristics of *I. goldmanii* and *I. umbellifera* phenolics showed a pronounced difference in binding efficiency. The simple, unsubstituted *I. goldmanii* phenolics were approximately four times more efficient than the substituted forms from *I. umbellifera*. This is almost certainly due to the presence in *I. goldmanii* phenolics of high molecular weight procyanidins. Nevertheless, *I. umbellifera* phenolics are potent growth inhibitors for larvae of *H. virescens*. Assuming that *in vitro* BSA precipitation approximates the *in vivo* interaction between procyanidin and dietary protein, our data are consistent with a multiple-mode view of phenolic bioactivity in *Inga*: oxidative stress due to the presence of *o*-diphenols, for example, and protein precipitation due to high molecular weight procyanidins. *H. virescens* is not a suitable assay organism for testing the multiple-mode concept. A rigorous bioassay will require a specialist herbivore, one that can tolerate diets containing the high levels of phenolics found in *Inga* leaves.

Simple procyanidins are likely the primitive form in this class of compounds. It appears that a novel set of selection pressures has led to a derived form of phenolic metabolites in *I. umbellifera*. The evolution of the delayed-greening/rapid expansion phenotype in the young leaves of *I. umbellifera* has likely imposed physiological constraints on the production

and storage of phenolics at the high concentrations seen in these species. The derived phenolics in *I. umbellifera* may have diverged from the ancestral form in parallel with the divergence in developmental strategy (Kursar and Coley, 2003). A more thorough knowledge of the phenolic chemistry of other delayed-greening *Inga* species, as well as an understanding of the whole-genus phylogenetic relationships, will certainly shed some light on this question.

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THE EFFECT OF POLLINATION ON FLORAL FRAGRANCE IN THISTLES

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Abstract—We investigated postpollination changes in fragrance composition and emission rates, as well as pollinator discrimination in hand-pollinated flower heads of two thistle species: Canada thistle (*Cirsium arvense*) and sandhill thistle (*C. repandum*). Following pollination, neither species emitted any novel compounds that could function as repellents. Scent emission rates declined in pollinated plants of both species by approximately 89% within 48 hr. This decline was evident in all 13 scent components of *C. arvense*. *Apis mellifera*, the dominant pollinator in the study population of *C. arvense*, was nearly three times more likely to visit an unpollinated rather than a pollinated flower head. A more complex pattern was observed for *C. repandum*, whose scent comprised 42 compounds. Quantities of aromatic and sesquiterpenoid volatiles declined after pollination, whereas two classes of scent compounds, fatty acid derivatives and monoterperpenoids, continued to be emitted. In *C. repandum*, discrimination against pollinated flower heads by *Papilio palamedes* (its primary pollinator) was not as marked. Unpollinated control plants of both species maintained moderate levels of scent production throughout this experiment, demonstrating that senescence and floral advertisement may be delayed until pollination has occurred. We expect postpollination changes in floral scent contribute to communication between plants with generalized pollinator spectra and their floral visitors. This study provides the first field study of such a phenomenon outside of orchids.

Key Words—Asteraceae, Canada thistle, *Cirsium arvense*, *Cirsium repandum*, dioecious, fragrance, herbivory, pollination, floral volatiles.

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INTRODUCTION

Floral fragrances advertise the site of reproductive structures (and rewards), luring pollinators from great distances (Metcalf and Metcalf, 1992). This manipulation of pollinators is often reciprocally beneficial, as floral scent promotes pollinator fidelity and pollinator efficiency (Marden, 1984; Dornhaus and Chittka, 1999). However, herbivores also navigate within a landscape of floral fragrances; plants that increase their apparency through floral display may be attracting their own enemies (Galen and Butchart, 2003). Advertising with visual and olfactory displays is only necessary prior to successful pollination. Following pollination, it may be beneficial to reduce the apparency of a flower to avoid attracting unwanted visitors. Research on pollination-induced changes has centered on documenting changes to the visual rather than the olfactory display. Fragrance is a complex trait. Over 150 compounds can be contained within a floral scent blend. However, it is also a quantifiable trait, and even small changes can be measured (Tollsten, 1993). As yet, pollination-induced changes to the *full set of components* in the fragrance blend have not been quantified from flowers other than orchids.

Postpollination changes, which can include withering, closing, or abscission of petals, color change, positional adjustment (becoming inaccessible to pollinators), cessation of nectar production, and scent alteration, have not been extensively tested across the angiosperms. There has been a widespread expectation that flowers almost universally undergo postpollination changes (Wheiss, 1995; see van Doorn, 1997 for exceptions). However, few studies directly test for pollination-induced changes (Gori, 1983; Primack, 1985; van Doorn, 1997), and when tested directly, color changes in flowers, for example, have often been found to be a result of floral aging, rather than a consequence of pollination (Casper and LaPine, 1984; Delph and Lively, 1989). Most studies on pollination-induced changes have centered on orchids, whereas only half of the remaining angiosperm families with animal-pollinated species have been examined for this phenomenon (van Doorn, 1997).

Scent changes induced by pollination have the potential to function similarly to color change, the best studied of the pollination-induced changes (Wheiss, 1995). First reported in the literature in the 1700s (Wheiss, 1995), color change allows flowers to continue contributing to the floral display after they are pollinated, in some cases even increasing visitation to a plant (Gori, 1989; Niesenbaum et al., 1999). Pollinators are recruited from a distance by the colorful display, but experienced pollinators avoid older flowers at closer proximity. The response of pollinators and/or nectar thieves to floral color change involves learning and represents an informed "decision". This has been demonstrated for a number of species (Wheiss, 1997). Wheiss (2001) suggests that for some plants, color change may not be advantageous, for example, if their

pollinators are incapable of associative learning. Interestingly, flowers pollinated exclusively by beetles do not undergo postpollination color changes.

Similar to the learned behavior associated with color changes, postpollination changes in floral fragrance could inform an experienced pollinator of the age and/or reward status of a flower. However, such a phenomenon need not depend upon associative learning, as floral scent can specifically target a pollinator's preconditioned behavior. In that case, the pollinator does not need to be experienced or have the ability to learn, as scent changes could result in signals that are already informative. This is the case for the sexually deceptive orchid, *Ophrys sphegodes*, in which Schiestl and Ayasse (2001) documented an increase in specific components that appear to act as pollinator repellents, rather than a general cessation of odor. This orchid species is pollinated through pseudocopulation by males of the solitary bee *Andrena nigroaenea*. The postpollination floral repellent, farnesyl hexanoate, mimics a compound produced by female bees and used in brood cells to decrease mating attempts (Bergström and Tengo, 1974). In the orchids, postpollination changes have been attributed to energy conservation (Arditti, 1979). However, the promotion of effective pollen deposition (outcrossing) is clearly the best explanation for scent change in this case, where pollinators must be duped not once but twice, and visits are rare events (Borg-Karlson, 1990).

Rather than altering in composition, fragrance may simply decline once pollination has taken place as the flower senesces, thus reducing herbivore attraction, discouraging ineffective pollen deposition, and conserving metabolic energy (Dudareva and Pichersky, 2000). In the orchid *Platanthera bifolia*, Tollsten observed a decrease in scent production just 24 hr after pollination, with a continual decline 2 and 5 d after, whereas the visual display remained unchanged for up to 1 wk (Tollsten and Bergström, 1989; Tollsten, 1993). The maintenance of floral display in *P. bifolia* suggests that factors besides energy conservation or floral senescence may mediate pollination-induced floral change in this orchid.

We sought to determine how the fragrance composition and total scent emissions might change in plants with generalized pollination biology. In such a system, postpollination signals are likely to act broadly on insect visitors rather than elicit behaviors from one specific pollinator. We conducted our study on two species of thistle, *Cirsium* (Asteraceae), whose flower heads attract and are pollinated by several species of insects. Would these thistles act similarly in spite of their different growing conditions, pollinator spectra, and fragrance chemistry? We sought to answer the following questions: (1) Are there pollination-induced changes in the floral scent in *C. arvense* and *C. repandum*? If so, what are the time scales and nature of these changes, i.e., are new compounds emitted, or does scent simply decline? (2) Do pollinators respond to changes in scent by avoiding pollinated flower heads? (3) Finally, in the absence of pollination, is senescence delayed and/or is the emission of fragrance enhanced as the risk of not getting fertilized increases?

METHODS AND MATERIALS

Natural History of Species. Cirsium repandum Michaux, sandhill thistle (Asteraceae), is native to the coastal plain of SE North America. It is an erect biennial or perennial that grows to 0.5–0.8 m tall and is found in sandy habitats common to North Carolina and South Carolina (Radford et al., 1968). Its range also includes parts of southeastern Virginia to eastern Georgia, but it is rare in these regions. The flower heads are protandrous, dark pink, with 1–5 flower heads per plant; flowering extends from May through July. Pollinators include Lepidoptera and Hymenoptera to a lesser degree. *C. arvense* (L.), Canada thistle, is also referred to as creeping thistle because of the creeping root system that leads to dense clonal stands. It is an erect perennial 0.3–2 m in height. *C. arvense* is an aggressive invasive species native to Europe, Western Asia, and North Africa, and was probably introduced into Canada in the early 17th century (Peschken and Derby, 1992). The plants have few to many flower heads comprising pink-purple disk flowers, which bloom from July through September and are pollinated by many species of generalist flower visitors in the orders Diptera, Hymenoptera, and Lepidoptera (Proctor et al., 1996).

Study Sites. C. repandum was studied at the Belle W. Baruch Institute of Coastal Ecology and Forest Science at the Hobcaw Barony located in Georgetown, SC, USA (79°18′W, 33°35′N). The field site is located in the shaded understory of a mixed loblolly pine and oak forest. *C. arvense* was studied at the U.S. Fish and Wildlife Wallkill River National Wildlife Refuge (Sussex, NJ, USA; 74°54′W, 41°26′N) established in 1990. Part of this refuge is situated on an old soccer camp, where our field site was located. The site is an open meadow with populations of both native and exotic invasive plants.

Hand Pollinations of C. repandum and C. arvense. To determine if there are pollination-induced changes in floral scent, flower heads restricted from natural pollination were hand-pollinated or kept unpollinated. Forty flower heads from each species of thistle—*C. repandum* and *C. arvense* (pistillate plants only)—were bagged in pairs just before florets had begun to emerge. Bags were made from insect screening 192 mesh (International Greenhouse Company, Sidell, IL, USA), with a strip of Velcro sewn at the base. In *C. repandum*, bagging began on June 10 and June 14, 2002. In *C. arvense*, bagging began on July 21 and July 23, 2002. Bagging occurred over 2 d to stagger data collection. Each flower head in a pair was randomly assigned to a pollination treatment. Three times a day, florets were brushed with cut flower heads that were producing pollen, and pollen-covered pipe cleaners. Bags covering unpollinated flower heads were similarly opened and reclosed three times a day to simulate the pollination treatment. Pollination manipulation was initiated on the 1st d that florets emerged and continued until all florets had emerged. The

two treatments, pollinated and unpollinated, were tested for pollination-induced changes either through observing pollinator behavior or scent changes (see below); flower heads were then rebagged and left to mature. Three wk after the experiment, flower heads were dissected, and the success of the pollination manipulation was established. Although *C. repandum* was not emasculated, self-fertilization did not occur in the unpollinated treatment.

Pollinator Observations. Half of the bagged pairs of flower heads were observed for differences in pollinator visitation. Observations were made 1 d after pistillate florets had entirely emerged, usually 2 d after pollinations began. Twenty flower heads from each species were unbagged and observed for 30 min at midday. All visitors and their bout lengths were documented to determine the number of visits per flower head, and the bout length for the two treatments. When possible, the pollinated and unpollinated treatments were observed simultaneously, otherwise observations were consecutive.

Scent Collections. To track postpollination scent changes, fragrance was collected from flower heads beginning after the entire flower head had emerged and had putatively entered the pistillate phase. All pollinations began as soon as the first florets emerged. In pistillate *C. arvense*, scent was collected after 24 hr, 1 d after the initial pollination (1 DAIP) and continuing until 3 d later (3 DAIP). In *C. repandum*, scent was collected on 2 DAIP and on 3 DAIP. In *C. arvense*, with the same sample size as *C. repandum*, we collected scent for 3 d, but only from an individual flower head for 2 consecutive d. From half of the plants we collected scent on 1 DAIP and on 2 DAIP. From the other half, we collected scent on 2 DAIP and on 3 DAIP. In *C. arvense*, pistillate maturation takes approximately 1 or 2 d. Therefore, in *C. arvense*, not all florets were necessarily pollinated in the pollination treatment on the 1st d of scent collection. In hermaphroditic *C. repandum*, pistillate maturation typically takes at least 2 d. We collected scent from *C. repandum only* after full pistillate maturity, which usually took place 48 hr after the initial pollination; we defined this day as 2 DAIP. Scent was collected from each individual flower head for 2 consecutive d.

Volatiles were collected using dynamic headspace sampling in the field. Intact flower heads were enclosed within a nylon resin oven bag (Reynolds Consumer Products, Richmond, VA, USA; Raguso et al., 2003). A glass cartridge was packed with 100 mg of the adsorbent Porapak Q (80–100 mesh). Ambient air was then pulled through an open hole into the bag across the flower head, through the cartridge and the adsorbent material at a flow rate of ca. 250 ml min^{-1} using a vacuum manifold, Air Check 52 or Air Check 2000 (SKC Inc., Eighty Four, PA, USA). Collections of ambient and vegetative volatiles allowed for identification of the floral compounds. Scent collections continued from 11:00 until 15:00 hr. Three flower heads were chosen to match the range of variation for the mass of the experimental plants. These prototypes were cut and dried (60°C) to estimate the dry weights. After a scent collection, cartridges

were eluted with 3 ml of hexane, at which time an internal standard of 3 μl of 0.01% anisole was added. After concentrating the samples to 75 μl, a 1-μl aliquot of sample was injected splitless onto a polar column [diam: 0.25 mm, length: 30 m, film thickness: 0.25 μm (EC WAX); Alltech Associates, Inc. Deerfield, IL, USA] at 240°C into Shimadzu gas chromatograph GC-17A equipped with a Shimadzu QP5000 quadrupole electron impact mass spectrometer as a detector. The carrier gas, helium, had a 1 ml min^{-1} flow rate and a split ratio of 12:1. The temperature program for the column began at 60°C for 3 min and was then ramped 10°C per min up to 260°C where it was held for 7 min. The pressure program was set at 60.6 (kPa) and then increased with a pulse of 400 (kPa) for 48 sec before returning to 60.6 for 2 min. Pressure was then increased at 3.9 (kPa) per minute until it reached 132.9 (kPa), where it was held for 7 min.

Compounds were identified through a combination of retention time (from previously injected standards) and mass spectral libraries [Wiley 1995 and NIST 1998 libraries (with more than 120,000 mass spectra)]. Quantification was achieved by relating mass ions of each scent compound to the mass ions of the internal standard, using previously run serial dilutions (Theis, 2003).

Statistical Analysis. Data were analyzed using SYSTAT 10.0 (Systat Software Inc., Richmond, CA, USA). For all scent data, neither relative amounts nor flux rates adhere to assumptions of normality, nor do insect distributions. Many of the compounds in the blend are produced by the same biosynthetic pathway and are correlated. In the absence of behavioral assays with specific compounds and/or blends, we expressed scent data in both relative amounts as well as flux rate: ng (g dry floral tissue)$^{-1}$ hr^{-1}. All compounds or compound classes (in the case of *C. repandum*) were tested for significant differences using the Mann–Whitney *U* test for pairwise comparisons. All *post-hoc* comparisons of individual days were tested using Bonferroni adjusted *P*-values (Sokal and Rohlf, 1995). In *C. arvense*, this resulted in a total of three *post-hoc* tests $\alpha < 0.017$, and two *post-hoc* tests for *C. repandum* $\alpha < 0.025$. A decline in scent due to aging was tested in unpollinated plants over days using the Mann–Whitney *U* test for *C. repandum*, and Kruskal–Wallis test for *C. arvense*. Pollinator visitation was analyzed using Fisher's exact test to differentiate between pollinated and unpollinated flower heads (received a visit/never visited). The Mann–Whitney *U* test was used for analyzing the number of pollinator visits and the bout lengths at pollinated and unpollinated flower heads.

RESULTS

Scent Reduction in C. repandum. Pollination of *C. repandum* flower heads resulted in a decrease in total scent production (Mann–Whitney *U* test, $U = 1.0$,

$P < 0.001$) (Figure 1; Tables 1 and 2). This drop was significant for either the relative or the absolute abundance of all five classes of compounds on 2 DAIP (1 DAIP was not recorded for this plant) (Table 2). Comparison of median flux rates from pollinated vs. unpollinated flower heads of the same age shows that pollination reduced scent production by 89% on 2 DAIP and by 92% on 3 DAIP. Age alone reduced scent by 43% in unpollinated flower heads (2 DAIP vs. 3 DAIP); this decline was restricted to the aromatics (Mann–Whitney U test, $U = 81.0$, $P = 0.003$). In pollinated plants, the decrease in total scent was a result of a decrease in three classes of compounds: aromatics, sesquiterpenoids, and 6-methyl-5-hepten-2-one, an irregular terpene, with the sharpest decline in the aromatics. The three dominant aromatic compounds, phenylnitroethane, phenylacetonitrile, and 2-phenylethanol, all decreased in flux rate based on pollination treatment (Table 1). In contrast, fatty acid derivatives did not decline with treatment and only some monoterpenoids declined. As a result, monoterpenoids and fatty acid derived compounds increased in relative abundance in pollinated flower heads, whereas compounds that did decline in flux rate maintained their relative abundance (except for 6-methyl-5-hepten-2-one, which declined in relative abundance).

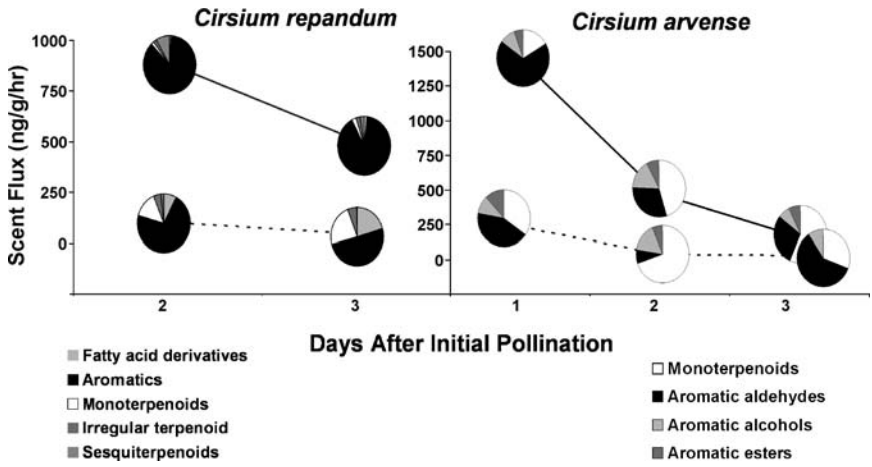

FIG. 1. Median postpollination scent changes in *Cirsium repandum* and pistillate *Cirsium arvense* beginning 1 d after the initial pollination. The solid line represents the total scent flux from unpollinated flower heads. The dashed line reflects scent flux from pollinated flower heads. Each pie graph depicts the relative contribution of the 13 compounds grouped into four classes for *C. arvense* and five classes for *C. repandum*. Sample sizes for *C. repandum*: pollinated 2 DAIP (9), 3 DAIP (9); unpollinated 2 DAIP (9), 3 DAIP (9). Sample sizes for *C. arvense*: pollinated 1 DAIP (5), 2 DAIP (10), 3 DAIP (5); unpollinated 1 DAIP (5), 2 DAIP (10), 3 DAIP (5).

TABLE 1. THE MEDIAN FLUX RATE (ng $g^{-1}hr^{-1}$) OF THE 42 COMPOUNDS, FROM FIVE CLASSES DETECTED IN *C. repandum*

Compounds	Retention time (min)	Pollinated[a]				Unpollinated[b]			
		DAIP 2		DAIP 3		DAIP 2		DAIP 3	
		Median	Quartile	Median	Quartile	Median	Quartile	Median	Quartile
Fatty acid derivatives (3)[c]		7.8	6.5–9.4	8.7	7.3–9.9	9.5	8.5–10.4	8.9	6.9–10.5
2-Hexanol (M)[d]	6.14	6.4	5.8–7.7	7.4	6.5–8.4	7.9	7.6–8.5	7.3	6.3–8.4
1-Hexanol (MR)[e]	8.38	1.4	0.6–1.4	1.4	0.8–1.4	1.0	0.8–1.2	1.3	0.5–1.6
(*E*)-Hex-3-en-1-ol (MR)	8.85	0	0–0.3	0	0–0.06	0.6	0–0.7	0.3	0.1–0.5
Aromatics (7)		71.5	44.1–144.8	21.4	12.6–45.3	770.9	541.7–1235.1	461.0	278.9–663.0
Benzaldehyde (MR)	10.95	8.0	5.0–11.5	3.7	2.9–6.0	36.3	27.5–54.1	27.7	19.4–47.9
Phenylacetaldehyde (MR)	12.48	3.2	2.5–5.3	1.7	1.1–3.8	18.8	12.0–21.6	11.6	8.8–12.5
Methyl salicylate (MR)	14.09	0	0–0	0	0–0	0	0–0	0	0–0
Benzyl alcohol (MR)	15.08	0	0–9.0	0	0–4.6	20.3	13.9–51.4	12.6	3.9–14.6
2-Phenylethanol (MR)	15.47	19.2	7.9–34.7	7.4	4.0–13.0	191.7	161.4–291.9	80.9	49.2–129.2
Phenylacetonitrile (MR)	15.71	12.8	8.5–24.8	3.8	1.7–11.4	281.7	202.2–365.5	216.2	131.1–268.5
Phenylnitroethane (M)	17.67	28.3	20.1–59.5	4.7	2.7–6.6	222.1	124.6–450.7	112.0	66.6–190.3
Irregular terpenoid (1)		4.8	4.0–6.9	2.3	0.7–2.9	16.5	10.1–18.0	12.7	7.8–20.1
6-Methyl-5-heptene-2-one (MR)	8.21	4.8	4.0–6.9	2.3	0.7–2.9	16.5	10.1–18.0	12.7	7.8–20.1
Monoterpenoids (10)		14.5	12.1–17.8	10.2	9.3–15.4	15.9	11.4–28.7	14.0	8.7–28.9
α-Pinene (MR)	2.73	5.5	4.2–6.3	4.8	4.6–5.6	5.1	3.7–6.1	a4.9	3.5–6.3
Camphene (MR)	3.42	0.4	0.3–0.4	0.3	0.2–0.5	0.4	0.3–0.5	0.3	0.2–0.7
β-Pinene (MR)	4.16	6.1	5.6–7.1	3.6	3.5–6.6	5.8	4.5–8.0	5.2	3.5–13.2
Sabinene (MR)	4.41	0.5	0.3–0.8	0.5	0.3–0.6	0.6	0.3–0.7	0.4	0.4–0.6
β-Myrcene (MR)	5.22	0.2	0.2–0.4	0.1	0–0.3	0.7	0.3–2.1	0.5	0.2–1.3
Limonene (MR)	5.85	1.6	1.3–2.0	0.8	0.7–1.6	2.1	2.0–4.6	2.2	0.8–2.3
E-β-Ocimene (MR)	6.81	0.2	0.1–0.3	0.1	0–0.2	0.3	0.1–0.4	0.2	0.2–0.2
α-Terpinolene (MR)	7.27	0	0–0	0	0–0	0.5	0–2.0	0.2	0–1.7
Linalool (MR)	11.76	0	0–0.4	0	0–0	0	0–1.3	0	0–1.5
α-Terpineol (MR)	12.94	0	0–0	0	0–0	0.4	0.2–3.0	0.2	0–1.0

Sesquiterpenoids (21)		1.6	0.3–6.5	0.2	0–0.5	76.9	16.0–253.9	13.3	1.8–119.7
α-Ylangene (MR)	10.43	0	0–0	0	0–0	0	0–0.4	0	0–0.4
α-Copaene (MR)	10.55	0	0–0.1	0	0–0	0.7	0.5–1.7	0.3	0.03–1.3
β-Elemene (MR)	11.85	0	0–0	0	0–0	0	0–4.3	0	0–0.6
β-Caryophyllene (MR)	11.97	0.06	0–1.1	0.02	0–0.4	2.7	0.8–23.4	1.0	0–11.3
β-Farnesene (MR)	12.73	0.1	0–0.5	0	0–0	0.9	0.6–5.1	0.5	0–2.2
α-Humulene (MR)	12.87	0	0–0	0	0–0	0.4	0.1–2.4	0	0–0.9
93(11),84(12),69(52), 53(10),41(100)[f]	13.38	0	0–0.4	0	0–0	1.4	1.3–2.4	0.4	0–2.3
α-Farnesene (MR)	13.97	0.1	0–0.1	0	0–0	6.0	0.4–12.2	1.9	0.1–8.3
(*Z*)-Geranylacetone (MR)	14.89	0.7	0–2.9	0	0–0	12.7	3.3–13.2	3.9	0.8–14.6
121(17),94(10),93(58),85(14), 81(10),80(26),69(79),68(50), 67(17),57(94),55(10),41(100)	14.93	0	0–0.2	0	0–0	0.05	0–0.2	0.03	0–0.2
(*Z*)-Nerolidol (MR)	16.28	0.6	0.3–1.0	0.2	0–0.2	8.1	6.3–31.8	2.1	0.9–18.0
Caryophyllene oxide (MR)	16.38	0	0–0.1	0	0–0	1.5	0.8–5.0	0.1	0–1.9
(*E*)-Nerolidol (MR)	16.75	0	0–0	0	0–0	23.0	1.7–30.6	2.9	0–19.2
121(10),110(12),109(17), 107(12),105(13),96(19), 95(23),93(28),91(17),83(10), 82(12),81(17),79(36)77(15), 71(10),69(32),67(23),55(35), 53(14),43(100),41(83)	17.33	0	0–0	0	0–0	0.6	0.3–2.0	0	0–1.5
207(11),165(22),164(44), 163(100),123(17),122(12), 121(37),111(10),109(41), 108(24),107(66),97(17), 95(54),94(12),93(25),91(23), 83(12),82(20),81(30),79(25), 77(15),69(24),67(27),57(19), 55(41),53(17),43(40),41(77)	17.80	0	0–0	0	0–0	2.0	0–6.4	0.1	0–3.1
(*Z*,*E*)-Farnesal		18.56	0	0–0	0	0–0	0.5	0–1.1	0.030–0.7

TABLE 1. THE MEDIAN FLUX RATE (ng g^{-1} hr^{-1}) OF THE 42 COMPOUNDS, FROM FIVE CLASSES DETECTED IN *C. repandum*

Compounds	Retention time (min)	Pollinated[a]				Unpollinated[b]			
		DAIP 2		DAIP 3		DAIP 2		DAIP 3	
		Median	Quartile	Median	Quartile	Median	Quartile	Median	Quartile
(*E*,*E*)-Farnesol	18.97	0	0–0	0	0–0	15.6	0–33.5	0	0–20.2
Farnesol (MR)	19.35	0	0–0	0	0–0	0	0–4.2	0	0–2.4
(*E*,*E*)-Farnesal (MR)	19.70	0	0–0	0	0–0	0.7	0–73.0	0	0–9.7
108(19),93(15),71(12), 55(14),43(100),41(27)	19.97	0	0–0	0	0–0	0.1	0–0.7	0.01	0–0.3
Farnesol isomer	20.27	0	0–0	0	0–0	0	0–0.4	0	0–0.6
Total (ng g^{-1} hr^{-1})		100.1	66.9–185.4	42.8	29.8–74.0	889.7	587.7–1546.1	509.8	304.1–842.1

[a] Pollinated plants 2 and 3 d after initial pollination (DAIP) $N = 9$.
[b] Unpollinated plants $N = 9$.
[c] Class with (#) of compounds in a class.
[d] Peaks identified using (M) mass spectral library with >90% identity.
[e] Peaks identified using (R) retention time of reference compounds by co-chromatography.
[f] The mass spectra of unidentified compounds are reported as the mass ion, with the percent relative to the base peak (100).

TABLE 2. CONTRASTING THE SIGNIFICANT DIFFERENCES IN SCENT EMISSIONS OF POLLINATED AND UNPOLLINATED FLOWER HEADS OF *C. repandum* 2 AND 3 AFTER THE INITIAL POLLINATION MANIPULATION

	Pollinated vs. unpollinated[a]				Aging in unpollinated[b]
	Absolute flux		Relative abundance		Absolute flux
Scent classes	DAIP 2	DAIP 3	DAIP 2	DAIP 3	DAIP 2 vs. DAIP 3
Fatty acid dervatives	0.2	0.514	<0.001**	<0.001**	0.806
Aromatics	<0.001**	0.001**	0.122	0.007*	0.003**
Monoterpenoids	0.35	0.191	0.001**	<0.001**	0.935
6-Methyl-5-hepten-2-one	0.003**	<0.001**	0.005**	0.462	0.624
Sesquiterpenoids	0.009*	0.001**	0.453	0.191	0.253

[a] Mann–Whitney *U* test for significant differences of each compound class with Bonferroni adjusted α-values for three comparisons: *$P < 0.025$, **$P < 0.005$, otherwise nonsignificant.
[b] Mann–Whitney *U* test for significant differences: *$P < 0.05$, **$P < 0.01$, otherwise nonsignificant.

C. arvense Scent Reduction. There was a reduction in total scent production in pistillate flower heads as a result of pollination (Mann–Whitney *U* test, $U = 54.0$, $P < 0.001$; Figure 1, Tables 3 and 4). One d after pollination (1 DAIP), scent from pollinated flower heads was 84% lower than scent from unpollinated flower heads. On 2 DAIP, scent from a pollinated flower head was 92% lower than from an unpollinated control, and 81% lower on 3 DAIP. These differences are attributable to the precipitous decline in scent produced by pollinated flower heads. Unpollinated flower heads also declined in scent production over time, although not as severely as pollinated ones; in fact, one anomalous unpollinated flower head increased in scent production from 2 DAIP to 3 DAIP. There was a decline of 89% in the median flux rate from an unpollinated flower head from 1 DAIP to 3 DAIP. This decline was significant only for phenylacetaldehyde (Kruskal–Wallis test, $df = 2$, $H = 6.21$, $P = 0.045$) and benzaldehyde ($H = 7.202$, $P = 0.027$, Table 4), aromatic compounds that dominate the floral scent blend in both pollinated and unpollinated *C. arvense* (Table 3). The greatest difference between the flux rates of pollinated and unpollinated flower heads occurred on 2 DAIP, when all 13 components of the scent blend were significantly lower in unpollinated flower heads (Table 4).

Pollinator Discrimination on C. repandum. Papilio palamedes accounted for 91% of all pollinator visits during the 30-min observation periods on pollinated and unpollinated flower heads of *C. repandum*. *P. palamedes* visited nine out of ten of the unpollinated flower heads and five out of nine of the hand-

TABLE 3. MEDIAN FLUX RATE (ng g^{-1} hr^{-1}) OF THE 13 COMPOUNDS DETECTED IN *C. arvense*

Compounds	Retention time (min)	Pollinated[a]						Unpollinated[b]					
		DAIP 1		DAIP 2		DAIP 3		DAIP 1		DAIP 2		DAIP 3	
		Median	Quartile	Median	Quartile	Median	Quartile	Median	Quartile	Median	Quartile	Median	Quartile
Monoterpenoids (5)		80.4	64.8–87.0	25.0	15.5–28.5	9.2	7.3–10.5	236.0	120.5–317.3	212.2	142.6–287.6	90.3	70.2–117.8
(*E*)-Furanoid linalool oxide[c]	9.68	0	0–0	0	0–0	0	0–0	1.0	0–1.2	0.9	0.4–1.1	0.2	0–0.7
(*Z*)-Furanoid linalool oxide	10.08	18.8	17.3–24.1	6.8	5.0–7.4	1.6	1.4–2.0	105.5	27.9–147.0	94.2	61.8–124.0	34.7	25.9–47.5
Linalool	11.07	1.5	0.8–1.7	0.0	0–0.3	0	0–0	13.2	1.5–13.3	11.3	4.2–17.3	2.2	1.9–5.3
(*E*)-Pyranoid linalool oxide	13.45	58.5	45.8–59.7	17.9	10.5–20.3	7.6	5.9–8.4	93.7	84.6–120.1	87.9	70.9–123.3	49.6	39.3–59.1
(*Z*)-Pyranoid linalool oxide	13.71	1.5	0.9–1.6	0.3	0–0.6	0	0–0.1	22.5	6.6–35.7	17.9	5.3–21.9	3.6	3.1–5.3
Aromatics (8)		151.6	98.9–400.3	10.9	4.8–54.2	20.7	20.1–26.9	1208.0	729.3–1365.5	256.5	118.1–687.3	69.5	46.5–81.6
Benzaldehyde	10.83	45.7	26.0–94.4	2.9	0.4–13.1	9.0	8.6–9.7	228.8	117.9–251.3	67.3	31.7–134.2	20.6	19.7–22.0
Phenylacetaldehyde	12.48	52.3	42.4–221.9	0.0	0–18.1	8.9	8.8–13.5	759.9	465.0–793.8	73.9	35.7–352.6	25.7	13.1–28.4
Methyl salicylate	13.99	6.5	1.3–13.0	0.0	0–0.1	0	0–0	20.2	19.2–25.7	2.5	1.1–18.3	0	0–0
Benzyl alcohol	14.97	17.8	4.3–25.2	0.6	0–7.3	0	0–0.5	94.4	46.3–127.1	43.4	16.3–61.7	6.3	1.3–7.1
2-Phenylethanol	15.37	6.4	2.8–10.5	5.2	4.1–7.1	2.9	2.8–3.0	41.2	33.4–50.8	34.5	16.2–51.4	5.5	2.3–9.6
p-Anisaldehyde	16.63	1.8	1.2–2.9	0.0	0–0.7	0	0–0.0	3.5	3.1–4.8	1.4	0.8–2.8	0	0–0.4
Dimethyl salicylate	16.97	18.9	18.8–24.2	1.7	0.3–5.9	0	0–0.2	32.8	20.7–51.0	16.1	7.6–30.3	5.1	4.1–5.4
Benzyl benzoate	22.04	2.2	2.0–8.2	0.5	0–1.9	0	0–0	27.3	23.7–61.0	17.3	8.7–36.1	6.4	6.0–8.6
Total		232.0	163.7–487.3	35.9	20.3–82.8	29.9	27.4–37.4	1444.1	849.9–1682.8	468.7	260.8–974.9	159.9	116.7–199.4

[a] Pollinated flower heads 1, 2, 3 d after initial pollination manipulation. Sample sizes: DAIP 1 (5), DAIP 2 (10) DAIP 3 (5).
[b] Sample sizes unpollinated: DAIP 1 (5), DAIP 2 (10), DAIP 3 (5).
[c] All compounds identified by cochromatography with known standards and matched mass spectral library with >90% identity.

TABLE 4. CONTRASTING THE DIFFERENCES IN SCENT PRODUCTION OF POLLINATED AND UNPOLLINATED FLOWER HEADS OF PISTILLATE *C. arvense* 1, 2, AND 3 D AFTER THE INITIAL POLLINATION MANIPULATION

	Pollinated vs. unpollinated[a]						Aging in unpollinated[b]
	Absolute flux			Relative abundance			Absolute flux
Scent compounds	DAIP 1	DAIP 2	DAIP 3	DAIP 1	DAIP 2	DAIP 3	DAIP 1 vs. DAIP 2 vs. DAIP 3
(*E*)-Furanoid linalool oxide	0.126	0.001**	0.054	0.126	0.001**	0.054	0.749
(*Z*)-Furanoid linalool oxide	0.028	0.001**	0.009*	0.465	0.888	0.016*	0.646
Benzaldehyde	0.117	0.002**	0.076	0.117	0.481	0.117	0.027*
Linalool	0.251	0.002**	0.005*	0.347	0.002**	0.005*	0.768
Phenylacetaldehyde	0.047	0.008*	0.175	0.047	0.054	0.465	0.045*
(*E*)-Pyranoid linalool oxide	0.175	0.001**	0.009*	0.028	0.014*	0.465	0.611
(*Z*)-Pyranoid linalool oxide	0.142	0.002**	0.008*	0.094	0.019*	0.008*	0.681
Methyl salicylate	0.402	0.004*	0.317	0.834	0.01*	0.317	0.126
Benzyl alcohol	0.076	0.001**	0.085	0.251	0.04	0.281	0.1
2-Phenylethanol	0.117	0.002**	0.602	0.347	0.139	0.016*	0.213
p-Anisaldehyde	0.175	0.008*	0.136	0.076	0.351	0.136	0.063
Dimethyl salicylate	0.602	0.003**	0.015*	0.028	0.622	0.026	0.147
Benzyl benzoate	0.251	0.001**	0.007*	0.251	0.021	0.007*	0.54

[a] Mann–Whitney *U* test for significant differences of each compound with Bonferroni adjusted α values for three comparisons: $*P < 0.017$, $**P < 0.003$, otherwise nonsignificant.

[b] Kruskal–Wallis test for significant differences: $*P < 0.05$, otherwise nonsignificant.

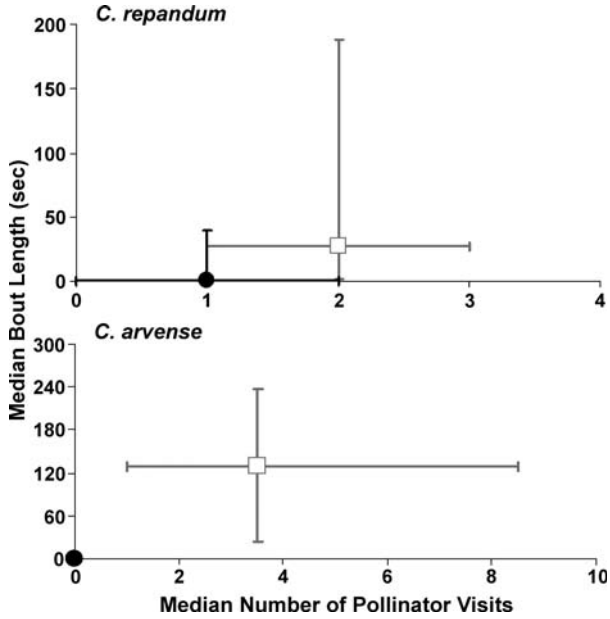

FIG. 2. Pollinator response to pollinated (●) and unpollinated (□) flower heads 2 d after initial pollination manipulation. The number of visits per flower head, per 30-min observation period, by the dominant pollinator (*P. palamedes* to *C. repandum*, *A. mellifera* to pistillate *C. arvense*) is shown (median, quartile). The bout length (sec), per 30-min observation period, by the dominant pollinator is also shown (median, quartile). In *C. repandum*, these response variables were not significant. In *C. arvense*, both bout length and the median number of pollinator visits were significant.

pollinated ones, but this difference was not significant ($P = 0.14$, two tailed Fisher's exact test). Other measures of pollinator preference, such as the number of pollinator visits and bout length, were not significantly greater at unpollinated flower heads (Mann–Whitney U test, pollinator visits: $U = 25.0$, $P = 0.095$, bout length: $U = 26.0$, $P = 0.11$; Figure 2).

Pollinator Discrimination on C. arvense. Pollinators discriminated between pollinated and unpollinated flower heads in *C. arvense*. The dominant visitor *Apis mellifera* made up 91% of all visits. *A. mellifera* visited 8 out of 10 of the unpollinated flower heads and only 2 out of 10 of the pollinated flower heads. This difference was significant ($P = 0.023$, two-tailed Fisher's exact test) as was the higher number of pollinator visits to unpollinated flower heads (Mann–Whitney U test, $U = 16.0$, $P = 0.007$), and longer bout length per visit (Mann–Whitney U test, $U = 15.5$, $P = 0.006$; Figure 2).

DISCUSSION

Fragrance emissions decreased in both *C. arvense* and *C. repandum* following pollination, whereas unpollinated plants maintained moderate levels of scent emissions. This decline is consistent with that demonstrated for the total scent blend in *Silene latifolia* (bladder campion) (Dötterl et al., 2005), the dominant component of *Nicotiana attenuata* (tobacco) (Euler and Baldwin, 1996), and both *Petunia hybrida* (petunia) and *Antirrhinum majus* (snapdragon), where pollen tubes reached the ovary at around 34 hr resulting in the decline of scent (Negre et al., 2003). In theory, scent compounds that are emitted at a higher flux rate following pollination could function as repellents (either to prevent additional pollinator visits or to deter predispersal seed predators). However, we did not detect any compounds emitted at significantly higher levels following pollination. In fact, in *C. arvense*, all components of the blend significantly declined following pollination. Interestingly, in *C. repandum*, the flux rate of the monoterpenoids and fatty acid derivatives did not decline as dramatically as in other classes, and the relative abundance of these classes increased. These compounds could potentially play a role in repellence of pollinators and herbivores. Given the chemical complexity of most floral scent blends, it cannot be assumed that all volatile components function solely as pollinator attractants (Raguso, 2003). Further tests of these compounds are necessary to determine if they have repellent qualities (e.g., Ômura et al., 2000). Perhaps it is not surprising that we did not detect novel repellent compounds following pollination in either thistle. Broadly repellent compounds are infrequently reported in the literature (Tollsten and Ovstedal, 1994), and, furthermore, compounds that have initially repellent properties can become attractive, as generalist insects show considerable plasticity, through learned behavior (Dobson, 1994). Repellent compounds emitted from pollinated plants, potentially informative to a variety of insects, could also inform herbivores of developing seeds.

Pollinators preferred to visit unpollinated flower heads. The most conservative estimate of pollinator behavior, whether or not a plant was visited at all, was consistent with other measures such as the "number of visitors", which may include pseudoreplication, and bout length, which is likely influenced by the amount of standing nectar. All three measures demonstrated a pollinator preference for unpollinated *C. arvense* plants. In *C. repandum*, there was more variation, and although there was a trend for pollinators to prefer unpollinated plants, these measures were not significant. A number of factors could account for the observed difference in pollinator discrimination between these two species of thistle. These thistle species have distinct pollinator guilds, different growing habits, and vastly different fragrance composition. *Post-hoc* explanations could be developed to explain the limited discrimination we observed on *C. repandum*, including the interplay between visual and olfactory floral cues

in swallowtail butterfly attraction (e.g., Wheiss, 1997). However, with a larger sample size, it is likely that we would be able to detect a significant preference for unpollinated flower heads of *C. repandum* by *P. palamedes*.

For the duration of this experiment, both species continued to produce scent from unpollinated flower heads, albeit at lower levels than unpollinated flower heads. Whereas scent in pollinated plants diminished almost entirely, scent from unpollinated plants declined but continued to be emitted; this decline was most apparent in the dominant aromatic compounds. Aging has a demonstrated degenerative effect on floral scent production (Tollsten, 1993; Pichersky et al., 1994; Dudareva et al., 1998). We speculated that scent might actually increase in unpollinated flower heads on the time scale of this experiment in order to solicit pollinators, but this was observed in just one flower head of *C. arvense*. In *C. repandum*, as unpollinated flower heads aged, there was a decrease in the relative abundance of the aromatics, including phenylacetaldehyde and phenylacetonitrile, compounds generally known to attract butterflies (Honda et al., 1998; Ômura et al., 1999; Andersson et al., 2002). We also observed a relative increase in the fatty acid derived compounds of the aging flower head, many of which are derived from the lipoxygenase pathway, and may convey a wound signal to insects (Hatanaka, 1993; Bate and Rothstein, 1998), including pollinators. Although we did not measure visitation to flower heads on the final day of the experiment, these changes are likely to have reduced discrimination between pollinated and unpollinated flower heads by *P. palamedes*. Our results suggest that there is a window of attraction for an aging flower head that has not yet been pollinated, albeit a brief one.

In both species of *Cirsium*, there appears to be a feedback mechanism for maintenance of the floral display based on pollination status. In dioecious *C. arvense*, this applies only to pistillate plants. In natural populations, staminate *C. arvense* flower heads do not demonstrate as dramatic a decline in scent production over time as pistillate flower heads do (Theis, 2003). In a prior study, we found that mature staminate flower heads, on average, emitted five times more scent per gram of dry tissue compared to pistillate flower heads. This difference increases dramatically to 9-fold in "past mature" flower heads (Theis, 2003). Feedback from pollination status (e.g., pollen tube growth or fertilization) in pistillate plants may be the mechanism behind the heightened disparity in scent production of older dioecious flower heads. Here, our results demonstrate that pollination can have direct impacts on floral longevity. In a literature survey, Primack (1985) found that for dioecious species there is more variation in floral longevity for pistillate than for staminate flower heads. Interestingly, two of the studies were performed in a greenhouse where zero variation is reported for pistillate flowers. Studies of floral longevity should take place under conditions matching those under which the plant is likely to have evolved, including pollinator assemblages (Ashman and Schoen, 1996). If pollination-induced

senescence is a factor for a given species, floral longevity will be directly related to pollination status. Results from our study demonstrate the importance of taking into account the effect of pollination in studies of floral longevity.

The proximate mechanism for a pollination-induced change has been identified as a coordinated genetic program involving ethylene, abscicic acid (ABA), and cytokinins (Van Staden et al., 1988; van Doorn, 1997; Chang et al., 2003); however, various explanations have been implicated to explain the ultimate mechanisms or the selection pressures that have led to these changes, including energy conservation and senescence physiology, herbivore avoidance, and pollinator signaling, or a combination of these factors (Gori, 1983). Of these, pollinator signaling for effective pollen transfer is the most common explanation. However, if pollinator efficiency were the driving force behind the cession of scent production in *C. arvense*, then like pistillate flower heads, staminate plants should also cease scent production after pollen has been removed. However, this is not the case. Older staminate flower heads lacking nectar or pollen continue to produce high levels of scent. In spite of this high emission rate, pollinators are not attracted to these flower heads either because of the altered ratios of the scent blend, or because of the visual changes that also occur (Theis, 2003). Perhaps the difference in scent production between staminate and pistillate flower heads could be better explained by energetics. Energy saving mechanisms may be more important in pistillate plants, as resources may be allocated away from scent production and toward seed production (Bierzychudek and Eckhart, 1988). However, the cost of the production of scent volatiles, which is measured in $\mu g\ d^{-1}$, is not very high and may not be as expensive to the plant as the potential ecological cost of attracting herbivores to developing seeds (Loughrin et al., 1992; Baldwin et al., 1997; Grison-Pige et al., 2001).

Floral herbivory, another explanation for pollinator induced scent changes, has largely been overlooked in understanding the evolution of floral display. In staminate *C. arvense*, the stage following pollen production is relatively impervious to harm, whereas after pollen has been deposited and fertilization has taken place, pistillate plants are vulnerable to herbivory. If pollinator visits are no longer required, scent emission is an unnecessary risk, and should be reduced. Therefore, decreased scent production following pollination in flowers would be crucial for pistillate plants, but not for staminate ones. Floral herbivores and their larvae can be very harmful by consuming or destroying floral tissue, pollen, and seeds (Augspurger, 1980; Louda and Potvin, 1995). Thus, although selection on floral display has typically been considered to be pollinator-mediated, floral herbivores may influence the evolution of floral traits directly, through tissue destruction, or indirectly, by deterring pollinators (Strauss, 1997; Krupnick et al., 1999).

In this work, we have presented the first nonorchid field experiment to quantitatively measure all of the components in the floral fragrance blend fol-

lowing pollination. We documented a clear pattern of decreased floral scent production following pollination. Flower heads that were not pollinated continued to produce moderate levels of scent. In *C. arvense*, the dominant pollinator *A. mellifera* preferentially visited those flower heads. In our analysis, we found no novel compounds emitted following pollination and no clear candidates for repellency. Scent production is just one component of the floral display, and both nectar reduction and color change also contribute to the apparency of the flower head. To measure the relative importance of olfactory display to reproductive fitness, we have undertaken field experiments with behavioral assays that manipulate scent quality while controlling for visual cues and nectar (Theis, 2003).

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EFFECTS OF DROUGHT STRESS AND NUTRIENT AVAILABILITY ON DRY MATTER ALLOCATION, PHENOLIC GLYCOSIDES, AND RAPID INDUCED RESISTANCE OF POPLAR TO TWO LYMANTRIID DEFOLIATORS

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Abstract—The growth–differentiation balance hypothesis (GDBH) postulates that variation in resource availability can increase or decrease allocation to secondary metabolism, depending on how growth is affected relative to carbon assimilation. Growth and leaf area of black poplar (*Populus nigra*) increased substantially in response to increased nutrient availability, while net assimilation rate and photosynthesis were less strongly affected. In response, total phenolic glycoside concentrations declined, which is consistent with GDBH. Drought stress decreased net assimilation rate and photosynthesis as well as growth, while increasing total phenolic glycoside concentrations. This pattern does not follow GDBH, which predicts lower secondary metabolism when resource limitation decreases both growth and carbon assimilation. However, there was a strong negative correlation between growth and total phenolic glycoside concentration consistent with a trade-off between primary and secondary metabolism, a key premise of GDBH. Drought decreased the growth of gypsy moth (*Lymantria dispar*) larvae but had no effect on white-marked tussock moth (*Orgyia leucostigma*). Increased nutrient availability had a positive linear effect on growth of whitemarked tussock moth, but no effect on gypsy moth. Treatment effects on gypsy moth corresponded closely with effects on total phenolic glycosides, whereas effects on whitemarked

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tussock moth more closely tracked changes in nutritional quality. Localized gypsy moth herbivory elicited rapid induced resistance to gypsy moth, with the effect being independent of water and nutrient availability, but did not affect whitemarked tussock moth, indicating that the effects of biotic and abiotic stress on insect resistance of trees can be species-specific.

Key Words—Growth–differentiation balance hypothesis, resource availability, allocation, phenotypic plasticity, gypsy moth, whitemarked tussock moth.

INTRODUCTION

Some studies have shown drought stress to increase secondary metabolism and decrease host quality of trees for insect herbivores (Ross and Berisford, 1990; Craig et al., 1991; Mopper and Whitham, 1992), whereas others have found the opposite (Wagner, 1986; Cobb et al., 1997; Roth et al., 1997). Fertilisation also has been shown to have divergent effects on the secondary metabolites of woody plants (Kytö et al., 1996; Koricheva et al., 1998a; Herms, 2002). The growth–differentiation balance hypothesis (GDBH) (Loomis, 1932; Lorio, 1986; Herms and Mattson 1992) provides a potential explanation by predicting a quadratic response of constitutive secondary metabolism across a resource gradient. Thus, increased resource availability can either increase or decrease secondary metabolite concentrations, depending on the initial status of the plant (Herms and Mattson, 1992).

Rapidly growing plants are predicted by GDBH to have low secondary metabolite concentrations as a result of a resource-based trade-off between primary and secondary metabolic pathways. However, moderate water or nutrient limitation slows growth more than carbon assimilation (Bradford and Hsiao, 1982; Luxmoore, 1991), which can result in the accumulation of carbohydrates in source leaves (Wardlaw, 1990; Geiger et al., 1996). This may increase the substrate available for secondary metabolism (Waterman and Mole, 1989), resulting in a negative correlation between growth and secondary metabolites (Herms and Mattson, 1992). However, when resource limitation is severe enough to depress carbon assimilation, secondary metabolism is predicted to fall because of energy and substrate constraints on biosynthesis. In this case, increased resource availability is predicted to increase both growth and secondary metabolite concentrations, generating a positive correlation between them (Herms and Mattson, 1992). Few studies have simultaneously quantified carbon assimilation rate, growth, and secondary metabolism across a range of conditions, which is necessary to test predictions of GDBH (Stamp, 2003, 2004).

Differential effects of resource availability on constitutive and induced resistance could influence host quality for herbivores (Lewinsohn et al., 1993;

Lerdau et al., 1994; Lombardero et al., 2000; Glynn et al., 2003). Rapid induced resistance is a form of host resistance in response to herbivory that impinges on insect generation causing the damage (Haukioja, 1990). The few studies that have addressed the effects of nutrient availability on rapid induced resistance of woody plants show that results vary depending on plant (Hunter and Schultz, 1995; Mutikainen et al., 2000) and insect species (Glynn et al., 2003). To our knowledge, effects of drought on rapid induced resistance of woody plants to folivores have not been investigated.

The objectives of this study were to test predictions of GDBH by quantifying effects of water and nutrient availability on carbon assimilation, growth, and total foliar phenolic glycoside concentration of black poplar, *Populus nigra* (L.), and on constitutive and rapid induced resistance to gypsy moth, *Lymantria dispar* (L.), and whitemarked tussock moth, *Orgyia leucostigma* (Smith).

MATERIALS AND METHODS

On March 29, 2003, 250 cuttings of the black poplar clone NC5271 were taken from stock plants, wrapped in moist paper, and stored at 4°C. Relative to other poplar clones, NC5271 is one of the fastest growing (Robison and Raffa, 1994) and most inducible to various elicitors including gypsy moth feeding (Havill and Raffa, 1999). Ten d later, cuttings were removed from storage, dipped into Hormex Rooting Powder No. 3® (Brooker Chemical, Hollywood, CA, USA) (0.3% indole-3-butyric acid), planted in Premier ProMix BX® (Premier Horticulture Ltd., Dorval, Quebec, CA) and maintained in a greenhouse (25°C, 12:12 L/D cycle). On May 16, rooted cuttings were transplanted to 10 l plastic pots containing the same medium and transferred to an outdoor nursery under 50% shade cloth to acclimate.

On June 2 (d 1), 180 of the most uniform cuttings were sorted into five blocks of 36, with assignment based on height and number of apical shoots. To quantify initial biomass and total leaf area, six plants in each block were selected at random for immediate harvest. Stems and foliage were harvested by cutting plants at ground level, and roots were extracted from container media with minimal damage using a low-pressure, high-volume air stream (Air Spade®, Concept Engineering Group, Inc., Verona, PA, USA). Shoots, leaves, and roots were oven-dried at 60°C for 96 hr and weighed to the nearest milligram. The remaining 150 plants were transferred to an outdoor gravel bed in full sunlight, where the 30 plants in each block were subjected to one of three fertilisation levels.

Fertility treatments were initiated by means of a computer-controlled fertigation (irrigation and fertilisation) system (Hansen et al. 2000): 30, 75, and 150 ppm N, with $N/P_2O_5/K_2O$, supplied in a ratio of 3:1:2 from calcium nitrate,

monoammonium phosphate, and potassium nitrate. Each plant was irrigated with 0.5 l of nutrient solution whenever the potting medium moisture tension (PMMT) dried to −4 kPa, as recorded by computer-monitored tensiometers (15 cm Mini "LT" Remote Sensing Units (0–25 kPa); Irrometer Company, Inc., Riverside, CA, USA) installed in one pot per treatment in the block containing the largest plants, because they dried out the fastest. Tensiometers, which were positioned to a depth of 8 cm midway between the pot wall and the plant, were also installed in one pot per treatment in two additional randomly selected blocks. This resulted in one tensiometer per treatment combination in three of five blocks, which provided a continuously monitored, experiment-wide estimate of mean PMMT for each treatment. On July 22 (d 51), 10 plants from each fertility treatment were destructively harvested as described above, with two selected randomly from each block, leaving a total of 120 plants in the experiment.

On July 24 (d 53), a drought-stress treatment was applied to half of the remaining plants in each fertility treatment by withholding irrigation until PMMT reached −25 kPa, whereas the rest were irrigated as described above, resulting in two discrete treatments that were maintained over the next 75 d. The irrigation of drought-stressed plants was controlled using scheduled events ($\approx$200 ml pot^{-1} event^{-1}), with approximately four daily events required to maintain PMMT at the required levels. Well-watered plants continued to be irrigated according to PMMT as described above. Tensiometer readings showed that mean PMMT of well-watered and drought-stressed plants generally remained between −3 and −4 kPa and −10 and −25 kPa, respectively, with precipitation having little effect (Figure 1).

On September 10 (d 101), half the plants in each treatment combination were randomly assigned a short-term, localized herbivory treatment designed to elicit rapid induced resistance. Three fourth-instar gypsy moths were confined within mesh sleeves on leaves 4–7 on the terminal shoot, with leaf 1 designated as the youngest leaf longer than 2 cm. Larvae were allowed to feed until at least 80% of the enclosed leaves had been consumed on all plants, whereupon sleeves and insects were removed. To control for potential effects of the enclosures on induced responses, corresponding leaves on the remaining (constitutive) plants were also enclosed by sleeves containing no larvae.

All remaining plants were harvested on October 7 (d 128). Thus, the first 52 d of the experiment consisted of three fertility treatments, whereas the final 75 d consisted of a 3 × 2 × 2 complete factorial design, with three fertility, two irrigation, and two defoliation treatments, with each of 12 treatment combinations replicated 10 times within each of five blocks, for a total of 120 plants.

Plant Growth and Dry Matter Allocation. Growth analysis (Hunt, 1978; Lambers and Poorter, 1992) was used to document treatment effects on plant relative growth rate and dry matter allocation. Relative growth rate (mg g^{-1} d^{-1}) was calculated as [(ln(final total mass − *ln*(initial total mass)] / time], with

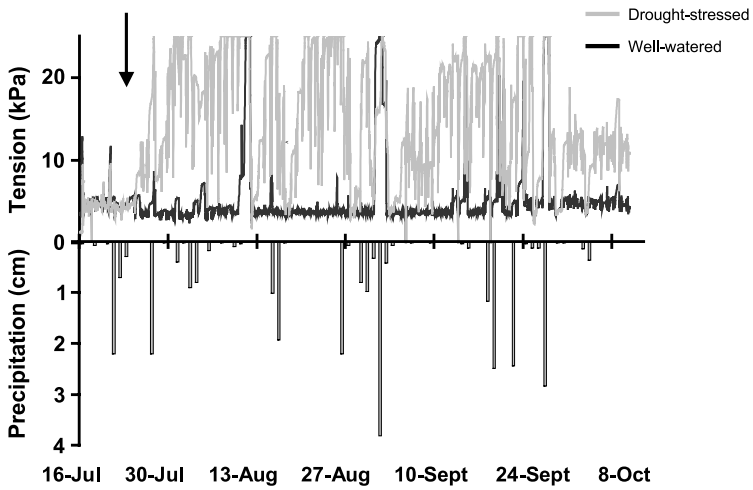

FIG. 1. Potting medium moisture tension (PMMT) for well-watered (dark line) and drought-stressed (gray line) plants over the course of the experiment (upper *y*-axis) and daily precipitation over the experimental period (lower *y*-axis). The arrow indicates date of initiation of the irrigation treatment.

initial and final mass determined from destructive harvests as described above. Specific leaf mass (g m^{-2}) was determined by measuring the area and dry weight of the leaves sampled for nitrogen analysis (described below). Leaf area was measured using a LI-3100 area meter (LI-COR, Inc., Lincoln, NE, USA). The dry weight of foliage sampled for foliar nitrogen and phenolic glycoside analyses (described below) was added to that subsequently obtained from the whole plant harvest. Total leaf area per plant (m^2) was estimated as total leaf biomass / specific leaf mass. Leaf area ratio (m^2 g^{-1}) was calculated as the quotient of total leaf area / total plant mass, and net assimilation rate (g m^{-2} d^{-1}) as relative growth / leaf area ratio. Percent root mass was calculated as (root mass / total plant mass) $\times$ 100. Initial mass for each plant (used to calculate relative growth rate and net assimilation rate) was estimated as the treatment mean for each block at the beginning experiment, as determined from destructive harvests before initiation of treatments.

Photosynthesis. Light-saturated net photosynthesis (μmol CO_2 m^{-2} sec^{-1}) was measured on d 23 and 48 (June 24 and July 19) prior to initiation of the drought-stress treatment on d 53, and on d 72 and 100 (August 12 and September 9) after initiation of the drought stress. Measurements were made using a portable photosynthesis system (LI-6200, LI-COR, Inc. Lincoln, NE, USA) on fully expanded, undamaged leaves from randomly selected plants, on cloud-free mornings between 08:30 and 11:30 EDT. Sample sizes were 10 for

each fertility treatment (two replicates from each block) on d 23 and 48 and five for each fertility–irrigation treatment combination (one replicate per block) on d 72 and 100.

Phytochemistry. To quantify the effects of nutrient and water availability on foliar nitrogen and phenolic glycoside concentrations, a subset of foliage was sampled just prior to whole-plant harvests on d 51 from all harvested trees and on d 128 from all trees not subjected to the defoliation treatment. For foliar nitrogen analysis, every fourth leaf was sampled from all branches on the tree. Leaves were dried at 60°C and then ground in a mill (Cyclotec EC 1093, Tecator AB, Hoganas, Sweden) to pass through a 0.4-mm mesh screen. Total foliar nitrogen content (mg g^{-1}) was determined using a Carbo Erba CNH analyser, Model NA 1500 (Daun and DeClerq, 1994). For foliar phenolic glycoside analysis, 12–15 fully expanded, undamaged leaves were sampled from between leaf positions 10–20 on random branches. Leaves were immediately frozen in liquid nitrogen and placed on ice before being transported to the laboratory where they were freeze-dried at −4°C within 1 hr of sampling. Dried leaves were subsequently ground to pass through a 0.4-mm mesh screen as described above.

Tremulacin and salicortin are dominant phenolic glycosides in *Populus* species (Lindroth et al., 1987; Clausen et al., 1989a,b; Lindroth and Hwang, 1996). We quantified their combined concentration using gas chromatography by first converting their cyclohexan-5-ene-2-one-1-ol carboxylate moiety to the volatile compound methyl 2-methoxybenzoate by extracting 1 g of each sample in 15 ml anhydrous methanol containing 2 mg of capric acid as an internal standard. Capric acid is converted to methyl decanoate under the reaction conditions, which has a similar retention time to that of methyl 2-methoxybenzoate. The solution was left to stand overnight, after which extracts were decanted and two drops (about 100 mg) of 98% sulfuric acid were added. After being left to stand for 12 d at room temperature, extracts were analysed using a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector and a 30 m × 0.25 mm bonded EC-1 column with a 0.25-μm film thickness (Alltech, Deerfield, IL, USA). The injector and detector temperatures were held at 275 and 300°C, respectively, the He flow rate was 2.5 ml min^{-1}, and the temperature program was set at 60°C for 3 min, 60–100°C at 7°C min^{-1}, 100–150°C at 5°C/min, and 150°C for 2 min. In all runs, 1.0 μl of sample was injected (splitless mode). Methyl 2-methoxybenzoate and methyl decanoate eluted at 12.62 and 12.47 min, respectively. Total phenolic glycoside concentration (mg g^{-1} dry weight) was calculated by summing the molar quantities of the two individual compounds (tremulacin plus salicortin) and expressing this as the percent mass of an equivalent molar amount of salicortin.

Insect Bioassays. Laboratory bioassays were conducted with first instar gypsy moth and whitemarked tussock moth to quantify treatment effects on

constitutive and induced resistance. Eggs of both species were obtained from the Canadian Forest Service, Insect Production Laboratory, Sault St. Marie, Ontario, CA. Three d after termination of the defoliation treatment, the two leaves immediately distal to those that had been confined in mesh sleeves were detached from plants and placed in separate Petri dishes (15 cm diam) with five neonate larvae of one of the two species. Larvae were reared in a growth chamber at 25°C with a 16:8 (L/D) photoperiod. To control for ontogenetic variation, half of the replicates for each insect species within a treatment combination received the oldest leaf (position 3), and the other half received the youngest (position 2). A plaster base in each dish saturated with distilled water throughout the bioassay maintained high humidity and leaf turgor. The five larvae in each Petri dish were weighed as a group at the start of the bioassay and again 72 hr later. Mean larval weight was calculated by dividing total weight by number of larvae. Larval growth (mg) was calculated as the difference between mean final and mean initial mass.

Data Analyses. Treatment effects on plant and insect variables were analysed using ANOVA (PROC GLM, Type III sums of squares; SAS Institute, Inc., 2000). All responses met assumptions of normality of residuals and homogeneity of variance. Data are reported as least square means ± 1 SE. Linear and quadratic contrasts were calculated to characterise significant effects of fertility level on treatment means (Chew, 1976; Mize and Schultz, 1985), with coefficients for the unequally spaced treatments calculated according to Robson (1959). Pearson correlation coefficients (PROC CORR; SAS Institute, Inc., 2000) were used to quantify relationships between dependent variables.

RESULTS

Plant Growth and Biomass Allocation. At the intermediate harvest on d 51, nutrient availability had significant linear and quadratic effects on plant growth and biomass allocation (Table 1). Total plant biomass, relative growth rate, and total leaf area increased by 50, 12, and 75%, and percent root mass and specific leaf mass decreased by 16 and 9%, respectively, as fertilisation rate increased from low to high (Table 2). Nutrient availability had no effect on net assimilation rate (Tables 1 and 2).

At the final harvest on d 128, nutrient availability continued to have significant linear effects on total leaf area and percent root mass. However, the effects of fertility on total plant biomass and relative growth rate varied within the two irrigation treatments (significant quadratic effect and fertility × irrigation interaction) (Table 3). The effect of nutrient availability of total plant mass was stronger in the drought stress than in the well-watered treatment,

TABLE 1. *F* VALUES FROM ANOVA FOR GROWTH AND DRY MATTER ALLOCATION OF POPLAR, *Populus nigra*, IN RESPONSE TO THREE FERTILITY LEVELS FOR 50 DAYS

Response variable	Fertility—main effect	Fertility—linear contrast	Fertility—quadratic contrast	Error *df*
Total biomass (g)	20.1***	35.9***	5.3*	22
Percentage root mass	12.7***	21.3***	5.0*	22
Relative growth rate (mg g^{-1} d^{-1})	21.8***	38.3***	56.6*	22
Total leaf area (m^2)	40.2***	70.6***	11.9**	22
Specific leaf mass (g m^{-2})	14.6***	23.2***	7.0*	22
Net assimilation rate (g m^{-2} d^{-1})	1.1	1.1	1.3	22

Level of significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

increasing 95 and 42%, respectively, as fertility increased from low to high (Table 4). In the drought-stress treatment, relative growth rate increased 29% as fertility increased. However, in the well-watered treatment, relative growth rate was less than 9% higher in the intermediate than in both the high and low fertility treatments (Table 4). When averaged across the two irrigation treatments, total leaf area increased 28% and percent root mass decreased 12% as fertility level increased from low to high (data pooled from Table 4). The effects of fertility on net assimilation rate were also dependent on the irrigation treatment (significant fertility × irrigation interaction). Nutrient availability had no effect on the net assimilation rate of drought-stressed plants, but had a quadratic effect on the well-watered plants, it being 11 and 24% higher in the intermediate treatment than in the high and low fertility treatments, respectively. Nutrient availability had no effect on specific leaf mass on d 128 (Tables 3 and 4).

Water availability had stronger effects on all measures of dry matter allocation compared to nutrient availability (Table 3). Relative to drought-stressed plants, the total biomass, relative growth rate, total leaf area, and net

TABLE 2. MEAN (±SE) GROWTH AND DRY MATTER ALLOCATION OF POPLAR, *P. nigra*, IN RESPONSE TO THREE FERTILITY LEVELS FOR 50 DAYS

Response variable	30 ppm N	75 ppm N	150 ppm N
Total biomass (g)	59.7 ± 3.5	80.6 ± 3.3	89.8 ± 3.3
Percentage root mass	20.7 ± 0.5	18.2 ± 0.5	17.4 ± 0.5
Relative growth rate (mg g^{-1} d^{-1})	69.5 ± 1.0	75.6 ± 1.0	78.0 ± 1.0
Total leaf area (m^2)	0.32 ± 0.02	0.49 ± 0.02	0.56 ± 0.02
Specific leaf mass (g m^{-2})	76.5 ± 1.0	70.6 ± 0.9	69.3 ± 1.1
Net assimilation rate (g m^{-2} d^{-1})	13.0 ± 0.4	12.4 ± 0.3	12.5 ± 0.3

F values and level of significance for the fertility treatment are reported in Table 1.

TABLE 3. *F* VALUES FROM ANOVA FOR GROWTH AND DRY MATTER ALLOCATION OF POPLAR, *P. nigra*, IN RESPONSE TO THREE FERTILITY LEVELS FOR 52 DAYS, FOLLOWED BY THREE FERTILITY LEVELS CROSSED WITH TWO IRRIGATION LEVELS FOR 75 DAYS

Response variable	Fertility—main effect	Fertility—linear contrast	Fertility—quadratic contrast	Irrigation	Fertility × Irrigation	Error *df*
Total biomass (g)	116.0***	187.0***	45.0***	929.0***	17.0***	110
Percentage root mass	19.0***	35.0***	2.0	27.0***	1.0	110
Relative growth rate (mg g^{-1} d^{-1})	6.5**	7.2**	6.3*	717.8***	11.9***	106
Total leaf area (m^2)	3.0	6.0*	0.0	119.0***	1.0	50
Specific leaf mass (g m^{-2})	0.8	0.4	1.0	7.0*	4.0*	50
Net assimilation rate (g m^{-2} d^{-1})	3.7*	2.1	5.7*	184.5**	4.1*	106

Level of significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

assimilation rate of well-watered plants were 148, 78, 170, and 76% higher, respectively, when averaged across all three fertility levels (data pooled from Table 4). Percent root mass of drought-stressed plants was 9% higher than for well-watered plants (data pooled from Table 4). In the two lowest fertility levels (30 and 75 ppm N), specific leaf mass was 15 and 22% higher in the well-watered relative to drought-stressed plants, respectively, but was not affected by irrigation in the high fertility (150 ppm N) treatment (fertility × irrigation interaction) (Tables 3 and 4).

Photosynthesis. Nutrient availability had no effect on photosynthesis on d 23, but had a positive linear effect on d 48 (Table 5) when photosynthesis rate was 28% greater in the high relative to the low fertility treatment (Table 6). On d 72, 19 d after the initiation of drought stress, neither treatment had any significant effect on photosynthesis (Table 5). However, on d 100, drought stress significantly decreased photosynthesis rate by 51% relative to the well-watered plants, whereas nutrient availability had a significant positive linear effect on photosynthesis across both irrigation levels, being 55% greater in the high relative to the low fertility treatment (Table 5, data pooled from Table 6). There were no significant interacting effects of the irrigation and fertility treatments on photosynthesis, and no significant quadratic effects of fertility (Table 5).

Phytochemistry. Foliar nitrogen content significantly increased with nutrient availability on both harvest dates and was decreased by drought stress on the final harvest date (Table 7). On d 51, foliar nitrogen concentrations averaged 30 ± 1, 36 ± 1, and 39 ± 1 mg g^{-1} for the 30, 75, and 150 ppm N fertility treatments, respectively. On d 128, foliar nitrogen concentrations of well-watered plants averaged 33 ± 1, 38 ± 1, and 36 ± 0.1 mg g^{-1} for the 30, 75, and

TABLE 4. MEAN (±SE) GROWTH AND DRY MATTER ALLOCATION OF POPLAR, *P. nigra*, IN RESPONSE TO THREE FERTILITY LEVELS FOR 52 DAYS, FOLLOWED BY THREE FERTILITY LEVELS CROSSED WITH TWO IRRIGATION LEVELS FOR 75 DAYS

	30 ppm N		75 ppm N		150 ppm N	
Response variable	Drought-stressed	Well-watered	Drought-stressed	Well-watered	Drought-stressed	Well-watered
Total biomass (g)	174.5 ± 17.1	476.0 ± 17.1	247.8 ± 17.1	741.3 ± 17.1	341.0 ± 17.1	675.8 ± 17.1
Percentage root mass	37.5 ± 0.7	32.8 ± 0.7	33.5 ± 0.7	31.4 ± 0.7	31.9 ± 0.7	29.5 ± 0.7
Relative growth rate ($mg\ g^{-1}\ d^{-1}$)	13.3 ± 0.5	26.5 ± 0.6	15.0 ± 0.5	28.3 ± 0.5	17.1 ± 0.5	25.9 ± 0.5
Total leaf area (m^2)	0.24 ± 0.07	0.90 ± 0.07	0.40 ± 0.07	0.89 ± 0.07	0.41 ± 0.07	1.05 ± 0.07
Specific leaf mass ($g\ m^{-2}$)	101.4 ± 4.9	116.8 ± 8.4	110.2 ± 3.7	134.9 ± 11.6	117.2 ± 5.5	112.3 ± 5.0
Net assimilation rate ($g\ m^{-2}\ d^{-1}$)	10.4 ± 0.7	16.8 ± 0.8	10.4 ± 0.7	20.8 ± 0.7	11.1 ± 0.7	18.7 ± 0.7

Sample sizes were 10 for each treatment, except for the 30 ppm N treatment in the intermediate harvest where the sample size was 9. *F* values and level of significance of treatment effects are reported in Table 3.

TABLE 5. *F* VALUES FROM ANOVA FOR NET PHOTOSYNTHESIS OF POPLAR, *P. nigra*, IN RESPONSE TO THREE FERTILITY LEVELS ON DAYS 23 AND 48 AND THREE FERTILITY LEVELS CROSSED WITH TWO IRRIGATION LEVELS ON DAYS 72 AND 100

Source of variation	Day 23[a]	Day 48[a]	Day 72	Day 100
Fertility (*F*)	1.6	21.2***	1.2	10.6***
F linear contrast	2.5	41.2***	0.4	20.9***
F quadratic contrast	0.6	0.9	1.1	0.3
Irrigation (*I*)	–	–	0.3	90.5***
$F \times I$	–	–	3.1	1.8
Error *df*	22	22	19	19

Level of significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.
[a]Dashes indicate where terms were not included in the model since the irrigation treatment had not yet been initiated.

150 ppm N fertility treatments, respectively. However, the foliar nitrogen concentrations for the drought-stressed plants averaged only 25 ± 1, 28 ± 1, and 31 ± 1 mg g^{-1}, respectively, as fertility increased.

On d 51, total phenolic glycoside concentrations were not affected by nutrient availability (Table 7). However, on d 128, fertility had a significant negative linear effect on total phenolic glycoside concentrations, with concentrations decreasing by 32%, averaged across both irrigation treatments, as fertilisation rate increased (data pooled from Figure 2). However, this effect was small compared to the larger effect of the irrigation treatment (Table 7), total phenolic glycoside concentrations being 89% higher in the drought-stressed relative to the well-watered plants (data pooled from Figure 2). There was no significant interaction between the drought stress and fertility treatments on total phenolic glycoside concentration (Table 7). Total phenolic glycoside concentrations were negatively correlated with total plant biomass (Figure 3).

TABLE 6. MEAN (±SE) NET PHOTOSYNTHETIC RATE (μ mol CO_2 m^{-2} sec^{-1}) OF POPLAR, *P. nigra*, IN RESPONSE TO THREE FERTILITY LEVELS ON DAYS 23 AND 48 AND THREE FERTILITY LEVELS CROSSED WITH TWO IRRIGATION LEVELS ON DAYS 72 AND 100

			Day 72		Day 100	
Fertility level	Day 23	Day 48	Drought-stressed	Well-watered	Drought-stressed	Well-watered
30 ppm N	25.7 ± 0.9	19.5 ± 0.7	11.0 ± 1.1	11.4 ± 1.7	6.6 ± 1.4	19.3 ± 1.5
75 ppm N	27.5 ± 1.4	22.6 ± 0.9	11.1 ± 1.5	9.9 ± 1.2	11.0 ± 1.4	19.0 ± 1.4
150 ppm N	27.4 ± 1.2	25.0 ± 1.1	10.3 ± 1.8	14.0 ± 1.0	14.0 ± 1.4	26.2 ± 1.6

Sample sizes were 10 for each treatment on days 23 and 48 and 5 for each treatment on days 72 and 100. *F* values and level of significance of treatment effects are reported in Table 5.

TABLE 7. *F* VALUES FROM ANOVA FOR FOLIAR CONCENTRATIONS OF TOTAL PHENOLIC GLYCOSIDES AND NITROGEN OF POPLAR, *P. nigra*, IN RESPONSE TO ONE OF THREE FERTILITY LEVELS FOR 52 DAYS, FOLLOWED BY THREE FERTILITY LEVELS CROSSED WITH TWO IRRIGATION LEVELS FOR 75 DAYS

Source of variation	Total phenolic glycoside concentration		Nitrogen concentration	
	Day 51[a]	Day 128	Day 51[a]	Day 128
Fertility (*F*)	3.4	5.7*	36.8***	12.7***
F linear contrast	1.4	11.2**	65.8***	18.1***
F quadratic contrast	0.3	0.3	9.7**	7.3**
Irrigation (*I*)	–	44.6***	–	87.0***
$F \times I$	–	0.1	–	4.2*
Error *df*	22	50	22	50

Level of significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.
[a]Dashes indicate where terms were not included in the model since the irrigation treatment had not yet been initiated.

Insect Bioassays. Drought stress significantly reduced the growth of first instar gypsy moth ($F_{1,100} = 42.01$, $P < 0.001$), which was 36% higher on well-watered plants (data pooled from Figure 4a). Previous defoliation by fourth instar gypsy moth also decreased the growth of first instar gypsy moth ($F_{1,100} = 12.46$, $P < 0.001$), which was 27% higher on the nondefoliated than on the induced plants (data pooled from Figure 4a). Rapid induced resistance to gypsy moth was expressed in both well-watered and drought-stressed trees and across all levels of

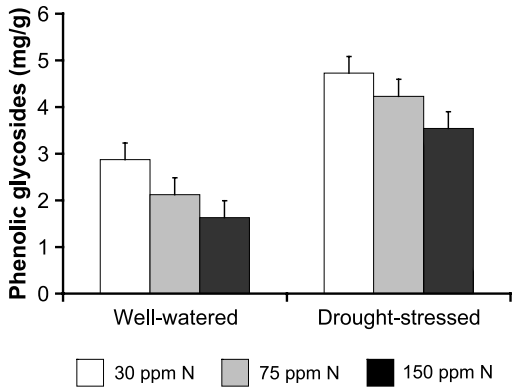

FIG. 2. Mean (±SE) total foliar phenolic glycoside concentration of poplar, *Populus nigra*, in response to three fertility levels for 52 d followed by three fertility levels crossed with two irrigation levels for 75 d. $P = 0.002$ for linear effect of fertility and $P < 0.001$ for main effect of irrigation; interaction, not significant.

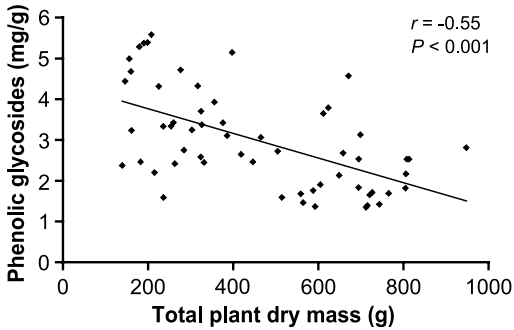

FIG. 3. Correlation between total biomass of poplar, *P. nigra*, and total foliar phenolic glycoside concentration in response to three fertility levels for 52 d followed by three fertility levels crossed with two irrigation levels for 75 d.

the fertility treatment (no significant interaction between defoliation and either the irrigation or fertility treatments). However, neither the irrigation nor the defoliation treatments had any effect on whitemarked tussock moth growth (Figure 4b). Fertility had no effect on gypsy moth growth, but growth of whitemarked tussock moth increased linearly as fertility increased ($F_{1,98}$ = 6.16, P = 0.015), being 18% greater in the high relative to the low fertility treatment (data pooled from Figure 5).

DISCUSSION

The patterns we observed conformed only in part with those predicted by GDBH (Herms and Mattson, 1992). Plant responses to nutrient availability were consistent, but effects of water availability diverged to some degree from responses predicted by GDBH. Consistent with GDBH, plant growth was more sensitive to nutrient availability than was carbon assimilation, as indicated by larger effects of the fertility treatment on plant biomass and leaf area than on net assimilation rate and photosynthesis. In response, total phenolic glycoside concentrations declined as nutrient availability increased. This observation is also consistent with previous studies on congeneric quaking aspen, *Populus tremuloides* (Michx.), in which phenolic glycoside concentrations also decreased as nutrient availability increased (Bryant et al., 1987; Hemming and Lindroth, 1999).

Plant responses to drought did not correspond as closely with the predictions of GDBH. Concentrations of total phenolic glycosides were low in the fast-growing, well-watered plants which is consistent with GDBH. However,

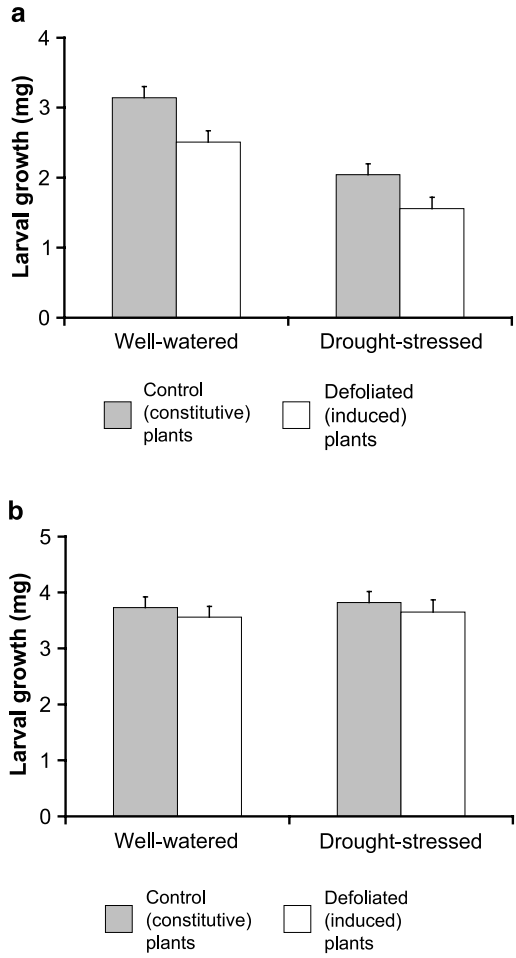

FIG. 4. Effects of short-term, localized gypsy moth herbivory and water availability on mean growth (±SE) of first instar (a) gypsy moth and (b) whitemarked tussock moth on poplar, *P. nigra*. For gypsy moth, $P < 0.001$ for main effect of irrigation and $P < 0.001$ for main effect of defoliation, interaction not significant; for whitemarked tussock moth, no effects significant. Data pooled across fertility treatments.

the high concentrations of total phenolic glycosides observed in response to drought stress were not consistent with GDBH, as both growth and carbon assimilation (as indicated by net assimilation rate and photosynthesis) were substantially reduced. In this contingency, GDBH predicts that foliar secondary metabolite concentrations should also decrease (Herms and Mattson, 1992). The

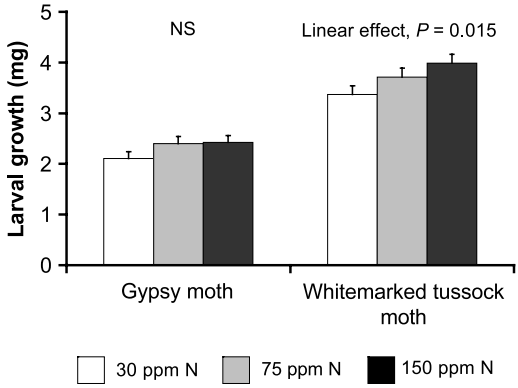

FIG. 5. Effects of fertility on mean growth (±SE) of first instar gypsy moth and whitemarked tussock moth on poplar, *P. nigra*. NS indicates effect is not significant. Data pooled across watering and defoliation treatments.

high total phenolic glycoside levels in drought-stressed plants contributed to a strong negative correlation with growth, which is consistent with the trade-off between primary and secondary metabolism predicted by GDBH (Herms and Mattson, 1992). Conclusions regarding effects on total phenolic glycoside concentration were not affected by expressing the data on a leaf area basis (mg cm^{-2}), as water and nutrient availability had smaller effects on specific leaf mass than on total phenolic glycoside content. Hence, treatment effects were attributable to changes in phenolic glycoside biosynthesis *per se*, rather than dilution or concentration effects resulting from variation in dry matter accumulation (Koricheva, 1999). Phenolic glycosides have been implicated as anti-Lepidoptera defences of *Populus* (Hemming and Lindroth, 1995, 2000; Hwang and Lindroth, 1997), and the adaptive advantage of high levels of defence was maintained even when drought-stressed plants were constrained by a smaller carbon budget, which would substantially increase the cost of producing and maintaining secondary metabolites. Contrary to our results, Roth et al. (1997) observed drought stress to decrease phenolic glycoside concentrations of quaking aspen.

Some studies have shown high fertility levels to amplify the negative effects of drought stress on plants (Walters and Reich, 1989; Miller and Timmer, 1994; Power et al., 1998) through such mechanisms as increased soil matric potential (Jacobs et al., 2004) or decreased root/shoot ratios (Linder and Rook, 1984; Linder et al., 1987), therefore, also potentially increasing the effects of drought stress on secondary metabolism and herbivore performance. However, we observed no evidence to support this hypothesis, even when plants were preconditioned to their respective nutrient treatment for 52 d prior

to exposure to drought stress. We did not observe a proportional reduction in growth of high fertility relative to low fertility plants when they were subjected to drought, nor were the nature of fertility × irrigation interactions on phytochemistry and insect performance consistent with enhanced effects of drought stress. Although percent root mass declined in response to increased nutrient availability, drought stress increased percent root mass, which may have had a counteracting effect. Both responses are consistent with theories of adaptive phenotypic plasticity (Bloom et al., 1985; Hirose, 1987; Ingestad and Ågren, 1991, 1992), which predict that plants will shift their allocation patterns in varying environments to increase acquisition of growth-limiting resources.

Outbreaks of phytophagous insects have been linked to droughts (Mattson and Haack 1987), although effects may be specific to particular feeding guilds (Larsson, 1989; Koricheva et al., 1998b; Huberty and Denno, 2004). This study did not provide any evidence to support the hypothesis that drought stress enhances folivore performance (White, 1984). In contrast, drought stress decreased the growth of gypsy moth, perhaps because drought also decreased foliar nitrogen and increased total phenolic glycoside concentrations, both of which are key determinants of gypsy moth performance on quaking aspen (Lindroth and Hemming, 1990; Hemming and Lindroth, 2000; Osier et al., 2000). Decreased foliar water content may also have contributed to lower insect performance (Scriber, 1977).

In contrast, drought stress had no effect on the growth of whitemarked tussock moth, perhaps because this species is less sensitive than gypsy moth to changes in phenolic glycosides, as whitemarked tussock moth has been shown to be relatively insensitive to other phenolic compounds (Karowe, 1989; Kopper et al., 2002). Environmental variation does not affect all secondary metabolites within a plant in the same way (Muzika, 1993; Reichardt et al., 1991; Kainulainen et al., 1996), which could also generate complex patterns of herbivore response. In two willow species, condensed tannin levels were decreased by mild drought, whereas simple phenolic compounds were not affected (Glynn et al., 2004). Additionally, positive effects of drought on nutritional quality, such as increases in free amino acids, soluble proteins, and soluble carbohydrates (Brodbeck and Strong, 1987; Mattson and Haack 1987), may counteract negative effects of increased secondary metabolite levels (Glynn et al., 2004). Nutrient availability had a positive linear effect on whitemarked tussock moth growth and foliar nitrogen, but had no effect on gypsy moth. This overall pattern of treatment effects on these two species is consistent with relatively high sensitivities of whitemarked tussock moth to nutritional quality and gypsy moth to phenolic glycosides.

Differential effects of resource availability on constitutive vs. induced resistance may be an important source of variation in herbivore performance (Lewinsohn et al. 1993; Lerdau et al. 1994; Lombardero et al., 2000). We are

not aware of other studies concerning effects of water availability on the expression of rapid induced resistance of woody plants to folivores, and are aware of only a few that have examined nutrient availability, all of which revealed some effect (Hunter and Schultz, 1995; Mutikainen et al., 2000; Glynn et al., 2003). In this study, however, rapid induced resistance to gypsy moth was not influenced by resource availability, it being expressed in all treatments. This study therefore emphasizes the difficulty in generalising about the effects of abiotic and biotic stress on host quality, even for insects within the same family.

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REVIEW ARTICLE

PLANT SURFACE PROPERTIES IN CHEMICAL ECOLOGY

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Abstract—The surface of the primary aerial parts of terrestrial plants is covered by a cuticle, which has crucial autecological functions, but also serves as an important interface in trophic interactions. The chemical and physical properties of this layer contribute to these functions. The cuticle is composed of the cuticular layer and the cuticle proper, which is covered by epicuticular waxes. Whereas the cutin fraction is a polyester-type biopolymer composed of hydroxyl and hydroxyepoxy fatty acids, the cuticular waxes are a complex mixture of long-chain aliphatic and cyclic compounds. These highly lipophilic compounds determine the hydrophobic quality of the plant surface and, together with the microstructure of the waxes, vary in a species-specific manner. The physicochemical characteristics contribute to certain optical features, limit transpiration, and influence adhesion of particles and organisms. In chemical ecology, where interactions between organisms and the underlying (allelo-) chemical principles are studied, it is important to determine what is present at this interface between the plant and the environment. Several useful equations can allow estimation of the dissolution of a given organic molecule in the cuticle and its transport properties. The implementation of these equations is exemplified by examining glucosinolates, which play an important role in interactions of plants with other organisms. An accurate characterization of physicochemical properties of the plant surface is needed to understand its ecological significance. Here, we summarize current knowledge about the physical and chemical properties of plant cuticles and their role in interactions with microorganisms, phytophagous insects, and their antagonists.

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Key Words—Attachment, chemical interaction, epicuticular waxes, glucosinolate, herbivores, host recognition, physicochemical properties, plant cuticle, protection.

INTRODUCTION

The plant surface is bordered by a cuticle, which plays an important role in the physiology and autecology of the plant, but also serves as the first contact zone in interactions of plants with other organisms at all trophic levels. The cuticle covers all aerial parts of higher plants, i.e., stems, leaves, petals, and fruits, with the exception of stems that have undergone secondary growth. It is a continuous layer where the only gaps are the pores of the stomata. To understand the different ecological functions of the cuticle, it is important to understand its chemical and physical nature. Current knowledge of such properties enables us to evaluate cues on the outer surface that are used by microorganisms or insects. For example, early work by Bernays et al. (1976) showed already in the 1970s that locusts and grasshoppers can discriminate between susceptible and nonsusceptible plants simply by contact of their chemoreceptors with surface waxes.

The physical structure and chemical composition of the cuticle have been subjects of earlier reviews (e.g., Jeffree, 1996; Riederer and Markstädter, 1996; Barthlott et al., 1998). Also, various reviews have focused on individual aspects of surface characteristics, such as wetting ability (Holloway, 1969, 1970, 1971; Otten and Heminghaus, 2004 and others) or optical characteristics (Barnes and Cardoso-Vilhena, 1996). Functions of the cuticle have been reviewed by Jeffree (1986), Schönherr and Riederer (1989), Riederer (1995), Riederer and Schreiber (1995, 2001), Jenks and Ashworth (1999), and Bargel et al. (2004). The specific role of the cuticle in interactions with other organisms was summarized in earlier reviews with regard to microorganisms (e.g., Kolattukudy et al., 1995; Mendgen, 1996), herbivorous insects (Eigenbrode and Espelie, 1995; Eigenbrode, 1996), and effectiveness of predatory insects (Eigenbrode, 2004). This review aims to bring together present knowledge about the plant surface from the different fields of plant (eco)physiology, microbiology, entomology, and ecology in an integrative and comprehensive way. We feel that the complexity of the cuticle and its various functions have been underappreciated in previous ecological literature. The first part of this review will outline the basic cuticular chemistry and physics involved, whereas the second part will deal with the relevance of these properties in a chemical–ecological context. We provide the chemical ecologist with an overview of the most recent literature on these topics, with emphasis on established methodology, and new approaches. Because we do not attempt to cover all aspects in depth, we refer to major reviews on particular topics.

PHYSICOCHEMICAL PROPERTIES OF THE PLANT SURFACE

Chemical Composition of Cutin and Waxes. The functionally important lipophilic fraction of the cuticle consists of cutin and waxes. Cutin forms the matrix of the cuticle and is a polyester-type biopolymer. It is composed mainly of two families of hydroxy and hydroxyepoxy fatty acids derived from the most common cellular fatty acids, as well as from C_{16} saturated and C_{18} unsaturated fatty acids (Holloway, 1982a). The chief members are 16-hydroxy and dihydroxy hexadecanoic acids, the latter having one hydroxyl moiety at the terminal carbon and another in a mid-chain position. The C_{18} family is represented by unsubstituted and 18-hydroxy octadec-9-enoic acids, 18-hydroxy-9-epoxy, and 9,10,18-trihydroxy octadecanoic acids. The monomers are cross-linked by ester and, in some species, by nonester bonds, although the exact chemical nature of these has yet to be elucidated (Schmidt and Schönherr, 1982; Riederer and Schönherr, 1988). The cutin monomers cross-linked by ester bonds can be released for identification by treatment with bases or by esterases (cutinases). The composition and amount per unit area of cutin vary considerably among plant species and may differ relative to plant organs and ontogenetic states (Riederer and Schönherr, 1984, 1988). This variation makes species- and organ-specific recognition possible.

Cuticular waxes are present in the lipophilic fraction of the cuticle. Waxes in the chemical sense are esters of long-chain fatty acids with long-chain primary alcohols. Here, the term waxes is used in a broad sense to include cyclic compounds such as pentacyclic triterpenoids (Riederer and Markstädter, 1996). The aliphatic fraction of cuticular waxes consists of a complex mixture of compounds having a hydrocarbon backbone with 21 to >40 carbon atoms. Their biosynthesis depends on the action of elongases on moieties derived from fatty acid synthetase. Further reactions, predominantly the addition of oxygen-containing substituents and the removal of one carbon, lead to the aliphatic compound classes present in cuticular waxes (Kolattukudy, 1996).

The major compound classes of plant cuticular waxes are *n*-alkanes (chain-length C_{21}–C_{35}) and smaller proportions of iso and anteiso homologues, primary alcohols (C_{22}–C_{40}), fatty acids (C_{20}–C_{24}), aldehydes (C_{24}–C_{36}), secondary alcohols (C_{21}–C_{35}), with a tendency for mid-chain hydroxylation, ketones (C_{21}–C_{35}), β-diketones (C_{22}–C_{36}), and *n*-alkyl esters (C_{36}–C_{60}) resulting from the combination of long-chain primary alcohols and fatty acids (Baker, 1982; Walton, 1990). Each class consists of a homologous series of isomers. In many cases, one compound dominates the total composition of the cuticular wax. The particular pattern is highly characteristic for a given plant species, plant part, or developmental stage. In addition to the aliphatic portion of the wax, various amounts of cyclic compounds, such as pentacyclic triterpene acids, triterpene alcohols, triterpene ketones, and triterpene esters, as well as hydro-

xycinnamic acid derivatives, flavonoids, and their respective glycosides, may be present.

Fine Structure of the Cuticle and Cuticular Surfaces. The most common structural model of the matrix portion of the cuticle is that of a *bilayered* cuticular membrane (Figure 1), where the two layers are distinguished by their ontogeny, ultrastructure, and chemical composition. The outermost portion (called the cuticle proper) forms a continuous layer that is separated from the outer epidermal cell wall. It is synthesized first and is composed of cutin with ester and, in some species, nonester bonds. These nonester bonds make the cuticle proper fairly resistant against the effects of esterases produced by fungi. The inner layer, called the cuticular layer, arises from impregnation of the outer epidermal cell wall by cutin and thus contains substantial amounts of various embedded cell wall polysaccharides. For reviews of cuticular structure and ontogeny, see Holloway (1982b) and Jeffree (1996).

The outer portion of the cuticle is encrusted with waxes (intracuticular waxes), and a thin film of epicuticular waxes covers the outer surface. In certain species, a more or less dense cover of epicuticular crystals may occur (Figure 2), and this is responsible for the distinctive visual appearance (see below). The epicuticular crystals are composed primarily of the single predominant component of the wax, and show species-specific, substance-dependent habits (Jetter and Riederer, 1994, 1995). For a thorough synopsis of a structural nomenclature for epicuticular wax crystals, see the review by Barthlott et al. (1998). Methods for mechanically separating the epicuticular from the intracuticular waxes have become available (Ensikat et al., 2000; Jetter et al., 2000), and these provide evidence that the actual leaf surface, which is first contacted by insects and microorganisms, may differ drastically in chemical composition from the internal wax.

The quantitative coverage and qualitative composition of the total wax as well as those of the epicuticular layer may vary according to a number of

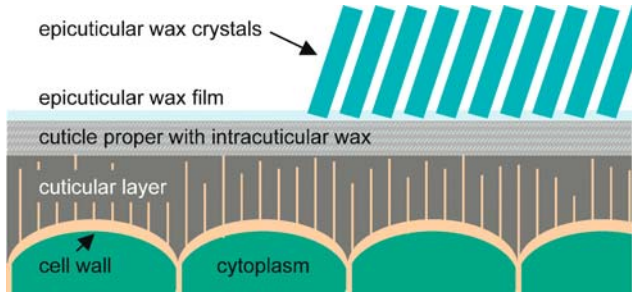

FIG. 1. Scheme showing the layering of the plant cuticle. The features displayed are not to scale. See text for further explanation.

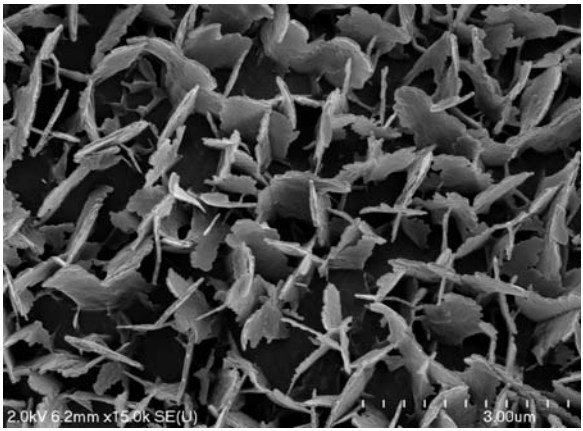

FIG. 2. Electron scanning micrograph of the upper leaf surface of pea (*Pisum sativum*). The upright crystals of cuticular wax consist mainly of 1-hexacosanol. This example is shown as representative for leaf surfaces where the very tips of epicuticular crystals represent the primary contact zone between an alighting organism and the leaf.

exogenous and endogenous factors. They may vary with season (Schütt and Schuck, 1973; Gülz et al., 1991; Gülz and Müller, 1992; Faini et al., 1999; Jenks et al., 2002), temperature (Reed and Tukey, 1982; Faini et al., 1999; Dodd and Afzal-Rafii, 2000; Dodd and Poveda, 2003), and light (Macey, 1974; Giese, 1975; Davis, 1978; von Wettstein-Knowles et al., 1980; Reed and Tukey, 1982; Steinmüller and Tevini, 1985; Upadhyaya and Furness, 1994; Shepherd et al., 1995). Erosion by wind and rain can also influence wax amounts and the density of cuticular wax blooms (Baker and Hunt, 1986; Hadley and Smith, 1989). Further exogenous factors of potential importance include carbon dioxide and ozone (Percy et al., 2002).

Endogenous factors influencing amount, composition, and distribution of the wax include ontogenetic states (Jetter and Schäffer, 2001; Ficke et al., 2004) and organ-specific regulation: differences in the cuticle exist between upper and lower leaf side (Premachandra et al., 1993; Kanno and Harris, 2000a,b; Müller and Hilker, 2001), and are especially pronounced in plants showing heteroblasty (Adati and Matsuda, 1993; Brennan et al., 2001; Steinbauer et al., 2004).

PHYSICAL PROPERTIES OF THE PLANT SURFACE

Hydrophobicity. The most important feature of primary plant surfaces is, because of their hydrophobicity, the repellence of water, aqueous solutions, polar mucilages, and small organisms with polar coatings. This feature is a

result of coverage by waxes that are essentially aliphatic and thus apolar. Therefore, wetting, i.e., the spreading of aqueous media on cuticular surfaces, is strongly impaired. The contact angle of pure water on such surfaces is approximately 112°.

Apolar surfaces become even more repellent when they are rough on a microscopic scale. This added repellency is due to epicuticular wax crystals and sometimes also by papillose epidermal surfaces or trichomes. Then, a sessile droplet of pure water may be completely round (contact angle about 180°), and the surfaces are beaded with water droplets that easily drip off (Barthlott and Neinhuis, 1997; Neinhuis and Barthlott, 1998). Higher wettability of the hydrophobic cuticular surface can be attained by apolar solvents or by aqueous solutions containing surface-active compounds such as synthetic or biological surfactants. Various aspects of leaf wettability have been the subject of reviews (Holloway, 1969, 1970, 1971; Hartley and Graham-Bryce, 1980; Watanabe and Yamaguchi, 1991a,b, 1992; Wagner et al., 2003; Otten and Herminghaus, 2004). Plant surfaces that are no longer pristine become increasingly wettable by aqueous solutions because of factors such as the deposition of salts (Klemm et al., 1987; Burkhardt et al., 2001) or the growth of microorganisms (Knoll and Schreiber, 1998). To assess the repellency of plant surfaces experimentally, various methods are available, the most prominent being contact-angle measurements (Pallas, 1997).

Mechanical adhesion of small particles, such as particulate matter from dry deposition, microorganisms, spores and conidia, as well as insect legs and eggs, may be impaired by the same factors that provide the surface with water-repellent properties (hydrophobic smooth and microrough surfaces). These biological consequences are discussed in a later section.

Optical Properties. Cuticular surface features also determine the optical properties of the primary parts of higher plants (Barnes and Cardoso-Vilhena, 1996) and can thereby influence their appearance for approaching insects (see below). On one hand, plant cuticles absorb ultraviolet radiation to varying degrees (Krauss et al., 1997), whereas they are translucent in the range of the photosynthetically active wavelengths of solar radiation. On the other hand, most radiation is reflected and scattered from cuticular surfaces. Leaf, fruit, and primary stem reflectance and scattering is partly due to roughness features on the surface with varying densities, dimensions, and refractivity and partly due to the cell walls of the underlying tissues (Slaton et al., 2001; Grant et al., 2003). Radiation emitted or reflected by plant surfaces may occur in the range from 280 nm to the infrared, including the wavelengths visible to the human eye and active in plant photosynthesis. In the visible range, reflection depends on the presence of epicuticular waxes and trichomes, which in extreme cases, may turn plant surfaces whitish as a result of broadband reflection in the visible part of the solar spectrum (Eller and Willi, 1977; Holmes and Keiller,

2002). In the ultraviolet range, radiation emission is influenced by the presence and dimensions (and thus scattering properties) of epicuticular wax crystals. When surface features have dimensions that approximate the wavelength of short-wave visible and UV radiation, these surfaces appear bluish and strongly emit in the ultraviolet (Clark and Lister, 1975; Riederer, 1989; Grant et al., 2003).

The radiation reflected from leaf surfaces has two principal components, i.e., diffuse and specular. The specular component is polarized to varying degrees, depending on microscopic leaf surface features (Grant et al., 1993; Brakke, 1994). Grant et al. (1993) have also shown that the polarized component may account for up to 90% of total reflection from adaxial leaf surfaces of some species. The visual system of some insects allows them to recognize and distinguish between various degrees of polarized light (see below).

Dissolution of Organic Molecules in the Cuticle. Plant surfaces not only have special physical properties that determine wetting and adhesion. They also interact with organic chemicals coming into contact with them either from the outside or from endogenous sources. These chemicals may be either in the gas or vapor phase or in solution. They may also be delivered to the plant surface by direct contact with a particle deposited there or with an organism alighting on the plant. Here, we deal with questions about types of chemicals that can move across the cuticle and factors that determine the sorption of extraneous material (e.g., pheromones) to the cuticle. Such movement will be discussed first from a physiological and physicochemical point of view to provide a basis for understanding their ecological implications.

The interaction of a molecule with the surface or the whole cuticle is referred to as sorption, which may involve adsorption and/or absorption (Schönherr and Riederer, 1989; Riederer, 1995). Sorption is a physicochemical phenomenon without involvement of any biological process. To understand sorption of organic compounds, we first have to consider their water solubility. The saturation concentration of a given compound in water ("aqueous solubility'') describes the interaction of a substance with an aqueous medium [e.g., a sessile droplet or water in the apoplast of the plant (Yalkowski and Banerjee, 1992)]. The tendency of a substance in an aqueous solution to move into and to accumulate in an adjacent organic phase such as the cuticle is expressed by the partition coefficient. This is a dimensionless property describing the degree of accumulation of a chemical in the organic phase relative to water. The most widely used measure for this accumulation tendency and, thus, the relative lipophilicity of a compound is the 1-octanol/water partition coefficient (K_{ow}) according to

$$K_{ow} = \frac{C_o}{C_w} \tag{1}$$

where C_o and C_w are the equilibrium concentrations of the substance in the 1-octanol and water phases, respectively (Hansch and Leo, 1979). When the organic phase is the cuticle, Equation 1 can be rewritten according to

$$K_{cw} = \frac{C_c}{C_w} \tag{2}$$

by substituting the equilibrium concentration in the cuticle (C_c) for the respective concentration in the 1-octanol phase.

Water solubilities and the 1-octanol/water partition coefficients are inversely related (Leo et al., 1971; Mackay et al., 1980; Yalkowski and Banerjee, 1992). With weak organic acids or bases, aqueous solubility and, thus, partition coefficients depend on the acidity constant pK_a and pH (Schwarzenbach et al., 1993). For volatile or semivolatile chemicals, the saturation vapor pressure (P_s in Pa) is an additional parameter determining the availability of a compound for absorption or adsorption to the surface of the cuticle. Values for water solubilities and octanol/water partition coefficients can be either obtained from the literature or estimated from fundamental properties of the compounds (Lyman et al., 1990).

Direct experimental measurement of the cuticle/water partition coefficient K_{cw} is feasible only for a small fraction of the large number of chemicals relevant in chemical ecology. Therefore, a quantitative property–property relationship (QPPR) was established between the cuticle/water partition coefficient and the 1-octanol/water partition coefficient (Schönherr and Riederer, 1989):

$$\log K_{cw} = 0.057 + 0.970 \log K_{ow} \quad (r = 0.987). \tag{3}$$

For illustrative purposes, Equation 3 can be used to estimate the cuticle/water partition coefficient of two glucosinolates, important contact allelochemicals in insect–plant interactions (see below). Brudenell et al. (1999) have measured log K_{ow} for 3-butenyl glucosinolate and allyl glucosinolate of -2.5 and -2.8, respectively. The values were independent of pH in the range from pH 7 to 4 because the pK_a of both compounds can be assumed to be much less than 0. The estimated log cuticle/water partition coefficients are -2.4 and -2.7, respectively. Thus, the equilibrium concentration of both glucosinolates in the cuticle will be 0.4 and 0.2% of that in the aqueous phase of the adjacent cell wall. As the corresponding wax/water partition coefficients are approximately one order of magnitude lower than K_{cw} (Burghardt et al., 1998), the equilibrium concentrations of glucosinolates in the outer wax layer will be negligible (0.02 and 0.04% for 3-butenyl glucosinolate and allyl glucosinolate, respectively). In addition, glucosinolates have only been localized in the phloem and in the

interior of cells (Chen and Andreasson, 2001; Chen et al., 2001), which suggests that the respective concentrations in the cell wall solution are extremely low or essentially zero.

These considerations demonstrate that only lipophilic uncharged organic chemicals can be sorbed onto plant surfaces. For volatiles, which are important in attraction or repellence of interacting organisms over a distance, lipophilicity and a low vapor pressure favor accumulation in the cuticle. The dissociated species of acids and bases and other polar organics such as carbohydrates or glucosylated substances as well as very small and volatile molecules in the vapor phase will not be absorbed or adsorbed to the cuticle, although transport across the cuticular pore is possible to some degree (see below). Also, such compounds might be removed easily from the plant surface. In contrast, the cuticle may act as a fixative for appropriate compounds enabling them to stay much longer on the surface and to exert their biological functions.

Transport. One of the main functions of the cuticle is to inhibit the loss and uptake of water and dissolved chemicals from and into the plant. Thus, it essentially controls the diffusive exchange between the plant and the surrounding environment (see below). From a physi(ologi)cal point of view, the cuticular pathway is the exclusive transport system for uptake into or release from the leaves and primary stems of compounds having specific characteristics. Liquids and relatively nonvolatile solutes contained either in the apoplast or in liquid or solid deposits as well as the unstirred air layer above the surface can use this pathway (Schönherr and Riederer, 1989; Riederer, 1995, 2004; Riederer and Schreiber, 1995). All apolar organics with low vapor pressures and sufficient lipid solubilities, including a wide variety of primary and secondary metabolites, belong to this class. Polar and charged compounds cannot partition into and diffuse across the lipid pathway of the cuticle. There is increasing evidence, however, that in some plant species, polar pathways exist that make possible the easy transport of small molecules (smaller than xylose with a molar volume ≤ 110 cm^3 mol^{-1} in *Hedera helix* leaf cuticles; Popp and Riederer, unpublished results) to the plant surface (Schönherr, 2002). In *H. helix* cuticles, transport of polar compounds above this size threshold was negligible.

Compounds with high volatility (e.g., gases, monoterpenes, glucosinolate-derived volatiles), in principle, can take two parallel paths between the interior and the outside as long as the stomata are open (Figure 3). The preferred pathway depends on the lipophilicity of the compound (i.e., its tendency to sorb in the cuticle) and on its saturation vapor pressure in the atmosphere above the cuticle (Riederer, 1990, 1995; Riederer et al., 2002). If stomata are closed or absent (in the case of some fruits), the diffusion of all volatiles and semivolatiles is restricted to the cuticular pathway.

Transport of material from the interior of a primary organ across the cuticle-covered plant/atmosphere interface or *vice versa* arises from a

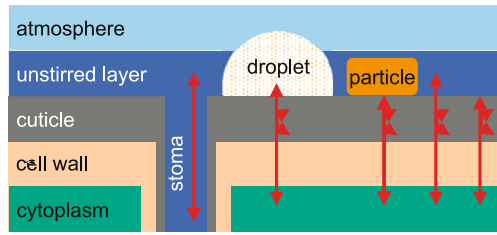

FIG. 3. Schematic representation of the compartments at the leaf/atmosphere interface and the pathways of diffusive transport connecting them. Compartments are not to scale, and only representative diffusion pathways are shown.

concentration gradient of solutes or vapors between the interior and the outside. However, from an ecological point of view, it is not important that a compound is transported at all, but how long it takes until biologically relevant doses are reached on the plant surface. From this, we can conclude that in an ecological context, the decisive function of the plant cuticle is not to act as an absolute barrier, but to exert kinetic control.

This overall process of transport across the cuticle can be visualized as the diffusion of a compound from a donor compartment (e.g., the solution in the apoplast or the vapor phase in the intercellular space) to a receiver compartment (e.g., the outer surface of the plant or the gaseous phase surrounding it). The compartments are separated by the cuticular transport barrier. Under steady-state conditions, the amount permeated linearly increases with time, and the flow N (mol a^{-1}) is given by

$$N = PA(C_d - C_r) \tag{4}$$

where C_d and C_r (mol m^{-3}) are the concentrations in the donor and receiver compartments, respectively, A (m^2) is the area of the cuticle exposed, and P (m sec^{-1}) is the permeance of the cuticle. At low concentrations and with nonelectrolytes, the gradient of chemical potential can be substituted by the difference of concentrations as driving force. In the steady state, when C_d = const. and $C_d >> C_r$, Equation 4 can be simplified to

$$N = PAC_d. \tag{5}$$

There is considerable variation among cuticular permeances determined for one chemical and the cuticles of a number of plant species. A study with 2,4-dichlorophenoxy acetic acid and the cuticles from 11 plant species showed that permeances may range over two orders of magnitude (Riederer and Schönherr, 1985).

The observation that cuticular permeances were correlated to partition coefficients, again, led to the attempt to establish quantitative relationships for predicting permeances of *Citrus aurantium* leaf cuticles (largest data set available) from more fundamental and easily accessible parameters. Such a correlation has been successfully established between the cuticular permeance and the 1-octanol/water partition coefficient (Schönherr and Riederer, 1989):

$$\log \mathrm{P} = 0.704 \log \mathrm{K}_{ow} - 11.2 \ (r = 0.91) \tag{6}$$

This equation can be used for estimating cuticular permeability for natural products not accessible to the direct experimental determination of permeances. Such calculations are useful to determine what an organism might find on a plant surface (see below). For instance, by using Equation 6, the cuticular permeances of 3-butenyl and allyl glucosinolate can be estimated from the experimentally determined log K_{ow} (Brudenell et al., 1999). The estimates for P are 9×10^{-14} and 5×10^{-14} m sec^{-1}, respectively. These values are much lower than any cuticular permeance measured so far. Given the very low or practically zero concentrations of glucosinolates in the cell wall solution, no glucosinolates will reach the cuticular surface via the cuticular pathway.

These predictions are supported by preliminary results from an experiment where a compartment containing a solution of 4-hydroxybenzyl glucosinolate was separated from a compartment with pure buffer by an isolated astomatous cuticle from *H. helix* leaves. Even after 14 d, no flux of the glucosinolate across the cuticle was observed (Popp and Müller, unpublished data). This demonstrates that glucosinolates are both too polar for the lipophilic pathway and too large for the polar pathway of *H. helix* cuticles. Future research will be needed to test whether this finding can be extended to the cuticles of other species.

FUNCTIONS OF PLANT SURFACES

Functions for Plants. The cuticle is the skin of plants and, as such, is both functionally and, in part, structurally analogous to the skin of vertebrates (Hadley, 1991). Cuticles are found with the oldest fossils of terrestrial plants (Edwards et al., 1996) indicating the reliance of plant life on this barrier to survive in the dry atmosphere. In this respect, the importance of the cuticular interface is equivalent to that of the arthropod cuticle (Hadley, 1989, 1991). Arthropods and higher plants share considerable similarities in the composition (waxes) and the function (low water permeabilities) of their respective cuticles (Hadley, 1981).

As a large driving force exists between the aqueous interior of a plant and its atmospheric surroundings, protection against uncontrolled water loss is the principal function of the cuticle. Plant cuticles are effective transpiration barriers reducing water loss to the same or even higher degree as would synthetic organic polymer sheets of comparable thickness (Riederer and Schreiber, 2001). This low (in many cases practically zero) water permeability allows plants to regulate the flow of water vapor almost exclusively by the stomatal pathway (Nobel, 1991).

Other common functions of skins and cuticles are retention of solutes in the body fluids, impeding the uptake of solutes and gases from the environment, protection against invasion by microorganisms, shielding of detrimental ultraviolet radiation, and, to a certain degree, mechanical defense (Figure 4).

To achieve these functions, the plant cuticle must have an array of suitable chemical and physical properties. The most important property is its hydrophobicity, which impedes the transport of water and the wetting of the outer surface (see above). Further hindrance of water loss is provided by the partial crystallinity of the cuticular waxes. The UV-shielding properties of plant surfaces result from phenolic compounds (flavonoids and hydroxycinnamic acid

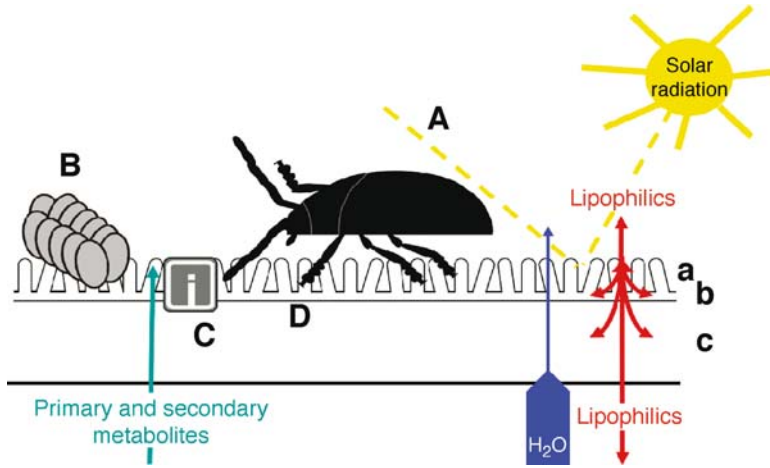

FIG. 4. Processes on the plant surface. (A) Reflectance, transmittance, and absorbance of solar radiation; (B) attachment of insect eggs (or spores of microorganisms, likewise); (C) physical and chemical cues used for host recognition by microorganisms and insects; (D) adhesion of insect legs and influences of surface characteristics on locomotion. Water and other metabolites diffuse in different degrees through the cuticular layer, formed by epicuticular wax crystals (a), the epicuticular wax film (b), and cutin and intracuticular waxes (c).

derivatives) associated with the cuticle or the outer epidermal cell wall (Krauss et al., 1997; Kolb et al., 2001, 2003). Many of these phenolics also have antimicrobial activity (Talley et al., 2002). A mechanical defense is provided by the relatively high tensile strength of the cuticle (Wiedemann and Neinhuis, 1998). The integrity of the cuticle may be compromised by microorganisms or insects making holes and by the mechanical action of wind (Hoad et al., 1992).

Interactions with Microorganisms. Under natural conditions, the cuticular surfaces in most cases are not as clean and pristine as usually shown by scanning electron micrographs. A variety of epiphyllic microorganisms such as filamentous fungi, yeasts, and bacteria colonize the leaf surface, forming a more or less dense biofilm. Under warm, humid conditions, the surface may also be covered with cyanobacteria and lichens (Blakeman, 1982; Andrews and Harris, 2000; Herrera-Campos et al., 2004).

The first requisite for microorganisms to become established on a plant is the ability to attach to the surface. To overcome the hydrophobic properties of the plant cuticle (see above), fungal spores and their hyphae excrete extracellular mucilage or release other liquids containing, for example, hydrophobic proteins that mediate an adhesive interaction with the leaf surface (Mendgen, 1996; Carver et al., 1999). Changes in wettability of leaf surfaces with age may be observed and are related to degradation of epicuticular wax crystals (Riederer, 1989) and to increasing coverage by epiphytic microorganisms (Knoll and Schreiber, 1998, 2000). Degradation of the waxes leads to higher leaf wettability, which offers, in general, a more suitable microhabitat for most phyllosphere organisms (Knoll and Schreiber, 1998), whereas surface wetness may hinder sporulation of others (Butler, 1996).

In addition to determining the wetting properties of the plant surface, cuticular waxes can serve as signals that induce spore germination (Kolattukudy et al., 1995) and can influence the growth pattern of the hyphae of biotrophic fungi (Mendgen, 1996; Mendgen et al., 1996). Specific long-chain alcohols and aldehydes are effective inducers (Ahmed et al., 2003; Dickman et al., 2003). These signals act as kairomones, allowing a pathogen to recognize its host plant. Also, cuticular waxes can relieve the self-inhibition of germination of conidia (Hegde and Kolattukudy, 1997). Cutin and long-chain fatty acids derived from the cuticular waxes may induce biochemical pathways that are required either for the pathogenicity of certain fungi (Ahmed et al., 2003; Dickman et al., 2003) or for the induction of plant defense reactions (Fauth et al., 1998). Thickness and three-dimensional structure of leaf wax crystalloids on leaves and fruits can contribute to resistance of plants against attack by fungal pathogens (Schwab et al., 1995; Ficke et al., 2004).

Colonization by epiphytic bacteria is, in part, limited by the availability of suitable carbon sources on the surface. These carbon sources are either carbohydrates or other small primary plant metabolites present at the surface

(Mercier and Lindow, 2000) or wax-derived hydrocarbons made available by biosurfactants (Georgiou et al., 1992). Several secondary metabolites present on the leaf surface exert antimicrobial properties and thus hinder colonization by microorganisms (Talley et al., 2002). Coumarins, flavonoids, monoterpenes, sesquiterpenes, and sesquiterpene lactones are primarily sequestered in glandular trichomes on the surface (Szafranek et al., 1998; Talley et al., 2002). These compounds are likely to be released onto the leaf surface as the cuticular sacs of the trichomes mature. In sagebrush populations, the quality and quantity of secondary metabolites on the leaf surface have been selected as a result of the abundance of fungi (Talley et al., 2002).

Mediation of Insect–Microorganism Interactions. The epicuticular waxes of leaves can affect germination of entomopathogenic fungi that infect herbivorous insects. Leaf surfaces of members of the Brassicaceae were shown to contain a mixture of stimulatory and inhibitory compounds to the entomopathogenic fungus *Metarhizium anisopliae*, influencing its virulence to the mustard beetle *Phaedon cochleariae* (F.). Surface leachates of nutrients increased the virulence (Inyang et al., 1999).

Spread of the fungal aphid pathogen *Pandora neoaphidis* was enhanced on pea plants with reduced compared to normal wax bloom. This effect was associated with better attachment of fungal conidia on the reduced wax surface (Duetting et al., 2003). Compounds produced from microorganisms such as yeast that accumulate on the leaf surface can have inhibitory effects on the oviposition by insects (Städler, 2002).

Interactions with Phytophagous Insects. After visual and/or chemical attraction to a host, all phytophagous insects will first come into contact with epicuticular wax layers when they land on aboveground primary parts of a higher plant. Here, they will encounter either a smooth waxy surface or epicuticular wax crystals. The chemical and physical properties of this contact interface differ drastically from those of the interior parts of the cuticle. Therefore, it is the outermost wax layer (except in the case of leaf surfaces having glandular trichomes) that harbors the physical and chemical cues involved in the primary step of the interaction between herbivores and the plant (Figure 4). These cues also can determine insect development and behavior. Such interactions mediated by surface properties have been studied mainly in crop plants such as cabbage, cereals, beans, and onion that are subject to infestation by several insect pests (Peter and Shanower, 2001). Only limited data are available on the role of the cuticle in nonfood plant–herbivore interactions (but see, for example, Edwards, 1982; Steinbauer et al., 2004).

Physical surface properties affecting plant–herbivore interactions include the microstructure of the epicuticular waxes. These wax properties determine optical characteristics by reflectance, transmission, or absorbance of specific wavelengths to varying degrees, as outlined above. The resulting visual

"Gestalt'' of a plant part offers an important cue in the host-finding process of insects, operating sequentially or simultaneously with chemical cues (Prokopy and Owens, 1983). For example, leaf color, determined by relative wavelength, significantly influences host preference of the potato leafhopper, *Empoasca fabae* (Harris) (Bullas-Appleton et al., 2004). As reflectance and scattering patterns change with senescence (Major et al., 1993), insects can use the optical information about age and, thus, the quality of their host. Insects with polarization-dependent color vision systems might be able to discriminate between shiny and matt surfaces (Hegedüs and Horváth, 2004). However, reflection polarization, as determined by the epicuticular wax structure, might also influence the color perception of insects visiting plants. Polarization of light can cause false perception of colors in *Papilio* butterflies, which have polarization-sensitive photoreceptors (Kelber et al., 2001).

Water droplets on the plant surface can be an important source of water for insects. They can also protect early instar larvae against desiccation or help them to stay on the plant (Raina, 1981). Contact with surface water offers a cue for termination of diapause in some species of Pyralidae (Lepidoptera) living in semiarid climates (Butler, 1996).

Primary metabolites, such as soluble carbohydrates, leak onto plant surfaces (see above) and can play a role as nutrients or stimulants for insects (Fiala et al., 1993; Derridj et al., 1996a,b). Sugar and sugar alcohols identified from apple fruit surfaces have been demonstrated to function as kairomones and evoke oviposition in female moths of *Cydia pomonella* L. (Lombarkia and Derridj, 2002). However, probably only relatively small- to medium-sized and uncharged organic molecules will leak to the outside (see above).

In interactions between phytophagous insects and plants, plant secondary metabolites provide major cues and understandably, therefore, have received the most attention as allelochemicals. A plethora of examples demonstrate how natural products act as feeding or oviposition stimulants, as deterrents, or in the case of volatiles, as attractants or repellents (Schoonhoven et al., 1998). Depending on their polarity and solubility (see above), secondary compounds can be released from plant tissues and accumulate either in the epicuticular waxes or in the cutin.

Volatiles may leave the inner leaf tissue either through the cuticle or the stomata. They may be absorbed by the cutin or adsorbed on the epicuticular wax layer. Such volatiles can attract or repel herbivores to or from a plant from a distance. For example, volatile isothiocyanates are attractive to specialist herbivores of Brassicaceae (Bartlet et al., 1997; Mewis et al., 2002), although their concentration in the headspace over undamaged leaves is extremely low (Tollsten and Bergström, 1988). However, volatiles also can be important in close-range orientation, acting synergistically with contact chemoreception cues (de Jong and Städler, 1999).

Nonvolatile secondary metabolites can be deposited on the plant surface *via* diffusion and associated with the waxes, or they can be stored in glandular trichomes (Bargel et al., 2004). The flavonol quercetin may be excreted from glandular trichomes on the leaf surface of tobacco, where it acts as a stimulant to the mirid *Tupiocoris notatus* (Distant; Roda et al., 2003). Many studies have addressed the presence of secondary metabolites on the plant surface of leaves, stems, or reproductive tissue (seeds and fruits) where they act as allomones or kairomones (Eigenbrode and Espelie, 1995; Brooks et al., 1996; Green et al., 2003). However, such compounds can also function as toxins for neonate larvae (Carter et al., 1989). Nonvolatile glucosinolates, the substrate from which isothiocyanates are formed, have been detected in surface extracts of Brassicaceae where they can stimulate feeding or oviposition (Städler, 1992; Hopkins et al., 1997; Griffiths et al., 2001; Marazzi et al., 2004). However, as pointed out above, the physicochemical properties of these polar compounds make it unlikely that they are present in the epicuticular layer itself. Several investigators have prepared "leaf surface extracts'' by dipping leaves in dichloromethane followed by methanol (e.g., Hopkins et al., 1997). However, this procedure may result in extraction of compounds from mesophyll or epidermal cells. Reifenrath et al. (2005) have recently suggested that glucosinolates are probably washed from the inner tissue when applying conventional extraction protocols, but that they are not naturally present on the leaf surface. When removing the epicuticular waxes mechanically from leaves of *Brassica napus* L. and *Nasturtium officinale* R. Br., they found no traces of these secondary compounds therein (Reifenrath et al., 2005).

To reexamine several of the examples described in the literature as well as for future studies, new techniques should be used that are available to probe the epicuticular layer selectively (Premachandra et al., 1993; Ensikat et al., 2000; Jetter et al., 2000). Stomatal aperture could be controlled by keeping plants shortly in darkness prior to extraction to avoid washing out of compounds Reifenrath et al. (2005). With these methods, the presence or absence of specific secondary compounds in the epicuticular layer might be unequivocally demonstrated.

If certain secondary compounds are virtually absent from waxes, insects might actually need to slightly damage the epicuticular layer by scratching it with their legs or ovipositor, thereby contacting compounds deeper in the cuticle or just beneath it (Städler, 2002). Indeed, an epicuticular layer with ubiquitously distributed aliphatic compounds could be adaptive for the plant, rendering it less detectable to specialists (Reifenrath et al., 2005).

Several insects use lipids of the wax layer, specifically various long-chain alkanes and alcohols, as cues in host plant selection (Adati and Matsuda, 1993; Lin et al., 1998; Powell et al., 1999; Morris et al., 2000; Coyle et al., 2003). For some insect species, lipids act only in synergy with secondary metabolites as

kairomones (Harris et al., 1987; Roessingh et al., 1992; Spencer, 1996; Spencer et al., 1999; Marazzi et al., 2004).

The quality and quantity of waxes can vary tremendously within a plant. Specialists can use these differences as information for orientation and for choosing a particular site for oviposition or feeding. This has been demonstrated, for example, in specialists on *Eucalyptus* spp. showing heteroblasty (Adati and Matsuda, 1993; Brennan et al., 2001; Steinbauer et al., 2004). Leaf-cutter bees make more cuttings on leaves of *Cercis canadensis* L. (Fabaceae) with crystalline adaxial surface wax than on glabrous leaves (Eigenbrode et al., 1999a). Also, differences between both leaf sides (abaxial or adaxial) in wax composition or structure can explain differences in attractiveness (Roessingh and Städler, 1990; Justus et al., 2000; Kanno and Harris, 2000a,b; Müller and Hilker, 2001). Hereby, recognition by the insect might be based on chemical and/or physical cues of the waxes. Coating with waxy substances can be a key cue in bioassays testing for stimulants, showing the importance of waxes as physical cues (Degen and Städler, 1997).

Conversely, substances contained in epicuticular waxes function as allomones, deterring oviposition, and feeding by herbivores (Juniper, 1995; Eigenbrode and Espelie, 1995; Eigenbrode, 1996), and leaf cutting by fungus-growing ants (Hubbell et al., 1984; Sugayama and Salatino, 1995; Salatino et al., 1998). In addition, they are potentially toxic to some insects (Espelie, 1996). It was found repeatedly that plant mutants or cultivars with an increased surface wax layer are more resistant to herbivores (Eigenbrode et al., 1996; Shepherd et al., 1999a). In other systems, the relative quantity of wax compounds could be correlated with plant resistance of different plant genotypes (Jones et al., 2002; Rapley et al., 2004). In the polyphagous grasshopper *Schistocerca americana* (Drury), rejection of unacceptable leaves was first dependent on biting the leaf, but subsequently occurred after palpation of the surface alone. Thus, leaf surface waxes can also be used as cues in associative learning (Woodhead and Chapman, 1986; Chapman and Sword, 1993).

Insect feeding can also induce wax production of the plant and change surface chemistry. *Beta vulgaris* leaves respond with increased wax development to the punctures of aphids (Bystrom et al., 1968). The amount of the triterpene squalene increases in apple leaves after infestation with leaf miners (Dutton et al., 2002).

Several insect species mark the spot of oviposition on a plant surface with an oviposition-deterring pheromone. Subsequent females arriving on the host will avoid oviposition at the same site, and thereby ensure that overcrowding on a limited resource does not occur. The persistence of these pheromones on the surface can vary from 1–2 hr up to 12 d (Anderson, 2002) and will depend on the saturation vapor pressure and the lipophilicity of the pheromone as well as the sorptive properties of the cuticular surface. Pheromones with low vapor

pressures and high lipophilicities will persist longer than those with high volatilities and a low tendency to accumulate in lipid phases (see above).

The surface chemistry of a herbivorous insect itself can vary drastically with different food sources. Larvae of *Manduca sexta* (L.) browsing on tobacco have high levels of duvatrienediols in their cuticular lipids. These compounds are probably transferred from the sticky globules of the host plant surface (Espelie and Bernays, 1989). Fatty alcohols with typical chain lengths of those on plant surfaces are present as white powder on some larvae of moths or sawfly species, suggesting that these waxes are of plant origin (Espelie and Bernays, 1989). The plant surface chemicals might be adopted (sequestered from the plant or synthesized *de novo*) by a herbivore, thus becoming chemically "camouflaged'' to predators, as a form of chemical mimicry (Espelie et al., 1991). In any case, such compounds could influence the behavior of foraging predators. It is even reported that the lipids of the seed and the fruit of apples (*Malus pumila* Mill.) are carried through to the lipids of the herbivore *Cydia pomonella* L. and further to its parasitoid *Ascogaster quadridentata* Wesmael (Espelie and Brown, 1990).

As mentioned above, the plant surface is capable of adsorbing and absorbing the vapors of organic compounds in the adjacent air layer. Sorption is enhanced by the unstirred air layers covering the plant surfaces. As a result, compounds of a herbivore that adsorb to or absorb in the plant surface can function as delayed signals for conspecifics. For example, the glandular defense secretion, (epi)chrysomelidial, of the mustard leaf beetle larvae of *Phaedon cochleariae* (F.) adheres to the leaf surface (Rostás and Hilker, 2002). Females of this species are deterred from feeding and oviposition by these secretions.

Epicuticular waxes also influence locomotion of herbivorous insects on a plant by determining the ability of tarsae to adhere to the surface (Woodhead and Chapman, 1986; Edwards and Wanjura, 1990; Eigenbrode, 1996). For example, epicuticular wax blooms can reduce the attachment forces of the mustard leaf beetle (Stork, 1980). Thus, glossy plants are often more heavily infested by herbivores than waxy ones, an important fact in choosing agricultural cultivars (Bodnaryk, 1992). The pedicles of flowers can be coated with "antirobbing'' devices, called the "greasy pole syndrome,'' thus preventing insects from reaching the reproductive tissue (Kerner, 1879; Juniper, 1995).

Interactions with Carnivorous Insects. Just as herbivores, which use visual cues that are characteristic of the plant surface, carnivorous insects also can respond to such cues for locating plants with potential prey. Recently, it was demonstrated that the parasitoid *Pimpla turionellae* (L.) can perceive changes in chromatic and achromatic plant surface characteristics evoked by infestation with endophytic hosts (Fischer et al., 2004).

Compounds from a herbivore that adsorb to the plant surface may also serve as host-finding kairomones for carnivorous arthropods. The sex phero-

mone of the moth *Mamestra brassicae* (L.) adsorbs on Brussel sprout leaves and increases the attractiveness for foraging females of *Trichogramma* egg parasitoids (Noldus et al., 1991). Small amounts of an unusual class of triacylglycerol were found on leaves visited by raspberry aphids. These compounds were derived from the aphid and probably arose from the presence of shed aphid skins on the leaf surface or by incorporation of cornicle secretions into the leaf wax (Shepherd et al., 1999a,b). Carnivores of aphids might use such cues for host exploitation.

Lipids of the plant bound to the herbivore (as explained above) might also be important in host finding for predators or parasitoids (Espelie and Bernays, 1989; Espelie and Brown, 1990; Espelie et al., 1991). Changes in surface chemistry induced by endophytic hosts such as leaf miners can help the parasitic wasp *Pholetesor bicolor* Ness (Hymenoptera: Braconidae) in host location (Dutton et al., 2002).

As for herbivorous insects, attachment of tarsae on plants is of primary importance for carnivorous insects. Adhesion of tarsae and thus predation efficiency is usually higher on plants with reduced amounts of epicuticular crystals, the same conditions that favor attachment of the prey (Eigenbrode, 1996; Eigenbrode and Kabalo, 1999; Eigenbrode and Jetter, 2002). This was demonstrated not only in the laboratory but also in field studies (Rutledge et al., 2003). Waxy blooms also influence parasitoids and can cause them to spend more time foraging on reduced epicuticular crystal cultivars (Chang et al., 2004). However, not all predators are impaired by epicuticular crystals (Eigenbrode et al., 2000). Different parasitoids of the same host insect are more or less sensitive to wax covers of the host plant (McAuslane et al., 2000). Therefore, the specific interactions of predators, parasitoids, and prey on crop plants need to be considered in planting strategies. Attachment of insects when they visit a plant has been elegantly quantified using centrifugal devices (Eigenbrode et al., 1999b; Federle et al., 2000; Eigenbrode and Jetter, 2002). For a review on the effects of epicuticular wax bloom on attachment and efficiency of predatory insects, we refer the reader to Eigenbrode (2004).

On *Macaranga* myrmecophytes, the epicuticular wax crystals allow only select symbiotic ants to walk on the stems. Thus, specialized ant species are protected mechanically against competitors that cannot climb the stems (Federle et al., 1997, 2000). The various physical properties of crystalline wax blooms of *Macaranga* species can be correlated to differences in chemical composition of the epicuticular triterpenoid blooms (Markstädter et al., 2000).

Cuticle Function in Carnivorous Plants. For pitcher plants that use insects as nitrogen and phosphorus sources, the structure of the epicuticular wax crystals within the trap plays an important role in preventing attachment of the

prey and thereby impairing escape from the trap (Knoll, 1914). Pitcher plants have a striking diversity of wax covers in the various zones of the pitcher. Peristomes of *Nepenthes* spp. traps are very slick for insects. Their slippery nature is due to water lubrication and anisotropic surface morphology. These physical properties lead to aquaplaning of a visiting insect, such as an ant (Bohn and Federle, 2004). The chemical composition of the surface of the peristomatal area is still not clear. In contrast, the chemical composition of the wax crystals in the zone below the peristome, the inner pitcher walls in the conductive zone, is mainly characterized by a polymer of C_{30} aldehydes. The exact mechanism by which this layer retains the insect prey within the pitcher has yet to be elucidated (Riedel et al., 2003). It has been conclusively shown that waxes do not play a role in the initial falling of the insect into the pitcher, but serve to retain caught insects within the trap (Gaume et al., 2002).

CONCLUSIONS

It is clear that the physicochemical and (eco)physiological characteristics of primary plant surfaces play a major role in chemical ecology. Our special reference to insect–plant relations allows us to reach general conclusions that are relevant to interactions of plants with a variety of organisms.

First, the physicochemical properties of the cuticle determine the wettability of a plant and its optical appearance. Furthermore, they influence the solubility as well as transport properties of molecules toward and away from the plant surface. These properties impact autecological features of the plant as well as interactions with other organisms (microbes, insects) that depend on first contact with the outermost surface.

Another important feature of the plant cuticle is the repellence of water, aqueous solutions, polar mucilages, and organisms with polar surfaces. The same physicochemical properties are involved in hindering attachment of microorganisms, herbivores, and carnivorous insects, as well as insect prey of carnivorous plants.

The presence or absence of chemicals in the epicuticular wax layer is determined primarily by their lipophilicity (1-octanol/water partition coefficient or aqueous solubility) and, in the case of volatiles, their saturation vapor pressure. Simple equations may be used to estimate the sorption in and diffusion of chemicals through the cuticle, and, thus, to assess the tendency of secondary metabolites to appear on plant surfaces. Polar and/or charged solutes that are readily soluble in water will not generally be found in or on the cuticle. Highly volatile, small substances will also not be present on primary plant surfaces for prolonged periods of time because they easily escape from the cuticle.

Microorganisms such as fungi may adopt hydrophobic qualities to enable them to attach to the surface. Furthermore, cuticular waxes can serve as signals in spore germination and growth of specific fungi, although secondary compounds on the surface may often have antimicrobial properties against an array of nonadapted organisms.

After approaching and landing on a plant, based on its optical characteristics and volatiles, an insect's behavior is influenced by water, primary and secondary compounds, and lipids present on the epicuticular waxes. However, only a limited range of compounds can be present at this outermost layer, and the waxes can even impair attachment as well as locomotion of insects to various degrees.

Methods are available for analyzing the composition of epicuticular separately from intracuticular waxes. These novel methods may now be used to clarify prior findings and to better understand the mechanisms whereby chemical surface cues may influence microorganism development or insect behavior.

The presence of chemical cues in or on the epicuticular layer does not only depend on the physical structure and chemical composition of the waxes *per se*. They may also result from transport of endogenous primary and secondary metabolites to the surface. In addition, exogenous volatiles or solutes adsorbed to the surface can serve as allelochemicals in the long and short range. The role of waxes as absorbents and release agents for biologically active material in chemical ecology has been underappreciated. Clearly, the interaction of epicuticular wax components with exogenous and endogenous substances makes the plant surface a chemical mosaic of signals that may evoke complex reactions in potential receivers such as insect herbivores, carnivores, or microorganisms.

The importance of plant surface properties as they relate to interactions with other organisms points to the need for future studies to focus on the role of the plant cuticle in chemical ecology. Present knowledge is based on the study mostly of crop plants and their pests. Such research should be expanded to include interactions of plants with their environments in natural ecosystems. In addition, the effects of changes in plant surface chemistry due to herbivore feeding or other stress factors may reveal additional information about the dynamics of surface chemistry in nature.

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CHANGES IN HEARTWOOD CHEMISTRY OF DEAD YELLOW-CEDAR TREES THAT REMAIN STANDING FOR 80 YEARS OR MORE IN SOUTHEAST ALASKA

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Abstract—We measured the concentrations of extractable bioactive compounds in heartwood of live yellow-cedar (*Chamaecyparis nootkatensis*) trees and five classes of standing snags (1–5, averaging 4, 14, 26, 51, and 81 years-since-death, respectively) to determine how the concentrations changed in the slowly deteriorating snags. Three individuals from each of these six condition classes were sampled at four sites spanning a 260-km distance across southeast Alaska, and the influence of geographic location on heartwood chemistry was evaluated. Cores of heartwood were collected at breast height and cut into consecutive 5-cm segments starting at the pith. Each segment was extracted with ethyl acetate and analyzed by gas chromatography. Concentrations of carvacrol, nootkatene, nootkatol, nootkatone, nootkatin, and total extractives (a sum of 16 compounds) for the inner (0–5 cm from pith), middle (5–10 cm from pith), and surface (outer 1.1–6.0 cm of heartwood) segments from each core were compared within each tree condition class and within segments across condition classes. Heartwood of class 1 and 2 snags had the same chemical composition as live trees. The first concentration changes begin to appear in class 3 snags, which coincides with greater heartwood exposure to the external environment as decaying sapwood sloughs away, after losing the protective outer bark. Within core segments, the concen-

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trations of all compounds, except nootkatene, decrease between snag classes 2 and 5, resulting in the heartwood of class 5 snags having the lowest quantities of bioactive compounds, although not different from the amounts in class 4 snags. This decline in chemical defense is consistent with heartwood of class 5 snags being less decay-resistant than heartwood of live trees, as observed by others. The unique heartwood chemistry of yellow cedar and the slow way it is altered after death allow dead trees to remain standing for up to a century with a profound impact on the ecology of forests in southeast Alaska where these trees are in decline.

Key Words—*Chamaecyparis nootkatensis*, snags, decay resistance, chemical defense, carvacrol, nootkatin, antifungal compounds.

INTRODUCTION

Yellow cedar, *Chamaecyparis nootkatensis* (D. Don) Spach, is a socially, ecologically, and economically valuable tree in southeast Alaska. Its wood is highly desired in foreign markets, especially Japan. Around 1880, yellow cedar growing near open bogs, or on semibog sites with poor drainage, began to die, initiating a forest decline that continues to this day (Hennon et al., 1990a). Circumstantial evidence suggests that this decline was triggered by climatic warming (Hennon and Shaw, 1994), with minimal involvement of biotic agents (Hennon, 1990; Hennon et al., 1990b,c,d). Interactions of site characteristics with maritime-continental climate patterns are associated with tree mortality (Hennon and Shaw, 1997) that has left standing dead trees (snags) on more than 200,000 ha widely spread across the landscape. Yellow-cedar trees can remain standing for up to a century after their death because of the heartwood's natural durability and superior strength. As a consequence of this slow deterioration and the progressive nature of the decline, standing snags accumulate on affected sites and can average about 65% of the yellow-cedar basal area (Hennon et al., 1990c). Although live trees are commercially valuable, snags have been used primarily for firewood. With snags scattered over large distances and with limited road access, their commercial salvage is not economically justified unless their wood properties are comparable with those of live trees.

In an attempt to develop products with greater value, investigators compare the various heartwood characteristics of snags with those of live yellow-cedar trees. Snags can be visually sorted into six classes with increasing mean time-since-death of 4, 14, 26, 51, and 81 years, respectively (class 6 not dated; Hennon et al., 1990c) by evaluating their retention of foliage, twigs, and branches. By class 6, the heartwood is decaying and the bole is broken, often close to the ground. Mechanical properties of snag heartwood do not change much through class 5 (McDonald et al., 1997; Green et al., 2002), but there is a modest reduction in recoverable wood and grade from snags in classes 4 and 5,

compared with live trees and younger snags (Hennon et al., 2000). Decay resistance of heartwood from class 5 snags is adequate for products used above ground, but may be less durable than the heartwood from live trees and younger snags when in contact with soil (DeGroot et al., 2000).

Heartwood durability and decay resistance in yellow cedar have been attributed to the tropolone, nootkatin, that inhibits growth of various wood decay fungi (Rennerfelt and Nacht, 1955). Reducing its concentration in the heartwood decreases the wood's resistance to decay (Smith, 1970; Smith and Cserjesi, 1970). Carvacrol is another major component in yellow-cedar heartwood with antifungal properties (Voda et al., 2003). Because the heartwood of class 5 snags is less resistant to decay than heartwood from live trees and younger snags, it is likely to have lower concentrations of one or more of these bioactive compounds. The objective of this study was to measure the concentrations of carvacrol, nootkatin, and other extractable constituents in heartwood of live yellow-cedar trees and the five classes of snags to determine how they changed over the course of 80 years or more, in the slowly deteriorating, standing dead trees. Live trees and snags were sampled at each of four sites spanning a 260-km distance across southeast Alaska to evaluate whether geographic location had an influence on heartwood chemistry.

METHODS AND MATERIALS

Study Sites. Live and dead yellow-cedar trees were sampled at four locations in southeast Alaska (Figure 1): Goose Cove, Baranof Island (57°30′35N, 135°30′48W); Halibut Point near Sitka, Baranof Island (57°05′26N, 135°22′43W); Nemo Point, Wrangell Island (56°16′58N, 132°19′38W); and Sal Creek, Prince of Wales Island (55°48′41N, 132°32′13W). Each of these locations represents forests typical of yellow-cedar decline.

Tree and Snag Selection. Three dead trees from each of the five snag classes and three live trees were selected for sampling at each of the four study sites, yielding a total of 72. Snag classes were identified by their retention of dead foliage, twigs, and secondary or primary branches as described by Hennon et al. (1990c). Diameters of each bole were measured at breast height (dbh at 1.4 m) on the uphill side. All trees and snags sampled at a site were in close proximity to one another; most were separated by less than 100 m and none more than 200 m apart. They were sampled as encountered and discarded as unacceptable if decay prevented the removal of a continuous heartwood core to the pith.

Heartwood Samples. Two cores of heartwood extending to the center of the live tree or snag were removed with an increment borer (5.0 mm i.d.) on opposite sides of the bole at breast height. The boundary between sapwood and

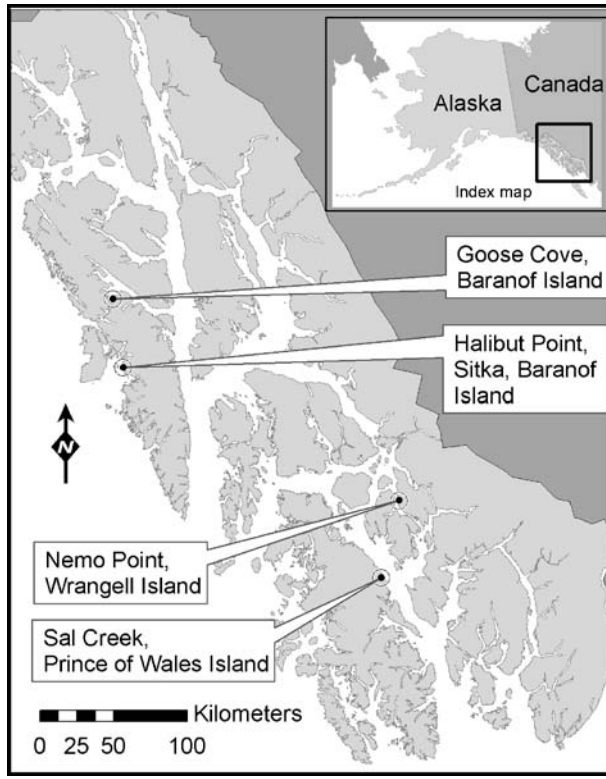


FIG. 1. A map showing the four sampling sites of yellow cedar in southeast Alaska.

heartwood was marked on cores from live trees and snags from classes 1 to 3 to assist the removal of sapwood in the laboratory. The pale yellow heartwood of yellow cedar is distinguishable from the white sapwood of live trees and black-stained or brown decayed sapwood of dead trees. Residual sapwood on class 4 snags was minimal (mean of 2–3 mm; Hennon et al., 2000) and readily detectable without marking. There was no sapwood remaining on class 5 snags. Each core was sealed in a plastic straw with tape (two straws for longer cores), placed inside a sealed plastic bag, and stored on ice in the field. Upon returning to the laboratory, cores were frozen at −36°C until analyzed.

Solvent Extraction. One increment core from each live tree or snag was removed from the freezer in random order and processed for extraction in a cold room at 7 ± 1°C. Cores were cut into consecutive 5.0-cm segments starting at the pith and progressing outward. The outermost heartwood segment was cut at the sapwood boundary (sapwood discarded) or terminated at the bole surface for

some class 4 and all class 5 snags. The two innermost segments from 0 to 5 and 5 to 10 cm from the pith are referred to as the inner and middle segments, respectively. The outermost or newest heartwood is referred to as the surface segment because it included the outer layer of heartwood left exposed after the sapwood had weathered away in class 4 and 5 snags. This surface segment varied in length. When greater than 1.0 cm, it was processed and extracted separately, but when 1.0 cm or less, it was combined with the previous segment yielding lengths ranging from just over 1.0 cm (11 surface segments between 1.0 and 1.9 cm) up to 6.0 cm. This resulted in seven small diameter individuals (one live tree, one class 1, two class 3, and three class 4 snags) having only two segments (inner and surface).

Each segment was sliced into disks 0.5–1.0 mm in thickness with a razor blade. The bulk of each sample was transferred to a preweighed vial (15-ml vol.) sealed with a Teflon®-lined screw cap for extraction. A small subsample was sealed in a preweighed 4-ml vial to measure the tissue water content. Both vials were adjusted to room temperature before recording tissue fresh weight. Four milliliters of ethyl acetate (Fisher Scientific, HPLC grade) were added to vials containing 3- to 6-cm length segments, whereas vials containing 2- to 3- or 1- to 2-cm segments were extracted with 2.0 and 1.0 ml of solvent, respectively. They were allowed to soak at room temperature (22 ± 1°C) for 7 d in the dark. Each extract was transferred by pipette into a 4-ml sealed vial containing anhydrous sodium sulfate (Mallinckrodt reagent grade, 0.68, 0.34, and 0.17 g/ 4-, 2-, and 1-ml solvent, respectively) to remove excess water. These were stored in a dark cold room until further processed for analysis. Just prior to analysis, 900 μl of room temperature extract were transferred into a 1.5-ml autosampler vial followed by 100 μl of ethyl acetate containing *R*-(+)-limonene (Aldrich, 99.7% purity) as the internal standard. Subsamples for water content measurements were dried at 102°C for 16 hr, then cooled to room temperature in a desiccator box before weighing.

Chromatography. Extracts were analyzed by gas chromatography using a Hewlett Packard 5890 Series II gas chromatograph (GC) equipped with a 6890 autosampler, flame ionization detector, and J&W Scientific DB-5 column (30 m × 0.25 mm, 0.25-μm film thickness). Helium was the carrier gas set at 1.0 ml/min flow through the column at the initial 100°C column temperature, with a split ratio of 1:10. The column oven was held for 1.0 min at 100°C, then increased to 150°C at 5°C/min, and then to 220°C at 3°C/min with no final temperature hold. Two microliters of extract were injected. Compound concentrations were calculated from a six-level standard curve of carvacrol (Sigma, 99%), with a limonene (Aldrich, 99.7%) internal standard at the same concentration as added to the extracts. All compound concentrations were calculated using the same response factor as carvacrol and normalized per gram dry weight using tissue water content measurements. Preliminary analysis indicated

that the response factors for carvacrol and nootkatin differed by less than 5%. Compounds were identified by GC–mass spectrometry using the same GC with an HP 5970 mass selective detector and a Phenomenex ZB-5 column (equivalent to the DB-5), with the oven conditions as above. Compounds were identified by comparison of their spectra with those in the spectral library or spectra obtained from authentic samples analyzed with this instrument.

Statistical Analyses. This sampling scheme is considered a balanced generalized randomized block because in all four blocks (sites), each of the six tree condition classes (live trees plus five snag classes) or treatments was independently received by three experimental units (trees or snags; Steele et al., 1997). This design allowed us to test whether any block (site) × tree condition class interactions were apparent. Each tree was repeatedly measured, once in each of the three heartwood core segments. Pearson's correlation coefficients among concentration estimates for the heartwood compounds provided information regarding the independence or correlation among these responses.

To assess whether there were systematic differences in dbh among snag age classes, the following one-way ANOVA model was fit to the data using SAS v8.2 PROC Mixed,

$$Y_{ijk} = m + \beta_i + a_j + \alpha_{ij} + \lambda_{ijk}$$

where Y_{ijk} is the dbh of the kth tree in the jth tree condition class in the ith block; m is the overall mean dbh for all trees; β_i is the random effect of block i ($i = 1, 2, \ldots, 4$); a_j is the effect of the jth tree condition class (j = live tree, snag class $1, \ldots, 5$); α_{ij} is the random error term that represents variability among sets of k trees ($k = 1, 2, 3$) from the same tree condition class among the blocks and is the error term used to test for condition class effect, $\alpha_{ij} \sim N(0, \sigma^2_\alpha)$; and λ_{ijk} is the random error term that represents variability among the k trees of the same tree condition class within the blocks, $\lambda_{ijk} \sim N(0, \sigma^2_\lambda)$, and is the error term used to test for site × tree condition class interaction.

The above model was expanded to model the concentration responses for the compounds described below using SAS v8.2 PROC Mixed (SAS Institute Inc., 1999),

$$Y_{ijkl} = m + \beta_i + a_j + \alpha_{ij} + \lambda_{ijk} + c_l + ac_{jl} + \varepsilon_{ijkl}$$

where Y_{ijkl} is the concentration of the compound of interest in the lth core segment of the kth tree in the jth tree condition class in the ith block; m, β_i, a_j, α_{ij}, and λ_{ijk} are defined as above; c_l is the effect of the lth core segment (l = inner, middle, surface); ac_{jl} is the interaction effect of the jth level of tree condition class and the lth core segment; and ε_{ijkl} is the random error term that

represents variability among core segments and is the error term used to test for effects of core segment and tree condition class by core segment interaction,

$$\varepsilon_{ijk} \sim \text{MVN}\left(\begin{pmatrix}0\\0\\0\end{pmatrix}, \begin{pmatrix}\sigma_{11}^2 & \sigma_{12}\sigma_{13}\\ \sigma_{12}\sigma_{22}^2\sigma_{23}\\ \sigma_{13}\sigma_{23}\sigma_{33}^2\end{pmatrix}\right)$$

and represents the covariance among core segments within a tree.

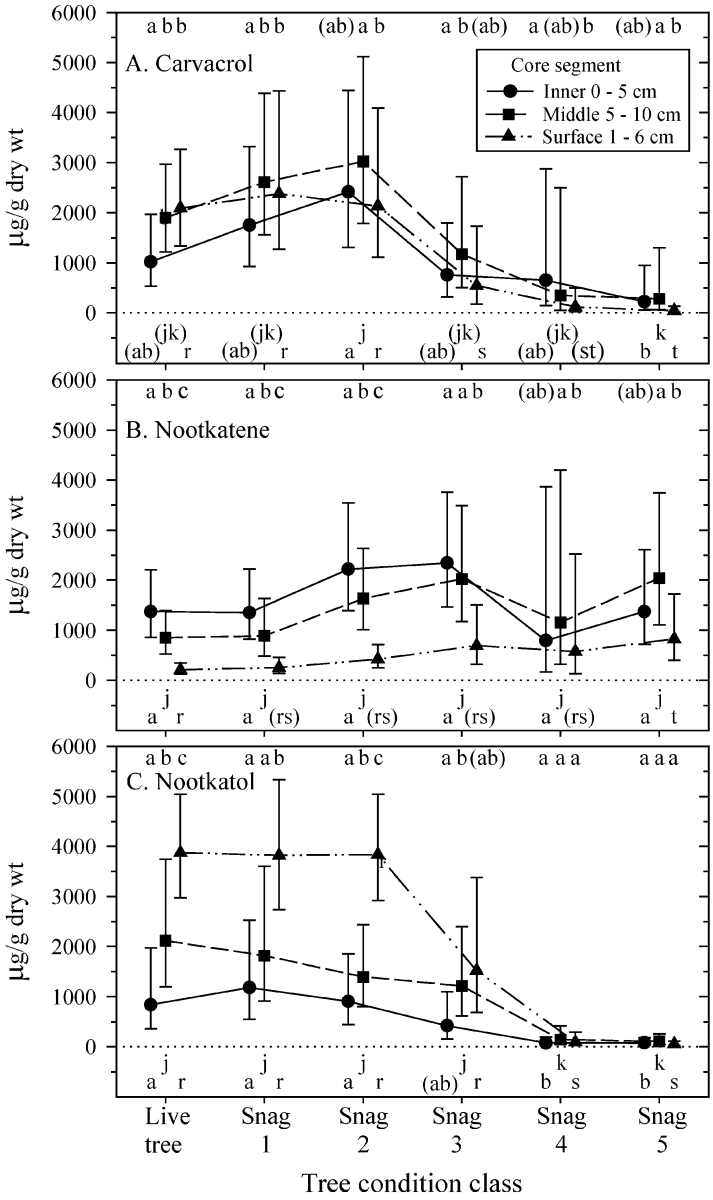
A heterogeneous variance model was fit allowing different estimates of the values of this covariance matrix for each tree condition class. The appropriate within-tree covariance structure was selected using the minimum Akaike's information criterion (AIC). All individual compounds had a fully unstructured covariance [UN(3) model] with unequal variance among core segments and unequal covariances between segments. The total extractives had a much simpler within-tree covariance model [TOEP(1) model] with equal variance among core segments and covariance among segments estimated to be 0. Each compound response was also modeled with dbh as a covariate.

Scatter plots of the initial data for each compound revealed a few samples with excessively high values (5–10 times the mean) that were removed as outliers. Homogeneity of variances and normality were evaluated prior to analysis by examining plots of the residuals (observed vs. predicted) and normal probability plots. All five individual compounds (but not the total) required natural logarithm transformation to meet these assumptions. For carvacrol, nootkatene, and nootkatol (all with interactions between core segments and tree condition class), the differences among means (on the log scale) of core segments within tree condition classes, and differences among tree condition classes within segments, were determined with pairwise comparisons using Bonferroni adjusted confidence intervals (CIs). For nootkatone and nootkatin (on the log scale), and the total extractives (all without interactions between core segments and tree condition classes), the differences among means of the tree condition classes were determined by pairwise comparisons using Tukey 95% CIs. Transformed means and their 95% confidence limits were back-transformed to medians for presentation.

An F statistic, treating block as a fixed effect, was calculated to assess effectiveness of blocking and site-specific differences in average response for all response variables, including dbh.

RESULTS

Sixteen compounds were selected for quantitation in the ethyl acetate extracts of yellow-cedar heartwood. Based on the results from the GC-MS analysis, including spectral comparisons with some authentic samples recently


A. Carvacrol
B. Nootkatene
C. Nootkatol
Core segment
Inner 0 - 5 cm
Middle 5 - 10 cm
Surface 1 - 6 cm
µg/g dry wt
Live tree
Snag 1
Snag 2
Snag 3
Snag 4
Snag 5
Tree condition class

isolated by Xioung (2000) or Khasawneh (2003), four compounds were identified as monoterpenes (3-carene, 4-terpineol, methyl carvacrol, and carvacrol), eight were sesquiterpenes (valencene, nootkatene, δ-cadinene, epi-nootkatol, nootkatol, valencene-13-ol, nootkatone, and valencene-11,12-diol), one was a tropolone (nootkatin), and three were unknown. Carvacrol, nootkatene, nootkatol, nootkatone, and nootkatin were selected for individual analysis (Figures 2 and 3) because of their biological activities, abundance, or chemical reactivity. A total extractives concentration using the combination of all 16 compounds was also analyzed (Figure 4). It is important to note that concentrations of the five individual compounds analyzed and the total extractives were related with one another as indicated by Pearson's correlation coefficients all greater than 0.62, except for nootkatene, whose coefficients with other compounds never exceeded 0.28. Differences in carvacrol, nootkatene, or nootkatol concentrations among core segments (Figure 2) were dependent on the tree condition class (significant interactions; Table 1), whereas concentrations of nootkatone and nootkatin (Figure 3) were dependent only on the tree condition class, with no differences among core segments within any of the condition classes (no interaction; Table 1).

Within live trees and class 1 snags, carvacrol concentrations in the inner core segments were significantly less than in the middle and surface segments (Figure 2A). This pattern began to reverse in class 2 snags resulting in surface core segments having lower concentrations than either the inner or middle segments, in all older snag classes. Within the surface core segments, carvacrol was significantly lower in class 5 snags than in live trees and all younger snags and significantly lower in class 4 than in live trees or class 1 and 2 snags. Concentrations of carvacrol within inner and middle core segments were significantly higher in class 2 snags than those in class 5.

Nootkatene concentrations within the middle and inner core segments were not different among any of the tree condition classes (Figure 2B). Quantities within the surface segments increased progressively starting in class 1, and by class 5, the concentrations were significantly higher than in live trees, with no other differences among classes. Within each tree condition class, the surface

FIG. 2. Median concentrations (±95% CIs) for the individual compounds carvacrol (A), nootkatene (B), and nootkatol (C) extracted with ethyl acetate from yellow-cedar heartwood of live trees and snags. Magnitudes of the CIs are indicative of the tree-to-tree variation about medians and are not accurate measures for identifying significant differences between medians. Within each tree condition class, the medians of core segments with similar letters above their symbols at the top of each graph are not significantly different. Within each core segment, the medians among tree condition classes with similar letters below their symbols at the bottom of each graph are not significantly different. In both instances, $\alpha = 0.05$.

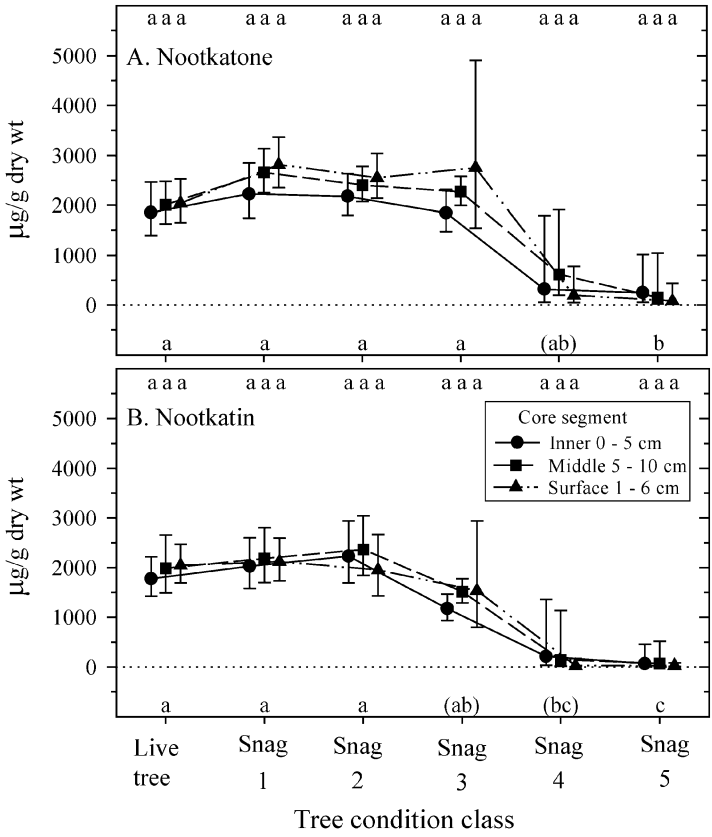


FIG. 3. Median concentrations (±95% CIs) for the individual compounds nootkatone (A) and nootkatin (B). The CIs and letters are the same as in Figure 2.

core segments contained significantly less nootkatene than the middle segments, and the inner segments in live trees and snags from classes 1 to 3. For live trees and class 1 or 2 snags, the inner core segments had significantly more nootkatene than the middle segments, with no differences between them in class 3–5 snags.

Nootkatol levels within live trees were highest in the surface core segments, followed by the middle and inner segments, with all differences being significant (Figure 2C). These relationships remained until class 3 when quantities generally declined, especially in the surface segments (but not significantly from levels in class 2). By class 4, nootkatol concentrations were the same in all core segments and significantly lower than in live trees and all previous snag classes. There were no differences in concentrations within segments or among segments for snags in classes 4 and 5.

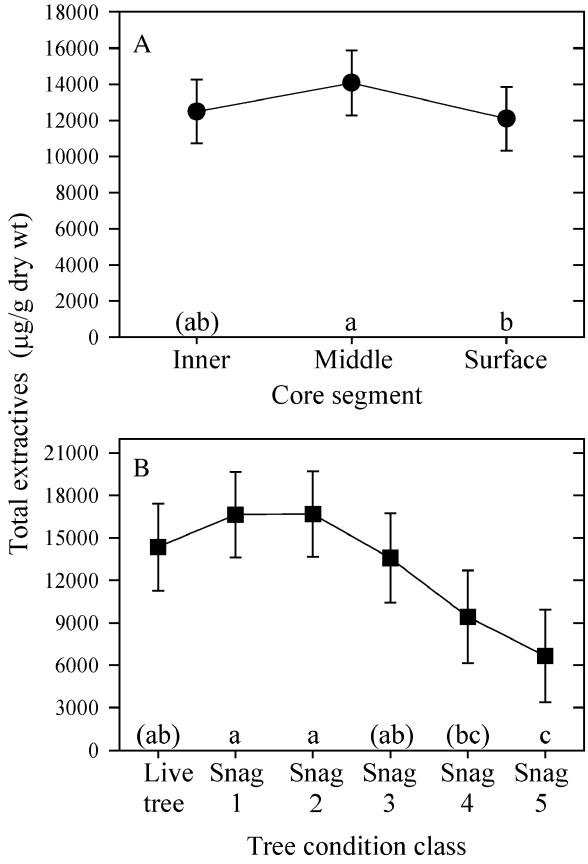


FIG. 4. Mean total extractives (±95% CIs) for 16 compounds removed with ethyl acetate from heartwood of live trees and snags of yellow cedar collected across southeast Alaska shown separately for core segments (A) and tree condition classes (B) because their interaction was not significant (Table 1). Means with the same letters at the bottom of the graph are not significantly different; $\alpha = 0.05$.

Nootkatone concentrations within all tree condition classes exhibited no significant differences among core segments (Figure 3A). Concentrations within segments remained unchanged from live trees to class 3 snags, followed by a nonsignificant decline in class 4. Class 5 snags had significantly less nootkatone than any of the more recent snags, except class 4.

Nootkatin exhibited a response similar to nootkatone, with no differences for core segments within any of the condition classes (Figure 3B). Within segments, the concentrations began to drop in class 3 rather than class 4.

TABLE 1. ANOVA RESULTS FOR EACH INDIVIDUAL COMPOUND AND THE TOTAL EXTRACTIVES FROM YELLOW-CEDAR HEARTWOOD

		Carvacrol		Nootkatene		Nootkatol		Nootkatone		Nootkatin		Total	
Factor in model	*df*[a]	*F*	*P*-value	*F*	*P*-value	*F*	*P*-value	*F*	*P*-value	*F*	*P*-value	*F*	*P*-value
Tree condition class (Tcc)	5, 15	6.51	0.002	2.30	0.097	22.58	<0.001	5.55	0.004	9.26	<0.001	8.75	<0.001
Core segment (Cs)	2, 173[b]	8.28	<0.001	103.64	<0.001	19.43	<0.001	1.41	0.246	2.87	0.059	4.68	0.011
Tcc × Cs	10, 173[b]	4.76	<0.001	4.32	<0.001	2.53	0.007	1.12	0.352	1.41	0.180	1.60	0.115

[a] Degrees of freedom for the numerator, denominator.
[b] Due to some missing values and removal of outliers, the denominator *df* is 172 for nootkatol, 171 for nootkatone and nootkatin, and 113 for the total extractives, which is much lower than for the individual compounds because of the different covariance structure used in the analysis.

Nootkatin concentrations in class 5 snags were significantly lower than all but class 4, and those in class 4 were lower than in live trees and class 1 or 2 snags.

Concentrations of the total extractives varied among core segments (Figure 4A), and among tree condition classes (Figure 4B), but without an interaction between them (Table 1). Middle core segments had significantly higher concentrations than the surface segments (Figure 4A). The class 4 snags had significantly lower amounts than class 1 and 2 snags, and the quantities in class 5 snags were lower than live trees, and all snag classes, except class 4 (Figure 4B).

The F statistic analysis showed that there were no differences among sites (all P values > 0.09) for the median concentrations of any compound or dbh. The analysis of variance (ANOVA) of compound concentrations with dbh as a covariate did not significantly reduce the variance, and the results were essentially unchanged from the ANOVA without dbh as a covariate. There was some evidence ($F_{5,15} = 2.57$, $P = 0.072$) that class 5 snags were smaller in diameter (averaging 25.6–33.2 cm dbh) than live trees or any of the other snag classes (averaging 31.2–41.3 cm dbh), with no differences among any of the latter.

DISCUSSION

Changes in compound concentrations in heartwood of yellow-cedar snags are associated with major physical changes of the boles as these standing dead trees deteriorate. Class 1 snags have intact outer bark and stained sapwood, whereas class 2 snags have sloughing bark and decaying sapwood. These changes, however, have minimal impact on heartwood chemistry, as the concentrations of most compounds in these snags remained the same as in live trees (Figures 2, 3, and 4B). By snag class 3, the outer bark is gone, the decaying sapwood is starting to slough away, and the heartwood surface begins to experience direct exposure to the external environment. This stage, some 25 years after tree death, marks the first substantial changes in heartwood chemistry. Because the outer heartwood is first exposed in class 3 snags, greater rates of chemical change might be expected to occur in the surface core segments compared with the middle and inner segments. This was observed, but only for nootkatol (Figure 2C). Tree to tree variation (as indicated by the larger confidence intervals) within the surface segments was greater in class 3 snags than in live trees or most younger snags for all individual compounds, except carvacrol. This probably reflects the variation in rates of sapwood decay and sloughing among class 3 snags, which results in different levels and durations of heartwood exposure to the environment. By snag class 4, all of the bark and most of the sapwood are gone from the bole, with checks or cracks penetrating about 3 cm deep into the heartwood (Hennon et al., 2002). Concentration changes that begin to appear in class 3 snags continue into class 4. Class 5 snags have no bark or sapwood

remaining and have deeper heartwood cracks (4 cm or greater) than in class 4. Heartwood of class 5 snags typically has lower concentrations of each compound (excluding nootkatene; Figure 2B) and total extractives than all younger snag classes, except class 4. Geographic location of the live trees and snags had minimal influence on their heartwood chemistry, probably because environmental parameters among the sites sampled in southeast Alaska were similar.

Potential mechanisms contributing to the concentration changes of heartwood components in aging yellow-cedar snags (Figures 2 Figures 3 Figures 4) are volatilization to the atmosphere, leaching, structural changes from reactions such as dehydration or oxidation, and possibly polymerization. As the heartwood surface becomes exposed to the elements, all of the compounds could be lost to some degree by volatilization, similar to the emissions of monoterpenes from the boles of lodgepole pine, *Pinus contorta murrayana* (Grev. and Balf.) Engelm. (Rhoades, 1990). Carvacrol is the most volatile of the individual compounds, and was likely impacted more by this mechanism than the other compounds, and partially responsible for the lower quantities measured in surface cores of class 4 and 5 snags with cracked and exposed heartwood. The forests of southeast Alaska are humid and wet (150- to 500-cm annual precipitation; Harris et al., 1974), so temperature and humidity both may function as the drivers of heartwood emissions to the atmosphere (Schade et al., 1999). Although these heartwood compounds all have limited water solubility, they could be gradually leached from the outermost layers of tissues exposed for many years to heavy rainfall.

Structural changes could be responsible for the decline of nootkatol, as a simple and facile dehydration reaction will yield nootkatene. Evidence supporting this rearrangement occurs in the heartwood of live trees, where the concentrations (transformed on the natural log scale) of these two compounds exhibit a nonlinear, inverse relationship (cubic polynomial; $R^2 = 0.567$). In addition, shifts in their relative concentrations appear to be associated with heartwood age. For example, surface core segments represent the youngest heartwood and contain 18.7 times more nootkatol than nootkatene (Figure 2B and C). In older, middle core heartwood, the concentrations of nootkatol are lower and the concentrations of nootkatene are higher than in surface core segments, resulting in nootkatol being only 2.5 times greater than nootkatene. The oldest heartwood from inner core segments of live trees contains the lowest nootkatol and highest nootkatene among the three segments, making the nootkatene 1.6 times more abundant than nootkatol. Loss of nootkatol from the surface cores is accelerated as the heartwood becomes more exposed between snag classes 2 and 4. Whereas nootkatene concentrations do increase in the surface core segments between these snag classes, the amount accumulated represents only a fraction of the nootkatol lost during the same period. Thus, nootkatol in exposed heartwood may be also changing to products other than

nootkatene (e.g., oxidation to nootkatone) or volatilizing to the atmosphere. Alternatively, nootkatene could be the initial rearrangement product of nootkatol, but is probably a transient intermediate because of the reactive conjugated double-bond system that is easily oxidized, thus minimizing the accumulation of nootkatene.

Longevity of yellow-cedar trees and the long persistence of standing snags in southeast Alaska are attributed to compounds in the heartwood functioning as a defense against disease and insects, with resistance to decay credited primarily to nootkatin, shown to have fungicidal activity at relatively low concentrations. This chemical defense also contributes to the durability of yellow-cedar wood used as lumber or other building materials (Rennerfelt and Nacht, 1955). Removal of nootkatin by the growth of a black-stain fungus, an organism that invades the wood of live trees and degrades this compound, dramatically decreased the resistance of the stained wood to decay fungi (Smith, 1970; Smith and Cserjesi, 1970). But other compounds also contribute to yellow-cedar durability. The antifungal properties of carvacrol were reported by Anderson in 1961 (cited in Barton, 1976), and it was one of the most active growth inhibitors of 22 compounds tested against the wood-decaying white rot, *Trametes versicolor*, and brown rot, *Coniophora puteana* (Voda et al., 2003). It also inhibits mycelial growth of various species of plant pathogens in the genus *Fusarium* (Thompson, 1996). Carvacrol is bactericidal toward a variety of human pathogens commonly found on meats, vegetables, rice, and dairy products (Ultee et al., 1999; Knowles and Roller, 2001; Friedman et al., 2002; Burt, 2004). Chamic acid and isochamic acid are two other monoterpenes that may contribute to decay resistance of yellow-cedar heartwood, but were not detected in our analysis because of their polarity. They have only 1/10th or less the activity of nootkatin (Rennerfelt and Nacht, 1955).

Extracts of yellow-cedar heartwood and some of the individual compounds are also biologically active toward a variety of invertebrates. Nootkatone repels and inhibits tunneling of Formosan termites, *Coptotermes formasanus* Shiraki (Maistrello et al., 2001; Zhu et al., 2001). Yellow-cedar fiber used in producing wall paneling was not eaten by eastern subterranean termites, *Reticulitermes flavipes* (Kollar), in free choice tests with five other panel wood products and pine sapwood as alternative choices (Kard and Mallette, 1997). However, in no-choice tests, the yellow-cedar fiber was severely damaged. Essential oil from yellow-cedar heartwood, nootkatone, carvacrol, valencene-13-ol, and nootkatol all have insecticidal and/or acaricidal activity toward various agricultural, stored products, or medicinal arthropod pests (Panella et al., 1997; Ahn et al., 1998; Khasawneh, 2003). Carvacrol also has nematicidal properties (Oka et al., 2000).

Loss of the antifungal compounds as dead trees deteriorate, as shown here, is most likely responsible for the reduced decay resistance of heartwood from class 5 yellow-cedar snags. In laboratory tests against the brown-rot fungus,

Gloeophyllum trabeum Pers. Ex. Fr., heartwood from class 5 snags lost significantly more weight (56.3%) from fungal decay than heartwood from live trees (21%) or class 3 snags (12%, not significantly different from live trees), when collected from individuals with a large diameter (425–535 mm or 17–21 in.) (DeGroot et al., 2000). A similar response was observed for smaller diameter snags (300–400 mm or 12–16 in.), but the average weight loss for class 5 heartwood (29.2%) was not significantly greater than heartwood from similarly sized live trees (15.9%). In a separate study of decay resistance, heartwood was placed in contact with soil for up to 4 years. As in the laboratory study described above, the wood from snag class 5 had significantly greater weight loss (i.e., decay) than wood from class 3 snags and live trees, which did not differ (Hennon, unpublished data). Furthermore, DeGroot et al. (2000) observed substantial tree-to-tree variation in decay resistance during laboratory bioassays, which probably results, in part, from the large tree-to-tree variation in concentrations of the antifungal compounds, as observed here. Because the composition and concentrations of chemicals in yellow-cedar heartwood protect it from fungal decay, it might be feasible to evaluate and rank the level of resistance for live trees or snags by quantifying the concentrations of nootkatin, carvacrol, and possibly other compounds. Development of an accurate predictive model for this purpose would require further testing of heartwood samples for decay resistance in conjunction with a quantification of their chemical constituents, as undertaken here.

Collectively, the studies of standing dead yellow-cedar trees suggest that changes in heartwood chemistry precede other major changes in wood properties. As demonstrated in this study, the heartwood constituents remain largely unchanged from live trees until snag class 3, about 25 years after tree death. Initiation of chemical changes corresponds with the class of snags experiencing increased heartwood exposure to external environmental parameters that can expedite these changes. Concentrations of most compounds, including those with antifungal activity, began to decline in class 3 snags. This gradual reduction in chemical defense continues as the snags age and subsequently leads to a decline in heartwood decay resistance. Whereas heartwood of class 5 snags is less decay-resistant than heartwood of live trees (DeGroot et al., 2000; Hennon, unpublished data), the strength properties of heartwood at the class 5 stage, some 80 years after death, remain unchanged from live trees (McDonald et al., 1997; Green et al., 2002). The unique heartwood chemistry and the slow way in which it is altered after tree death have profound ecological and economic implications. Dead yellow-cedar trees across southeast Alaska remain standing as snags for up to a century, and because the heartwood stays strong, hard, and undecayed, they probably offer little in the way of habitat for cavity-nesting animals. This limited deterioration and surprising persistence of wood properties also offer opportunities for recovering valuable wood products.

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IMPACT OF PHENOLIC COMPOUNDS AND RELATED ENZYMES IN SORGHUM VARIETIES FOR RESISTANCE AND SUSCEPTIBILITY TO BIOTIC AND ABIOTIC STRESSES

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Abstract—Contents of phenolic compounds and related enzymes before and after sorghum grain germination were compared between varieties either resistant or susceptible to biotic (sooty stripe, sorghum midge, leaf anthracnose, striga, and grain molds) and abiotic (lodging, drought resistance, and photoperiod sensitivity) stresses. Independent of grain germination, sorghum varieties resistant to biotic and abiotic stresses had on average higher contents of proanthocyanidins (PAs), 3-deoxyanthocyanidins (3-DAs), and flavan-4-ols than susceptible varieties. Results show that content of 3-DAs is a good marker for sorghum resistance to both biotic and abiotic stresses because it correlates with resistance to all stresses except for photoperiod sensitivity. The second good marker for stress resistance is content of PAs. Total phenolic compounds and the activities of related enzymes are not good markers for stress resistance in sorghum grains.

Key Words—Sorghum, proanthocyanidins, 3-deoxyanthocyanidins, phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, stress, biotic, abiotic.

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INTRODUCTION

Sorghum [*Sorghum bicolor* (L.)] Moench is a C4 plant grass of the hot, semiarid tropics and genetically diverse. On the basis of genetic diversity, some sorghum varieties are more resistant to stresses than others (Tao et al., 1993, 2003; Deu et al., 1994). The constraints of sorghum production are both biotic and abiotic stresses. Among biotic stresses, leaf anthracnose caused by the fungus *Colletotrichum graminicola*, sooty stripe incited by the fungus *Ramulispora sorghi*, and striga caused by the parasitic plant *Striga hermonthica* (Del.) Bent are the most damaging pests in West Africa in general, and particularly in Burkina Faso (Tenkouano, 1995; Neya and Le Normand, 1998; Trouche et al., 2001). In addition, sorghum midge caused by insects (*Contarinia sorghicola*, *Sitodiplosis moselana*, *Stenodiplosis sorghicola*, etc.) attack developing sorghum grains (Sharma and Hariprasad, 2002; Tao et al., 2003). Also, grain mold is a high constraint of sorghum production, and several molds are associated with sorghum caryopse damage (Melake-Berhan et al., 1996; Waniska et al., 2001). In Africa, the absence of grain mold resistance has been cited as a constraint to adoption of improved cultivars (Audilakshmi et al., 1999).

Important abiotic stresses limiting sorghum production in West Africa are drought, photoperiod sensitivity, and lodging (Tenkouano, 1995; Trouche et al., 2001). Lodging may be indirectly related to fungal infections, which weaken the plant (Waniska et al., 2001).

An increase in the activities of phenolic related enzymes and the accumulation of phenolic compounds have been correlated with resistance of cereals to biotic stresses (Mohammadi and Kazemi, 2002). Plant resistance to biotic and abiotic stresses is often regulated by the metabolism of phenolic compounds. Sorghum phenolic compounds, e.g., phytoalexins (3-deoxyanthocyanidins) or allelochemicals (*p*-hydroxybenzoates, *p*-coumarates, and flavanols), are involved in plant resistance to all kind of stresses (Lo et al., 1999; Weston et al., 1999, Weir et al., 2004).

Both biotic (fungi, insects, viruses, etc.) and abiotic (drought, temperature, photoperiod, nutrient deficiencies, etc.) stresses induce phenylalanine ammonia lyase (PAL; EC 4.3.1.5) synthesis (Chalker-Scott and Fuchigami, 1989; Tovar et al., 2002). PAL activity has been detected in the green shoots and leaves (Stafford, 1969; Mohan et al., 1988) of sorghum, and in sorghum, the infection of the plant with pathogens involves a rapid accumulation of PAL mRNA (Cui et al., 1996).

Also, peroxidases (POXs, EC 1.11.1.7; donor: H_2O_2 oxidoreductase) play an important role in stress-related resistance. One of the important physiological roles of POXs is the synthesis of cell-wall polymers (lignin and suberin), which constitute physical barriers for both biotic and abiotic stresses (Cosgrove, 1997).

In sorghum, POXs are involved in thermal tolerance (Choudhary et al., 1993) and resistance to fungal infection (Luthra et al., 1988).

Polyphenol oxidases (PPOs, EC1.14.18.1; monophenol, 3,4-*L*-dihydroxyphenylalanine: oxygen oxidoreductase) play an important role in plant defense via the oxidation of endogenous phenolic compounds into *o*-quinones, which are toxic to invading pathogens and pests (Mohammadi and Kazemi, 2002). PPO activity in plants increases under abiotic stress conditions (Mayer and Harel, 1991) and upon fungal infections (Luthra et al., 1988).

Several studies in other plant species have shown that the levels of phenolic compounds, and the activities of PAL, POX, and PPO are different between resistant plants and plants susceptible to stresses (Lo et al., 1999; Mohammadi and Kazemi, 2002). Comparing the effects of germination on the levels of phenolic compounds (Dicko et al., 2005a) and the activities of phenolic compounds related enzymes (Dicko et al., 2005b), the levels of these compounds were found to be highly variable in sorghum varieties. Whether the levels of phenolic compounds and related enzymes in sorghum grain could be linked to the grain or plant resistance or susceptibility to stress is unknown. The aim of the present study was to identify possible markers for the grain or plant resistance or susceptibility to these stresses. This was done by comparing the levels of endogenous phenolic compounds and related enzymes in ungerminated and germinated sorghum kernels of known grain or plant agronomic properties for resistance or susceptibility to biotic and abiotic stresses.

METHODS AND MATERIALS

Chemicals. 4-Hydroxyanisole (4-HA) and gallic acid (3,4,5-trihydroxybenzoic acid) were obtained from Aldrich (Steinheim, Germany). 3,4-Dihydroxyphenylpropionic acid (DHPPA) was from Acros Organics (Geel, Belgium). Folin-Ciocalteu's reagent, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), insoluble polyvinylpyrrolidone (PVP), bovine serum albumin (BSA), and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) were from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide was from Merck (Darmstadt, Germany). Sorghum apigeninidin, isolated and characterized by Kouda-Bonafos et al., (1996), was a gift of Dr. Eloi Palé from the Laboratory of Natural Substances, University of Ouagadougou, Burkina Faso. Cyanidin chloride was from Extrasynthèse (Lyon, France). Apple ciders procyanidin oligomers (average degree of polymerization = 7.4) were kindly provided by Dr. Stephanie Prigent (Wageningen University, Wageningen, The Netherlands) and Dr. Catherine M. G. C. Renard (INRA, Rennes, France). These procyanidins were purified by RP-HPLC and characterized by thiolysis-HPLC as described by Guyot et al. (2001). All other chemicals were of analytical grade.

TABLE 1. SORGHUM VARIETIES AND THEIR AGRONOMIC PROPERTIES[a]

Code	Variety name	Gen.[b]	Country of origin	Race	Grain testa	Color of grain/ glume/plant	Known particular properties
V1	Ajabsido	LR	Sudan	C	+	W/R/R	Preflowering drought resistant
V2	BF 88-2/31-1	IL	Burkina Faso	C	−	W/P/tan	–
V3	BF 88-2/31-3	IL	Burkina Faso	C	−	W/R/tan	Preflowering drought susceptible, sooty stripe susceptible, grain mold susceptible, postflowering drought resistant, lodging resistant
V4	BF 89-18/139-1-1	IL	Burkina Faso	C	−	W/P/tan	Postflowering drought susceptible
V5	Cauga 108-15	IL	Burkina Faso	GC	−	W/P/R	Photoperiod insensitive
V6	Cauga 22-20	IL	Burkina Faso	GC	+	W/P/R	Photoperiod sensitive
V7	CE 180-33	IL	Senegal	C	+	W/R/tan	Leaf anthracnose susceptible, photoperiod insensitive, lodging resistant
V8	CEF 322/53-1-1	IL	Burkina Faso	C	−	W/P/R	Postflowering drought resistant
V9	CEF 395/9-2-3	IL	Burkina Faso	GC	−	W/P/tan	Hard grains (PSI < 10)
V10	CEF 396/12-3-1	IL	Burkina Faso	GC	−	W/P/R	Hard grains (PSI < 10)
V11	CEM 326/11-5-1.1	IL	Mali	GC	−	W/P/tan	Leaf anthracnose resistant, photoperiod insensitive, postflowering drought susceptible, lodging resistant, Hard grains (PSI < 10)
V12	CGM 1/19-1-1	IL	Mali	G	−	W/P/R	Sorghum midge susceptible
V13	CGM 19/9-1-1	IL	Mali	G	−	W/B/R	Striga susceptible
V14	CGM 19/9-1-2	IL	Mali	G	−	W/P/R	–
V15	CK 60	IL	USA	K	−	W/P/R	Striga susceptible
V16	F2-20	IL	Burkina Faso	C	−	W/R/tan	Leaf anthracnose resistant, sorghum midge resistant, photoperiod sensitive, lodging resistant
V17	Farkakofsi 781	LR	Burkina Faso	G	+	R/B/R	–
V18	Framida	IL	South Africa	KC	+	R/C/R	Striga resistant, photoperiod sensitive
V19	G 1296	LR	Burkina Faso	GC	−	R/R/R	Good for dyeing, grain mold resistant

V20	G 1414	LR	Burkina Faso	G	−	W/P/R	Photoperiod sensitive
V21	G 1636	LR	Burkina Faso	G	−	W/P/tan	Photoperiod sensitive, Soft grains (PSI > 16)
V22	ICSV 1002	IL	Burkina Faso	C	−	W/P/tan	Leaf anthracnose resistant
V23	ICSV 1049	IL	Burkina Faso	C	−	W/P/tan	Sooty stripe resistant, photoperiod insensitive, postflowering drought resistant, lodging resistant
V24	ICSV 745	IL	India	C	−	W/P/tan	Sooty stripe susceptible, sorghum midge resistant
V25	IRAT 10	IL	Niger	C	−	W/B/R	Grain mold susceptible, drought resistant, photoperiod insensitive, lodging resistant
V26	IRAT 174	IL	Burkina Faso	C	−	W/C/R	Photoperiod sensitive, lodging resistant
			Senegal	C	+	W/R/tan	Preflowering drought resistant, photoperiod insensitive, lodging susceptible
V27	IRAT 202	IL					
V28	IRAT 204	IL	Senegal	C	−	W/P/tan	Sooty stripe and anthracnose susceptible, postflowering drought susceptible, photoperiod insensitive, lodging susceptible
V29	IRAT 277	IL	Burkina Faso	C	−	W/R/tan	Leaf anthracnose resistant, photoperiod insensitive, Soft grains (PSI > 16)
V30	IRAT 9	IL	Cameroon	C	+	R/C/R	Grain mold resistant, photoperiod insensitive
V31	IS 15401	LR	Cameroon	GC	−	W/P/R	Striga resistant
V32	Kaapelga	LR	Burkina Faso	G	−	W/P/tan	Photoperiod insensitive, postflowering drought resistant, Hard grains (PSI < 10)
V33	Kapla-57	LR	Burkina Faso	G	+	R/P/R	Sorghum midge susceptible
V34	Kokologho	LR	Burkina Faso	C	+	W/B/R	Postflowering drought resistant
V35	90L1235	IL	USA	GC	−	W/B/R	Sorghum midge resistant
V36	Magadji 1-509	LR	Burkina Faso	GC	−	R/B/R	Photoperiod insensitive
V37	Nafo-Natogué 775	LR	Burkina Faso	G	−	R/B/R	–
V38	Nazongala tan	IL	Burkina Faso	G	−	W/B/tan	Soft grains (PSI > 16)
V39	Nongomsoba	LR	Burkina Faso	G	−	W/B/tan	Soft grains (PSI > 16)
V40	S 29	LR	Burkina Faso	G	−	W/R/R	Striga susceptible
V41	Sariaso 10	IL	Burkina Faso	C	−	W/R/R	Postflowering drought resistant, photoperiod insensitive

TABLE 1. CONTINUED

Code	Variety name	Gen.[b]	Country of origin	Race	Grain testa	Color of grain/ glume/plant	Known particular properties
V42	Sariaso 11	LR	Burkina Faso	G	+	W/P/tan	Sooty stripe resistant, leaf anthracnose sensitive, striga resistant, postflowering drought resistant, photoperiod sensitive
V43	Sariaso 12	LR	Burkina Faso	G	+	W/B/R	Photoperiod insensitive, postflowering drought resistant
V44	Sariaso 14	IL	Burkina Faso	C	−	W/P/tan	Grain mold resistant, sorghum midge resistant, postflowering drought resistant, photoperiod insensitive, thumbtacks sensitive, lodging resistant
V45	Sariaso 9	LR	Burkina Faso	G	−	W/B/R	Sooty stripe resistant, photoperiod sensitive
V46	Segaolane	IL	Botswana	C	−	W/P/R	Preflowering drought resistant
V47	SRN 39	IL	Sudan	C	−	Y/P/tan	Striga resistant
V48	Tiamassie 289	LR	Burkina Faso	G	+	W/B/R	–
V49	Tx 7000	IL	USA	C	−	W/P/R	Postflowering drought susceptible
V50	Zugilga	LR	Burkina Faso	G	+	R/B/R	–

[a] C = Cautadum; G = Guinea; GC = Guinea-Caudatum; D = Durra; K = Karif; KC = Karif-Caudatum, R = red; W = white; Y = yellow; IL = inbred line; LR = Landrace; PSI = particle size index. Grain with (+) or without (−) pigmented testa layer; (–) = not known.
[b] Gen. = genetic type.

Sorghum Grains. Fifty sorghum varieties were grown during the rainy season of 2002 at the experimental station of Saria, in Burkina Faso (West Africa). The environment was semiarid (temperature: 30–42°C; annual precipitation: 850 mm). Growth conditions have been described previously (Dicko et al., 2005a). For convenience, the sorghum varieties were classified in alphabetic order of their name followed by arabic numbers: V1 to V50 (Table 1). Mature grains (≥60 days after anthesis) were harvested, surface-sterilized, and germinated as described previously (Dicko et al., 2002, 2005a). Germinated and ungerminated sorghum grains were dried, ground, and stored (Dicko et al., 2005a). Flours of both germinated and ungerminated sorghum varieties were analyzed. The varieties were grouped according to their resistance or susceptibility to biotic stresses, e.g., sooty stripe, sorghum midge, leaf anthracnose, striga, and grain molds; and abiotic stresses, e.g., lodging, drought resistance, and photoperiod sensitivity (Table 2). Information on resistance and susceptibility to these specific stresses was obtained from sorghum breeders from the experimental station of Saria and Farakoba (Institut National pour l'Environnement et la Recherche Agronomique, Burkina Faso).

Extraction and Quantification of Phenolic Compounds. Sorghum phenolic compounds were extracted and quantified as previously described (Dicko et al., 2002, 2005a). Results were expressed as phenolic equivalent per gram of flour (w/w), on dry matter basis. Phenolic compounds were extracted from 50 mg of sorghum flour by continuous stirring with 1.5 ml of 1% (v/v) HCl in methanol at 25°C for 20 min, followed by centrifugation (5000 *g*, 10 min, 25°C), and supernatant collection. The residue was reextracted with 0.5 ml HCl/methanol as described above, the supernatants were pooled, and denoted total phenolic extract. The total phenolic extract was used directly for analysis or kept in the dark at −30°C. The same extract was used for quantification of total phenolics, proanthocyanidins (PAs), 3-deoxyanthocyanidins (3-DAs), and flavan-4-ols. The total phenol content was determined using the Folin-Ciocalteu's method adapted to a 96-well plate assay. To 10 µl of extract, 25 µl of Folin-Ciocalteu's reagent (50%, v/v) were added. After 5 min of incubation, 25 µl of 20% (w/v) sodium carbonate solution and 165 µl water were added. Blanks were prepared for each sorghum sample by replacing Folin-Ciocalteu's reagent with water. Gallic acid was used as a standard. The standard was always freshly prepared. The absorbances were measured after 30 min at 760 nm.

PAs and flavan-4-ols were assayed essentially as described by Melake-Berhan et al. (1996) with miniaturization to adapt the assay to a 96-well plate format as follows. To determine flavan-4-ols, 50 µl of the extract were added to 700 µl of reagent A (30%, v/v 12 M HCl in butan-1-ol) or to 700 µl of reagent B (15%, v/v 0.1 M acetic acid; 15%, v/v, methanol and 70%, v/v, butan-1-ol). The sample in reagent A was mixed by vortex and left at 25°C, for 1 hr, to allow formation of anthocyanidin pigments derived from flavan-4-ols (Melake-Berhan

TABLE 2. AGRONOMIC PROPERTIES OF SORGHUM VARIETIES

Type of stress	Plant/grain agronomic properties	Variety code
Biotic	Sooty stripe resistant ($N = 3$)	V23, V42, V45
	Sooty stripe susceptible ($N = 3$)	V3, V24, V28
	Anthracnose resistant ($N = 4$)	V11, V16, V22, V29
	Anthracnose susceptible ($N = 3$)	V7, V28, V42
	Sorghum midge resistant ($N = 4$)	V16, V24, V35, V44
	Sorghum midge susceptible ($N = 2$)	V12, V33
	Striga resistant ($N = 4$)	V18, V31, V42, V47
	Striga susceptible ($N = 3$)	V13, V15, V40
	Grain mold resistant ($N = 3$)	V19, V30, V44
	Grain mold susceptible ($N = 2$)	V3, V25
Abiotic	Lodging resistant ($N = 7$)	V3, V10, V16, V23, V25, V26, V44
	Lodging susceptible ($N = 2$)	V27, V28
	Preflowering drought resistant ($N = 3$)	V1, V27, V46
	Preflowering drought susceptible ($N = 1$)	V3
	Postflowering drought resistant ($N = 8$)	V3, V23, V32, V34, V41, V42, V43, V44
	Postflowering drought susceptible ($N = 4$)	V4, V11, V28, V49
	Photoperiod insensitive ($N = 14$)	V5, V7, V11, V23, V25, V27, V28, V29, V30, V32, V36, V41, V43, V44
	Photoperiod sensitive ($N = 7$)	V6, V18, V20, V21, V26, V42, V45

et al., 1996). Aliquots of the mixture (150 µl) were put in duplicate into a 96-multiwell plate, and the absorbance was read at 550 nm to quantify anthocyanidins formed from flavan-4-ols (Melake-Berhan et al., 1996). Cyanidin was used as standard to estimate the total amount of the anthocyanidins derived from flavan-4-ols. For quantification of PAs, the sample remaining in the tube with reagent A was further heated at 100°C, for 2 hr. Under these conditions, PAs are converted to anthocyanidins, and the unstable pigments formed from flavan-4-ols are destroyed. After cooling, 200 µl of the sample were put in duplicate in a 96 multiwell plate, and the absorbances of anthocyanidin compounds derived from PAs were read at 550 nm. Sample mixtures with reagent B, which were not heated, served as blanks for the quantification of both PAs and flavan-4-ols. Apple procyanidins with an average degree of polymerization of 7.4 and treated as indicated above were used as standards for sorghum PA quantification (Dicko et al., 2005a). For direct spectrophotometric quantification of 3-DAs, 50 µl of the total phenolic extract were mixed with 150 µl of methanol and the absorbances were read at 475 nm (Melake-Berhan et al., 1996). Sorghum apigeninidin was used as standard.

Enzyme Extraction and Determination of Protein Concentration. Enzyme extraction and total protein quantification were performed as described

previously (Dicko et al., 2002, 2005b). Enzyme extracts were prepared by mixing 250 mg of sorghum flour with 1.2 ml of 50 mM Tris–HCl buffer (pH 7.3) containing 0.5 M $CaCl_2$ and 2% (w/v) polyvinylpyrrolidone, at 4°C for 1 hr. The homogenate was centrifuged (14,000 *g*, 4°C, 45 min), and the resulting supernatant was denoted enzyme extract of PPO, POX, and PAL. Total protein was quantified by the linearized method of Bradford, using bovine serum albumin as standard.

Determination of Enzyme Activities. PAL activity was evaluated by measuring *trans*-cinnamic acid formation from L-phenylalanine as described previously (Tovar et al., 2002). Commercial sodium *trans*-cinnamate was used as standard. The spectrophotometric assay for PPO was performed as described previously (Dicko et al., 2002). 4-Hydroxyanisole (4-HA) and 3,4-dihydroxyphenylpropionic acid (DHPPA) were used as substrates to determine the monophenolase and *o*-diphenolase activities of PPO, respectively. The enzyme extract (10 μl) was incubated with 150 μl 50 mM sodium acetate, pH 5.5, 10 μl 40% (v/v) DMF and 10 μl 50 mM 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) at 25°C for 5 min. The reaction was started by addition of 20 μl 100 mM of the phenolic substrate (prepared in 0.15 mM phosphoric acid). The reaction was monitored at 500 nm. Blanks, in which the enzyme extracts or substrates were replaced by buffer, were performed. One unit of PPO activity (U) is defined as the amount of enzyme producing 1 μmol MBTH-*o*-quinone adducts/min resulting from the oxidation of 4-HA or DHPPA. Prior to determination of POX activity, extracts from ungerminated and germinated sorghums were diluted 400- and 1000-fold, respectively, in 50 mM Tris–HCl, pH 7.3, containing 0.5 M $CaCl_2$ and 1 mg/ml bovine serum albumin. POX activity was measured spectrophotometrically by monitoring the H_2O_2-dependent oxidation of ABTS, at 25°C (Dicko et al., 2002). The reaction mixture consisted of 10 μl of 200-fold diluted crude enzyme extract, 20 μl of 100 mM ABTS, 10 μl of 100 mM H_2O_2, and 160 μl of 50 mM sodium acetate, pH 4.0. Blanks, in which the enzyme extract or substrates were replaced by buffer, were performed. The reaction was monitored at 414 nm. One unit of POX activity (U) is defined as the amount of enzyme releasing 1 μmol ABTS radical/min under the assay conditions.

Statistical Analyses. Statistical analyses were carried out as previously described (Dicko et al., 2002, 2005a). All spectrophotometric assays were carried out in 96-well microtiter plates (Nunclon, Denmark) using a multiwell plate reader (μQuant Bio-Tek Instrument, Inc.) interfaced with a personal computer. Absorbances and slopes of absorbances per min (OD/min) were automatically recorded using KC junior software version 1.31.5 (Bio-Tek Instrument, Inc, USA). All assays were carried out at least in duplicate. Results were subjected to ANOVA performed with Statistica version 4.5 software for Windows. Significant differences in mean performance for each composition among sorghum varieties were determined by Student's *t*-test, where $P < 0.05$ implies significance.

RESULTS AND DISCUSSION

The contents of phenolic compounds and the activities of related enzymes in varieties grouped according to their agronomic properties are presented in Table 3. Difference in composition was determined using Student's *t*-test between varieties resistant and varieties susceptible to specific biotic or abiotic stresses (Table 4). Furthermore, the levels of significance were confirmed with ANOVA.

Correlations between Phenolic Compounds and Related Enzymes and Resistance or Susceptibility to Biotic Stress. In the present study, independent of grain germination, no significant difference in total phenolic compound content between sorghum varieties susceptible and those resistant to biotic stresses was found. However, leaves of sorghum resistant to fungi contained a higher content of total phenolics than leaves of susceptible ones upon pathogen challenge (Luthra et al., 1988). This suggests that total phenolic compounds content in sorghum grains, which are not challenged by pathogens, is not a good indicator for resistance to biotic stress.

When focusing on individual classes of phenolic compounds, it can be seen that PA levels were higher on average in varieties resistant to sooty stripe, sorghum midge, and grain mold than susceptible ones. In contrast, resistance to anthracnose and striga was not correlated with content of PAs. The high content of PAs in varieties resistant to mold is in agreement with previous observations showing that high PA containing sorghums are generally resistant to grain molding and weathering (Waniska et al., 2001). Sorghum midge resistance is also associated with PAs content in another study (Sharma and Hariprasad, 2002).

3-DA content is higher in all varieties resistant to the referred biotic stresses compared to that of susceptible ones. This indicates a function as phytoalexins (Weiergang et al., 1996; Lo et al., 1999). For example, the content of 3-DAs, notably apigeninidin, is an indicator of grain resistance to fungi such as *Colletrichum graminicola*, *Fusarium oxysporum*, *Gibberelle zeae*, and *Gliocladium roseum* (Schutt and Netzly, 1991) and of sorghum resistance to anthracnose (Lo et al., 1999). Colored phenolic compounds, probably 3-DAs, were suggested to be involved in sorghum resistance to striga (Arnaud et al., 1999). Recently, the importance of phenolic compounds as allelochemicals, involved in plant–parasite interactions, were indicated (Weir et al., 2004). Our results suggest that the content of 3-DAs in sorghum grain is not only an indicator of resistance to mold, but also to sooty stripe, anthracnose, sorghum midge, and striga.

The content of flavan-4-ols is on average higher in varieties resistant to mold than in susceptible ones. This agrees with previous findings showing that flavan-4-ol content is an indicator of grain resistance to molds (Schutt and Netzly, 1991; Melake-Berhan et al., 1996). Moreover, the results presented here show that

independent of germination, the flavan-4-ol content was on average higher in varieties resistant to sooty stripe, sorghum midge, leaf anthracnose, and striga than in susceptible ones. This suggests that the flavan-4-ol content in sorghum grain is a good indicator of plant resistance to sooty stripe, sorghum midge, and striga. For leaf anthracnose, no correlation could be found with the grain content of flavan-4-ols.

Before germination, PAL activity was higher in varieties resistant to sooty stripe and sorghum midge than in susceptible ones. However, after germination there was no difference. PAL activity in germinated varieties resistant to anthracnose was higher than in susceptible ones. This supports previous findings that high PAL activity in sorghum is associated with resistance to *Colletotrichum* species (Kamida et al., 2000).

POX and PPO activities before germination could not be linked to resistance or susceptibility to biotic stresses. Germination did not affect this trend for POX. After germination, the mono-PPO activity increased in the sooty stripe resistant varieties and decreased in anthracnose resistant varieties. These results are in agreement with findings that PPO may confer resistance to some fungal species such as *R. sorghicola* (sooty stripe agent) (Luthra et al., 1988).

Correlations between Levels of Phenolic Compounds and Related Enzymes, and Resistance Susceptibility to Abiotic Stress. Varieties resistant to lodging had significantly lower PAs than susceptible ones, both before and after germination. From these results, it may be inferred that the PA content of the grain is a marker for plant resistance to lodging. Independent of grain germination, varieties resistant to both preflowering and postflowering drought contained apparently more PAs, 3-DAs, and flavan-4-ols than susceptible ones. Results indicate the importance of polyphenols in drought resistance.

For other plants (*Mangifera indica*), the phenolic compound content is influenced by their response to light (Tovar et al., 2002). However, no significant difference in content of total phenolic compounds, PAs, 3-DA, or flavan-4-ols was found between the grain of sorghum varieties resistant or susceptible to photoperiod.

POX activity in ungerminated grains was not significantly different between varieties resistant or susceptible to abiotic stresses. After germination, POX activity appeared to be on average higher in varieties resistant to lodging than in susceptible ones. High activity of POX in varieties resistant to lodging could be related to the role of POX in the formation of physical polymeric barriers such as suberin and lignin (Cosgrove, 1997; Quiroga et al., 2000), which might confer the plant with high rigidity.

Specific POX isoenzymes in leaves, notably the cationic ones, are correlated with photoperiod sensitivity in sorghum, and play a role in plant adaptation (Pao and Morgan, 1988). However, in the present study POX activity of the grain could not be linked to the plant sensitivity to photoperiod.

TABLE 3. PHENOLIC COMPOUNDS AND PHENOLIC ENZYMES IN GROUPS OF SORGHUM VARIETIES RESISTANT AND SUSCEPTIBLE TO STRESSES

Type of stress		Total phenolics (%)[a]	PAs (%)	3-DAs (%)	Flavan-4-ols (%)	PAL (mU/mg)[b]	POX (U/mg)	Mono-PPO (mU/mg)	Dipheno-PPO (mU/mg)
					Ungerminated grains				
Biotic	Sooty stripe resistant ($N = 3$)	0.79	0.22	0.06	0.18	6.2	24.5	0.9	47.3
	Sooty stripe susceptible ($N = 3$)	0.78	nd	nd	nd	2.3	20.7	0.7	43.6
	Anthracnose resistant ($N = 4$)	0.73	nd	0.02	nd	6.0	34.9	0.9	42.9
	Anthracnose susceptible ($N = 3$)	0.76	0.09	nd	nd	4.7	36.2	1.0	47.7
	Sorghum midge resistant ($N = 4$)	0.74	0.45	0.05	0.16	6.3	30.8	0.8	45.5
	Sorghum midge susceptible ($N = 2$)	0.62	0.05	nd	nd	2.7	55.1	1.0	41.4
	Striga resistant ($N = 4$)	1.06	0.39	0.05	0.24	6.7	37.6	1.1	48.1
	Striga susceptible ($N = 3$)	0.70	0.13	0.04	nd	9.0	43.2	0.9	41.8
	Grain mold resistant ($N = 4$)	1.65	1.31	0.25	0.35	4.5	21.7	0.8	48.0
	Grain mold susceptible ($N = 2$)	0.77	0.05	nd	nd	4.0	19.2	0.9	41.0
Abiotic	Lodging resistant ($N = 7$)	0.68	0.06	0.03	nd	5.4	29.7	0.9	46.4
	Lodging susceptible ($N = 2$)	1.08	0.53	nd	nd	2.2	15.5	0.9	46.8
	Preflowering drought resistant ($N = 3$)	0.80	0.24	0.04	nd	nd	40.2	1.0	45.2
	Preflowering drought susceptible ($N = 1$)	0.72	nd	nd	nd	2.3	17.4	0.7	39.3
	Postflowering drought resistant ($N = 8$)	0.71	0.26	0.04	0.17	4.9	22.6	0.9	46.4
	Postflowering drought susceptible ($N = 4$)	0.69	0.07	nd	nd	3.2	38.1	0.7	46.0
	Photoperiod insensitive ($N = 14$)	0.82	0.23	0.05	0.23	4.5	38.6	0.9	45.2
	Photoperiod sensitive ($N = 7$)	1.03	0.44	0.04	0.22	4.6	28.6	0.9	47.6

					Germinated grains				
Biotic	Sooty stripe resistant ($N = 3$)	0.70	0.14	0.04	0.12	7.06	64.3	1.3	31.8
	Sooty stripe susceptible ($N = 3$)	0.59	nd	nd	nd	6.80	50.9	0.8	22.8
	Anthracnose resistant ($N = 4$)	0.72	nd	nd	nd	3.04	89.3	0.4	16.8
	Anthracnose susceptible ($N = 3$)	0.68	0.05	nd	nd	11.81	66.6	1.0	25.9
	Sorghum midge resistant ($N = 4$)	0.73	0.34	0.07	nd	8.54	85.0	1.1	34.7
	Sorghum midge susceptible ($N = 2$)	0.89	0.06	0.02	nd	8.37	96.3	0.9	29.0
	Striga resistant ($N = 4$)	1.00	0.29	0.04	0.17	7.49	87.9	1.6	28.3
	Striga susceptible ($N = 3$)	1.02	0.17	0.05	nd	12.38	97.6	1.0	16.7
	Grain mold resistant ($N = 4$)	1.59	1.02	0.14	0.32	8.86	82.0	1.6	27.0
	Grain mold susceptible ($N = 2$)	0.71	0.05	nd	nd	6.10	89.2	0.8	20.8
Abiotic	Lodging resistant ($N = 7$)	0.76	0.09	0.03	nd	5.33	92.3	0.8	22.1
	Lodging susceptible ($N = 2$)	0.92	0.28	nd	nd	6.47	16.0	0.7	16.1
	Preflowering drought resistant ($N = 3$)	0.96	0.16	0.05	nd	7.59	54.2	1.0	22.6
	Preflowering drought susceptible ($N = 1$)	0.69	nd	nd	nd	9.86	79.9	0.8	24.4
	Postflowering drought resistant ($N = 8$)	0.78	0.24	0.05	0.15	10.65	58.7	1.4	35.6
	Postflowering drought susceptible ($N = 4$)	0.64	0.06	nd	nd	7.71	71.8	0.8	20.1
	Photoperiod insensitive ($N = 14$)	0.84	0.17	0.03	0.20	9.39	74.0	1.1	24.2
	Photoperiod sensitive ($N = 7$)	0.96	0.32	0.04	0.16	8.04	79.4	1.1	22.5

PAs = proanthocyanidins (w/w, procyanidin equivalents); 3-DAs = 3-deoxyanthocyanidins (w/w apigeninidin equivalents); PAL = phenylalanine ammonia lyase; POX = peroxidase; mono-PPO = monophenolase activity of polyphenol oxidase (PPO); dipheno-PPO = diphenolase activity of PPO.

[a] Total phenolic compounds (gallic acid equivalents).

[b] All enzymes activities are expressed in terms of specific activities (U/mg of protein in the crude extracts).

TABLE 4. STUDENT *t*-TEST RESULTS INDICATING LEVEL OF SIGNIFICANCE IN COMPOSITION OF PHENOLIC COMPOUNDS AND RELATED ENZYMES FOR PAIRED COMPARISON BETWEEN SORGHUM VARIETIES RESISTANT AND SUSCEPTIBLE TO STRESSES

Type of stress	Group of varieties	Total phenolics	PAs	3-DAs	Flavan-4-ols	PAL	POX	Mono-PPO	Di-PPO
					Ungerminated grains				
Biotic	Sooty stripe resistant ($N = 3$)	NS	+	+	+	+	NS	NS	NS
	Sooty stripe susceptible ($N = 3$)	NS	−	−	−	−	NS	NS	NS
	Anthracnose resistant ($N = 4$)	NS	−	+	NS	NS	NS	NS	NS
	Anthracnose susceptible ($N = 3$)	NS	+	−	NS	NS	NS	NS	NS
	Sorghum midge resistant ($N = 4$)	NS	+	+	+	+	NS	NS	NS
	Sorghum midge susceptible ($N = 2$)	NS	−	−	−	−	NS	NS	NS
	Striga resistant ($N = 4$)	NS	NS	+	+	NS	NS	NS	NS
	Striga susceptible ($N = 3$)	NS	NS	−	−	NS	NS	NS	NS
	Grain mold resistant ($N = 4$)	NS	+	+	+	NS	NS	NS	NS
	Grain mold susceptible ($N = 2$)	NS	−	−	−	NS	NS	NS	NS
Abiotic	Lodging resistant ($N = 7$)	NS	−	+	NS	+	NS	NS	NS
	Lodging susceptible ($N = 2$)	NS	+	−	NS	−	NS	NS	NS
	Preflowering drought resistant ($N = 3$)	NS	+	+	NS	−	NS	NS	NS
	Preflowering drought susceptible ($N = 1$)	NS	−	−	NS	+	NS	NS	NS
	Postflowering drought resistant ($N = 8$)	NS	+	+	+	NS	NS	NS	NS
	Postflowering drought susceptible ($N = 4$)	NS	−	−	−	NS	NS	NS	NS
	Photoperiod insensitive ($N = 14$)	NS	NS	NS	NS	NS	NS	NS	NS
	Photoperiod sensitive ($N = 7$)	NS	NS	NS	NS	NS	NS	NS	NS

					Germinated grains				
Biotic	Sooty stripe resistant ($N = 3$)	NS	+	+	+	NS	NS	+	NS
	Sooty stripe susceptible ($N = 3$)	NS	−	−	−	NS	NS	−	NS
	Anthracnose resistant ($N = 4$)	NS	−	+	NS	+	NS	−	NS
	Anthracnose susceptible ($N = 3$)	NS	+	−	NS	−	NS	+	NS
	Sorghum midge resistant ($N = 4$)	NS	+	+	NS	NS	NS	NS	NS
	Sorghum midge susceptible ($N = 2$)	NS	−	−	NS	NS	NS	NS	NS
	Striga resistant ($N = 4$)	NS	NS	+	+	NS	NS	NS	NS
	Striga susceptible ($N = 3$)	NS	NS	−	−	NS	NS	NS	NS
	Grain mold resistant ($N = 4$)	NS	+	+	+	NS	NS	NS	NS
	Grain mold susceptible ($N = 2$)	NS	−	−	−	NS	NS	NS	NS
Abiotic	Lodging resistant ($N = 7$)	NS	−	+	NS	NS	+	NS	NS
	Lodging susceptible ($N = 2$)	NS	+	−	NS	NS	−	NS	NS
	Preflowering drought resistant ($N = 3$)	NS	+	+	NS	NS	NS	NS	NS
	Preflowering drought susceptible ($N = 1$)	NS	−	−	NS	NS	NS	NS	NS
	Postflowering drought resistant ($N = 8$)	NS	+	+	+	NS	NS	+	+
	Postflowering drought susceptible ($N = 4$)	NS	−	−	−	NS	NS	−	−
	Photoperiod insensitive ($N = 14$)	NS	NS	NS	NS	NS	NS	NS	NS
	Photoperiod sensitive ($N = 7$)	NS	NS	NS	NS	NS	NS	NS	NS

PAs = proanthocyanidins; 3-DAs = 3-deoxyanthocyanidins; PAL = phenylalanine ammonia lyase; POX = peroxidase; mono-PPO = monophenolase activity of polyphenol oxidase (PPO); dipheno-PPO = *o*-diphenolase activity of PPO.
NS, not significant ($P > 0.05$); +, significantly high ($P < 0.05$); −, significantly low ($P < 0.05$).

Before grain germination, PPO activities could not be linked to abiotic stresses. After germination, both the monophenolase and *o*-diphenolase activities of PPO were higher in postflowering drought resistant varieties than in susceptible ones. This suggests a role of PPO in postflowering drought resistance, in agreement with earlier findings (Mayer and Harel, 1991). Independent of grain germination, PPO activities could not be related to resistance to lodging or photoperiod. PAL activity in the grain cannot be used as a marker for resistance to lodging, drought, and photoperiod variation as well.

Overall Impact of Phenolic Compounds and Related Enzymes in Stress Resistance. Results in Table 4 show that 3-DA content is a good marker for sorghum resistance to both biotic and abiotic stresses because 3-DAs are positively correlated with resistance to all stresses except for photoperiod sensitivity. The second marker for stress resistance is PA content. Total phenolic compounds and the activities of related enzymes are not good markers for stress resistance in sorghum grains. For photoperiod sensitivity, none of the screened biochemical compounds could be used as a marker of resistance in sorghum grain.

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WHOLE PLANT RESPONSE OF LETTUCE AFTER ROOT EXPOSURE TO BOA (2(3*H*)-BENZOXAZOLINONE)

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Abstract—The goal of our work was to expand the knowledge about plant stress response to the allelochemical 2(3*H*)-benzoxazolinone (BOA). We focused on physiological processes that are affected by this secondary metabolite. Physiological and biochemical characteristics of plants exposed to BOA help us to better understand its mode of action and open the gate to the use of allelochemicals as "natural" herbicides. Measurements on photosynthesis, fluorescence, water relations, antioxidant enzymes (superoxide dismutase, peroxidase), ATPases, and lipid peroxidation indicated that a phytotoxic effect follows BOA exposition. This effect was intense enough to interfere with plant growth and development and to produce "induced senescence." Based on this, we propose a multifaceted mode of action for BOA with effects at different levels and in different parts of the plant.

Key Words—Allelopathy, plants, benzoxazolinone, mode of action, biopesticide, allelochemical, botanical compound, lettuce, *Lactuca sativa.*

INTRODUCTION

Plant compounds have been suggested as alternatives to conventional herbicides (Dayan et al., 1999). Understanding the mechanisms of whole plant-induced responses to allelopathic compounds will provide information about their application. 2(3*H*)-Benzoxazolinone (BOA) occurs naturally as an anti-*Fusarium* compound in rye seedlings (Virtanen et al., 1957), and is found in other grass species (Sánchez-Moreiras et al., 2004). BOA has been associated

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with dose-dependent germination inhibition and growth reductions (Chiapusio et al., 1997), and with effects on plant energy metabolism (Friebe et al., 1997). Phytotoxic effects of BOA have been found on root ultrastructure, where packed multiple columns of cells failed to lengthen at the root tip of cucumber plants (Burgos et al., 2004). Microarray technology has detected a range of defensive and detoxification processes when roots are exposed to BOA (Baerson et al., 2005).

The objective of this study was to analyze the biochemical and physiological mechanisms of BOA inhibition in a 15-d experiment to understand the relationships between molecular mode of action and physiological effects. Lettuce was used as an assay species, and several different metabolic and physiological processes were measured so that the complex stress response on whole-plant development after exposure to BOA could be better understood.

METHODS AND MATERIALS

Plant Material and Growth Conditions. Lettuce seeds (*Lactuca sativa* L. cv. Great Lakes, California) were germinated in plastic trays with 5 cm depth layer perlite, to which 500 ml 1:1 Hoagland's solution (Hoagland and Arnon, 1950) was added every other day. Seedlings were germinated in a darkened, temperature-controlled (20°C) chamber. For seedling growth, the environmental conditions were as follows: 12:12 (L:D) hr photoperiod, 18:8°C temperature, 60 ± 5% relative humidity, and 200 μmol m^{-2} sec^{-1} irradiance. One mo after germination (when plants had three fully expanded leaves), they were transferred to pots containing perlite (13 cm) to stimulate root system development, with the same mineral solution and growing conditions. One wk later, roots were exposed to 1:1 Hoagland's solution (control), and the same solution + 1 mM BOA (treatment) for 15 d.

In vivo Measurements. Photosynthesis was measured with an infrared gas analyzer LI-6400 (Li-Cor model 6400, Lincoln, NE, USA) in the first fully expanded leaf from six replicates per treatment after exposure. Photosynthesis values were recorded in leaves at 800 μmol m^{-2} s^{-1} quantum flux density (provided by 6400-02B led light source), 20°C leaf temperature, and 400 μmol s^{-1} CO_2 flow value. These parameters were fixed for all measurements. Values of transpiration (mol m^{-2} s^{-1}) were recorded. Any damaged plant was discarded.

Immediately after gas exchange measurements, the influence of BOA (1 mM) on the functional state of the photosynthetic apparatus was investigated by using different parameters of the *in vivo* chlorophyll fluorescence of whole plants. Pulse-modulated and continuous fluorescence were recorded for the same six replicates per treatment used for gas exchange measurements. Three values per plant were recorded for both fluorescence measurements. The pho-

tochemical yield of Photosystem II (PSII) was established by measuring two parameters (Genty et al., 1989), the first of which was the quantum efficiency of open Photosystem II centers (F_v/F_m ratio), recorded using a Plant Efficiency Analyzer from Hansatech Instruments Ltd. (Norfolk, UK). Lettuce leaf pieces were kept in darkness for 20 min and illuminated with a flash of saturating light. Parameters used were intensity of light (1800 μmol m^{-2} s^{-1}) and record length (3 sec). The other photochemical quenching parameter was the quantum yield of Photosystem II photochemistry (Φ_{PSII}), measured with a Fluorescence Monitoring System from Hansatech Instruments Ltd.

Plant Growth and Water Status. Information about plant growth was obtained by measuring plant height, root length, number of fresh and dry leaves, leaf and root biomass, fresh and dry weight, and specific leaf area. Following these measurements, leaf samples were ground and used for total C, N, and H content determination (w/w, dry basis %) with an elemental CHN analyzer (EA 1110 Automatic Elemental Analyzer; Fisons Instruments, Milan, Italy). Calculations were made to obtain the C/N ratio.

Plant water status of control and BOA-treated plants was evaluated by measuring the stomatal density (Meister and Bolhàr-Nordenkampf, 2001), relative water content (Reigosa and González, 2001), water potential (Ψ_w) with a pressure chamber technique (Scholander chamber, SoilMoisture Equipment, Santa Barbara, CA, USA; González, 2001), and leaf osmotic potential with a calibrated vapor pressure osmometer (Wescor 5500, Logan, UT, USA). Osmotic values were expressed in terms of osmolality (mmol kg^{-1}). All measurements were recorded for eight replicates per treatment. Leaves and roots from plants were harvested and separately stored at −80°C for later analyses.

Plant extracts were obtained for superoxide dismutase and peroxidase activities as two of the main components of the antioxidant defense system. Five replicates per treatment were used for leaf and root analyses. Superoxide dismutase activity was measured by the nitroblue tetrazolium reaction according to the method described by Beauchamp and Fridovich (1971). Superoxide dismutase leaf and root activities were measured in the sample at 560 nm with a WPA Spectrophotometer (Cambridge, UK). The enzyme unit was determined with the value of the nitroblue tetrazolium inhibition percentage. Superoxide dismutase activity was given in relation to the dry weight of the sample. Peroxidase activity was measured using 0.5% w/v guaiacol as hydrogen donor (Silva and Nogueira, 1984) in the presence of 0.1% v/v H_2O_2 at 25°C. To monitor the peroxidase reaction, measures were recorded for 7 min. The absorbance difference between final and initial reading was given per milligram of dry weight and per minute of measuring.

The level of lipid peroxidation was measured by the thiobarbituric acid reaction based on the method described by Dhindsa et al. (1981). With this method, we measured the quantity of malondialdehyde acid (MDA) present in

the sample, which is a product of lipid peroxidation and can be correlated with the lipid status of the sample. Lettuce leaves and roots from five replicates per treatment were employed. Malondialdehyde acid values were expressed in mmol/g DW using the extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968). Sulfhydryl groups were determined by Ellman's reactive, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) in roots from five living plants per treatment. Color change was measured at 412 nm with a WPA Spectrophotometer (Prudencio-Ferreira and Areas, 1993). The extinction coefficient used for calculations was 13.6 mM^{-1} cm^{-1} (Peters et al., 1988).

ATPase activity was assayed according to the method described by Cruz-Ortega et al. (1990) in leaf and root microsomal fractions from five plants per treatment. Microsomal fractions were obtained according to the method of Friebe et al. (1997). Protein content of this fraction was assayed via the Bradford method (Bradford, 1976) with BSA as standard. Slight modifications to the Bradford method, suggested by Stoscheck (1990) to decrease variability in the chromogenic responses of different proteins, were used. Total, organular, plasma membrane, vacuolar, and mitochondrial ATPase activity were measured using the following inhibitors: Na_3VO_4 (plasma membrane ATPase inhibitor), NaN_3 (mitochondrial ATPase inhibitor), KNO_3 (tonoplast ATPase inhibitor), and Na_2MoO_4 (acid phosphatase inhibitor). ATP hydrolysis occurred at 30°C for 1 hr. Six repetitions were tested for each case. After hydrolysis, released inorganic phosphate was measured according to the method of Chifflet et al. (1988). Absorbance was read at 850 nm with a Spectronic® 20 Genesis™ Spectrophotometer (Spectronic Analytical Instruments, Leeds, UK). KH_2PO_4 was used as standard. Finally, nmol P_i mg^{-1} min^{-1} was calculated for each sample by considering the absorbance values, the quantity of protein, and the incubation time.

Total protein was quantified by using the spectrophotometric Bradford assay (Bradford, 1976) in lettuce leaves from five replicates per treatment. Commercial bovine seroalbumin (BSA) was used as standard. Values were expressed per gram of dry weight. In the same way, leaves from five replicates per treatment were used for free proline content determination according to the method described by Pedrol et al. (2000) and Ramos and Pedrol (2001). Commercial purified proline was used as standard. Values were expressed per gram dry weight. Free polyamines were determined by thin layer chromatography according to Pedrol and Tiburcio (2001). Commercial purified spermine, spermidine, and putrescine solutions were used as standards (Sigma). Separation of dansylamines was developed in a plate of silicagel 60 Å (Whatman LK6D) using 100 ml of 5:4 v/v cyclohexane/ethyl acetate solvent system. Thirty μl per extract were applied on the plate. Chromatography was allowed at 4°C for 40 min. Spermidine, spermine, and putrescine marked spots were excised and eluted in 2 ml ethyl acetate. Sample fluorescence was measured with a fluorescence spectrophotometer with excitation at 350 nm and emission at 495 nm. Spermine, spermidine, and putres-

cine contents were expressed as nmol g^{-1}. Polyamine contents were used for Pas/Das index (Spermine + Spermidine/ Putrescine) calculation.

Statistical Analyses. In all cases, there was a comparison between control and treatment using Student's *t*-tests.

RESULTS AND DISCUSSION

Data from 1 mM BOA-exposed plants were analyzed and compared to the control. Values and significance of differences between control and BOA-exposed plants for all variables are detailed in Table 1 (general measures) and Table 2 (stress metabolites and enzymatic activities).

BOA Effects on Plant Growth, Water Status, and Photosynthetic Efficiency. Plants growing in the presence of 1 mM BOA were smaller, with less fresh biomass and a significant increase in dry leaf biomass with respect to the control. Effects of BOA on plant appearance became obvious 3 d after application, and were evident after 1 wk of exposure (Figure 1). The previously detected effects of BOA on root growth (Chiapusio et al., 1997) were corroborated. As confirmed by statistical analysis in Table 1, lettuce plants had shorter and typically browning roots without hairs after 1 mM BOA treatment. However, BOA-exposed lettuce roots were also characteristically thicker, leading to a similar root biomass when compared to control. These results are consistent with those reported by Burgos et al. (2004), who found that increased width of cucumber cortical cells resulted in increased root diameter. When root/leaf ratio is analyzed, the results show a large increase in this ratio in BOA-exposed plants, reflecting the decrease in leaf biomass and the insignificant changes on root biomass.

This increase in the leaf/shoot ratio was positively correlated with an increased C/N ratio in the samples. In dry matter, the total C content analyzed was higher in BOA-treated plants compared to control after 15 d treatment, whereas total N was significantly lower. It is well known that plants with decreased growth from typical stresses (e.g., mild water or nutrient stress; Bradford and Hsiao, 1982) have reduced nitrogen concentrations and increased levels of immobile carbon-based secondary metabolites. This relative surplus of carbon results in increased lipid production and a thicker cuticle, whereas a diminution in nitrogen content can induce a decrease in amino acids, proteins, and chlorophyll present in plants (Chapin et al., 1986). Observed alterations could imply a higher rigidity of cell wall, thus preventing cell elongation and concomitant cell growth. These changes in dry matter, which also resulted in increased C/N ratios, may cause plants to have a higher production of fibers, lignins, and cell wall material (Larcher, 1980), and thus small leaves with lower leaf water content and more permeability.

TABLE 1. BIOMETRIC, PHOTOSYNTHETIC, AND WATER STATUS CHANGES INDUCED BY EXPOSURE OF LETTUCE PLANTS TO 1 mM BOA DURING 15 D

Parameter	Control	BOA	Percent	Significance
Element analysis				
Total C (%)	38.91	41.67	107.1	***
Total N (%)	4.72	3.63	76.9	***
C/N	8:1	12:1	150.0	***
Biometric measurements				
Plant height (cm)	16.58	13.08	78.9	***
Fresh leaf biomass (g)	17.19	8.8	51.2	***
Dry leaf biomass (g)	0.32	0.88	273.2	***
Dry weight/Fresh weight	0.051	0.069	134.8	**
Dry biomass/Nitrogen fresh leaves	0.016	0.024	156.4	**
Root length (cm)	18.43	12.82	69.6	*
Root biomass (g)	1.84	1.23	66.8	n.s.
Dry leaf biomass/dry root biomass	0.17	0.71	408.7	***
Shoot length/Root length	0.90	1.02	113.4	**
Specific leaf area ($m^2\ g^{-1}$ DW)	573.38	561.32	97.9	n.s.
In vivo measurements				
P_n: net photosynthetic rate ($\mu mol\ m^{-2}\ s^{-1}$)	6.3	3.78	60.0	***
Transpiration ($mol\ m^{-2}\ s^{-1}$)	1.09	0.27	24.5	***
F_v/F_m, variable/maximal fluorescence	0.84	0.83	98.4	n.s.
Φ_{PSII}, Photosystem II quantum efficiency	0.80	0.77	96.4	*
Water relations				
Relative water content (%)	83.17	76.30	91.7	**
Ψ_w (MPa)	−1.80	−3.23	179.4	**
Ψ_s ($mmol\ kg^{-1}$)	323.0	340.0	105.3	n.s.
Stomatal density	18.4	15.8	86.1	*

Data were analyzed using *t*-tests. Level of significance in BOA data with respect to control: ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, n.s. ≥ 0.05.

As observed in Table 1 ("water relations"), a nearly 10% decrease in relative water content was correlated with a decline in leaf water potential (Ψ_w) from −1.8 in control to −3.23 MPa in BOA-exposed lettuce. It was directly correlated with a decrease in transpiration rate to one fourth in BOA-exposed plants when compared to respective controls. Osmotic potential (Ψ_s) in leaves of the treated plants remained unaltered, and proline was not affected (not showing osmoregulation). Short-term responses such as closing stomata or longer-term responses by reducing stomatal density (which decreased from 18.4 stomata per field in control to 15.8 in BOA-exposed plants) could be effective ways to reduce transpiration. Even so, Ψ_w in these plants was severely

TABLE 2. ENZYMATIC AND METABOLIC CHANGES INDUCED BY EXPOSURE OF LETTUCE PLANTS TO 1 mM BOA DURING 15 D

Parameter	Control	BOA	Percent	Significance
Stress markers				
Free proline (μmol g^{-1} DW)	4.16	3.73	89.7	n.s.
Spermine (nmol g^{-1} DW)	35.38	20.21	57.1	***
Spermidine (nmol g^{-1} DW)	143.76	90.12	62.7	**
Putrescine (nmol g^{-1} DW)	58.39	67.87	116.2	n.s.
Polyamines/Diamines (ratio)	3.23	1.76	54.5	*
Total leaf proteins (mg g^{-1} DW)	4.64	2.84	61.1	*
Total root proteins (mg g^{-1} DW)	4.35	4.86	111.7	n.s.
Enzymatic activities and oxidative measurements				
Leaf LP (mmol MDA g^{-1} DW)	112.1	191.5	170.9	***
Leaf SOD (% SOD g^{-1} DW)	593.3	478.3	80.6	**
Leaf POD (units POD g^{-1} DW)	1.49	0.79	53.4	**
Leaf TATPase (nmol P_i mg^{-1} min^{-1} g^{-1} DW)	13.58	12.79	94.2	n.s.
Leaf OrgATPase (nmol P_i mg^{-1} min^{-1} g^{-1} DW)	5.08	2.76	54.3	n.s.
Leaf MPATPase (nmol P_i mg^{-1} min^{-1} g^{-1} DW)	8.48	10.03	118.3	n.s.
Leaf VATPase (nmol P_i mg^{-1} min^{-1} g^{-1} DW)	2.43	2.98	122.6	n.s.
Leaf MATPase (nmol P_i mg^{-1} min^{-1} g^{-1} DW)	1.84	1.87	101.6	n.s.
Root LP (mmol MDA g^{-1} DW)	210.0	433.0	206.3	***
Root SOD (% SOD g^{-1} DW)	1612.0	815.0	50.6	***
Root POD (units POD g^{-1} DW)	2.55	2.94	115.2	n.s.
Root TATPase (nmol P_i mg^{-1} min^{-1} g^{-1} DW)	28.38	14.17	49.9	**
Root OrgATPase (nmol P_i mg^{-1} min^{-1} g^{-1} DW)	9.88	4.09	41.4	n.s.
Root MPATPase (nmol P_i mg^{-1} min^{-1} g^{-1} DW)	18.50	10.07	54.4	**
Root TATPase (nmol P_i mg^{-1} min^{-1} g^{-1} DW)	2.76	2.39	86.6	n.s.
Root MATPase (nmol P_i mg^{-1} min^{-1} g^{-1} DW)	3.58	3.22	89.9	n.s.
–SH Sulfhydryl groups (mmol SH g^{-1} DW)	17.07	70.61	413.6	***

Data were analyzed using *t*-tests. Level of significance in BOA-exposed with respect to control plants: ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, n.s. ≥ 0.05.
Polyamines/diamines: index polyamines/diamines; LP: lipid peroxidation; MDA: malondialdehyde acid; SOD: superoxide dismutase activity; POD: peroxidase activity; ATPase: ATPase activity; TATPase: total ATPase activity; OrgATPase: organelle ATPase activity; MPATPase: plasma membrane ATPase activity; TATPase: tonoplast ATPase activity; MATPase: mitochondrial ATPase activity; VATPase: vacuolar ATPase.

decreased, revealing a strong loss of turgor in the plant cell, thereby limiting stomatal opening and growth expansion.

Net photosynthetic rate shows an almost twofold decrease in BOA-exposed plants. Decreased transpiration could be affecting P_n due to the limitation of CO_2 assimilation because of stomatal closure. *In vivo* chlorophyll fluorescence provided additional information: the photochemical yield of PSII (F_v/F_m ratio), typically affected by photoinhibition (Epron et al., 1992), was not affected by BOA; however, the quantum yield of electron flow through PSII *in vivo* (Φ_{PSII})

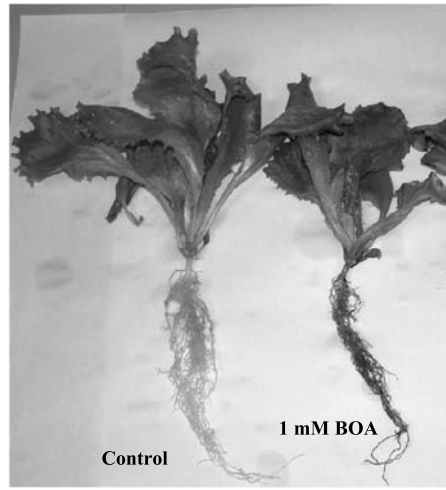

FIG. 1. Control and 1 mM BOA-exposed plants. Changes in leaf biomass and in root biomass were detected with highly significant differences after 15 d treatment.

was slightly affected. The unaltered F_v/F_m data suggest no changes on the intrinsic efficiency of PSII and, therefore, no direct damages on the PSII reaction centers. Nevertheless, the inhibition of Φ_{PSII} values could be a result of a weaker efficiency of PSII reaction centers, and it may indicate an alteration of the rate of linear electron transport (Nilsen and Orcutt, 1996), suggesting that the proportion of photons absorbed by PSII and used for photosynthesis was not the same as in control. This altered PSII efficiency, as well as the water unbalance observed on BOA-exposed plants, could be explained through an alteration on membrane properties.

Oxidative Damage after BOA Exposition. The antioxidant enzymes superoxide dismutase and guaiacol peroxidase were analyzed after 15 d BOA exposure in lettuce leaves and roots. Superoxide dismutase activity was severely inhibited in leaves and roots of the allelochemical-targeted plants with special relevance in roots, where the activity was reduced to 50%. Guaiacol peroxidase activity also suffered a strong decrease in lettuce leaves, although no effect was detected in roots (Table 2). The function of these two enzymes is essential for maintaining the equilibrium of free radicals, thus preventing oxidative damage (Zeng et al., 2001). This activity reduction can be produced by direct damage in the structure of enzymes, but it might also be due to a senescence process (Dhindsa et al., 1981). Decreased levels of both enzymes can imply increased levels of $O_2^{\cdot -}$, H_2O_2, and $OH^{\cdot}$ resulting in increased levels of lipid peroxidation and solute leakage (Dhindsa et al., 1981), which play a key role in the senescence process. $O_2^{\cdot -}$ that accumulated in the absence of superoxide dismutase

contributes to membrane damage, and it plays a key role in specific chains of signal transduction (Jabs et al., 1997). An excessive presence of H_2O_2 in the surroundings of the cell wall has been related to reduced plant cell expansion mediated by cell wall-associated peroxidases (Bacon, 1999). H_2O_2 diffusing into the continuous space of the cell wall is a limiting factor for its extensibility.

The low activities of superoxide dismutase and peroxidase, which suggest a negative effect of BOA on the protective ability of plant tissues against oxidative damage, can lead to an increased lipid peroxidation, which is highly significant for leaves as well as for roots in the BOA-targeted plants in the current experiment. The malondialdehyde acid (MDA) content of the samples treated with 1 mM BOA increased almost 100% in roots and 80% in lettuce leaves. Lipid composition of membranes is one of the main cellular targets susceptible to damage by free radicals (Davies, 1987). Lipid hydroperoxides, formed in the process of lipid peroxidation, have been found to alter membrane properties by increasing hydrophilicity of the internal side of the bilayer (Frenkel, 1991) with the consequent increase in membrane permeability and the alteration of nutrient balance. The structure of such important organelles as chloroplasts or mitochondria is also affected (Zeng et al., 2001). Lipid degradation products participate in the signal transduction cascade of the stress response (Blokhina et al., 2003). The reactive oxygen species (ROS) not only cause peroxidation of lipid membranes, but also "a depletion of reduced glutathione, oxidation of protein thiol groups and membrane depolarization" (Blokhina et al., 2003). The observed increase in soluble sulfhydryl groups due to BOA can be the result of ROS-induced damage on protein structure. Similar effects produced by other cyclic hydroxamic acids have been previously reported (Niemeyer et al., 1982; Friebe et al., 1997).

Total and plasma membrane ATPase activity are significantly reduced on lettuce roots exposed to BOA because of a decrease in plasma membrane ATPase activity. The inhibition of these primary pumps can disrupt the transport of ions, amino acids, sugars, and other molecules through plasma membranes. H^+-ATPase has been reported as an unlikely target site for nonspecifically interacting xenobiotics causing cytotoxicity by impairment of the plasma membrane (Palmgren, 1998). Inhibition of ATP synthesis in the presence of BOA via a direct action on the ATPase complex was first reported by Niemeyer et al. (1987). They attributed BOA's inhibitory effects to an impairment in the ATP synthesis as a result of BOA action on the mitochondrial electron transport and ATPase complex. An alteration of this enzymatic activity can result in an altered redox status by blocking proton pumping with a consequent decrease in the internal pH. This modification in the redox status can lead to the activation of the phenylpropanoid pathway and the subsequent lignification of the cell wall. Cell wall modifications are a common response of plants to environmental stress, limiting elongation and growth by altering the water balance. Alkalinization of apoplastic sap due to

reduced ATPase activities can be responsible for ABA-dependent restriction in leaf elongation rate (Bacon et al., 1998). In this sense, an increase in external pH due to alterations in the proton gradient will reduce cell wall expansins, whereas the cell wall-associated peroxidases will increase (Bacon, 1999).

Stress Markers. Reduction of total soluble leaf proteins in BOA-exposed plants could be the consequence of the increase in lipid peroxidation, which has been related with high levels of denatured proteins. Senescent plants have shown a diminution in their protein content by increasing proteolysis as well by reducing protein synthesis (Wen et al., 1996). This reduction of protein synthesis has been previously observed for BOA (Burgos et al., 2004) and other allelochemical compounds (Cameron and Julian, 1980).

Polyamines and diamines (Table 2) have been also largely implicated in free radical scavenging and plasma membrane stabilization, as well as in structural functions and protein and DNA synthesis (Schipper et al., 2000). Free spermine and spermidine have been reduced in BOA-exposed plants, whereas putrescinde content did not change significantly. Decrease in polyamines can lead to an alteration in the plasma membrane (Bors et al., 1989). Absence of free spermine and spermidine in the cell has been also reported as a common trait in apoptosis (Nitta et al., 2001), and can accelerate senescence (Borrell et al., 1997). The role of polyamines in the cell cycle control and cell membrane stabilization can lead to cell cycle arrest and direct or indirect apoptosis (Li et al., 1999), loss of cell integrity, and cell death (Schipper et al., 2000). Such alterations on free polyamine contents have been also found in the study of paraquat, a common herbicide known for its toxicity by way of oxidative damage (Kao, 1997). The protective effect observed here could be "specific to the pathway inducing apoptosis and may be mediated by activation of transcription of genes required for cell proliferation and survival" (Kilpeläinen, 2002).

Proposed Mode of Action. We attribute the results to the effect of BOA alone, although they could also be due to degradation products, both inside the plant and in the pots. We performed a parallel experiment to determine the fate of the allelochemical in solution (data not shown), and noted that it remained stable for 2 wk. Even so, some of the observed effects could still have been due to both BOA and degradation products.

BOA is acting at different physiological levels, and these effects are mutually correlated. BOA shows multiple modes of action where different altered metabolic processes must be considered in an interrelated manner. A scheme of the effects of BOA on lettuce metabolism is proposed (Figure 2). Besides particular metabolic effects, BOA exposure leads to a significant inhibition of plant growth and reduction in vigor.

As shown in Figure 2, BOA affects plant metabolism via oxidative damage. The data suggest that an alteration of cell redox status by acting on the oxygen protective cellular mechanisms (enzymatic system, polyamines, etc.) is

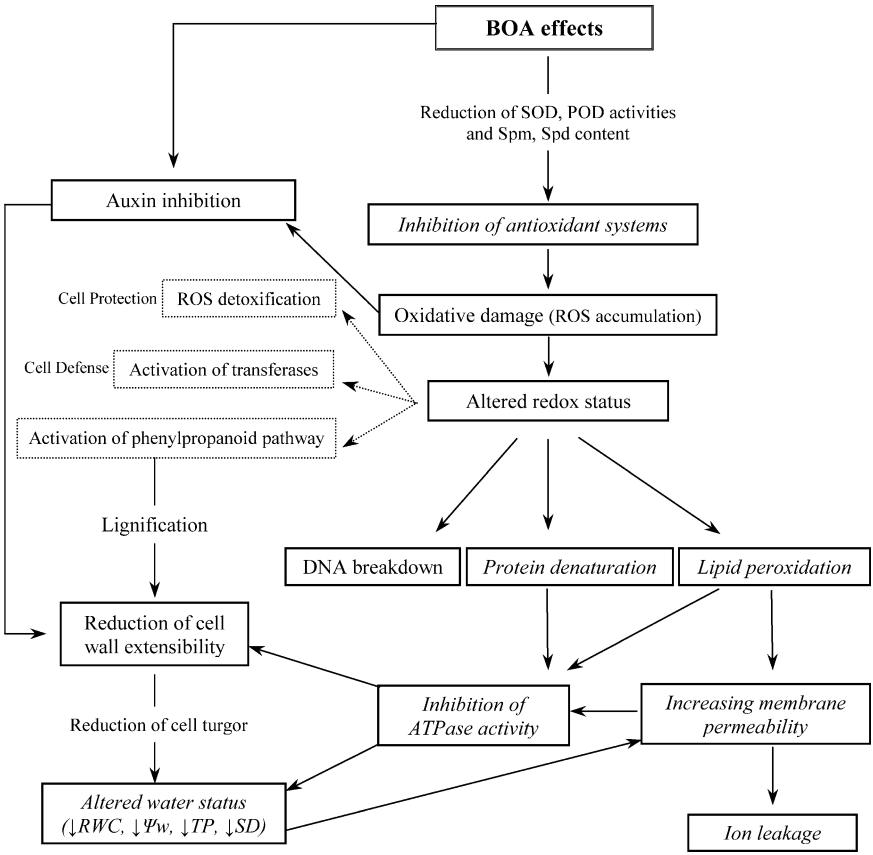

FIG. 2. Schematic representation of BOA toxicity via oxidative damage, auxin inhibition, or interference of both effects. Cell damage due to free radicals generated by BOA, and reductions in superoxide dismutase and peroxidase activities and spermine and spermidine contents lead to membrane alterations and water imbalance with drastic consequences for the cell. This proposed scheme is inspired by another theory proposed by Einhelig (1986) for the possible multiple mode of action of allelochemicals.

a direct metabolic consequence of BOA's presence. BOA could be interfering with key factors of the oxidative metabolism. Some effects on sulfhydryl groups of enzymes and on polyamine content should be considered important. The BOA redox cycle and its effects on antioxidant enzymes could generate the accumulation of free radicals that are toxic for the cell by direct action, increasing lipid peroxidation. In this sense, Burgos et al. (2004) found increased vacuolation and lipid content, concluding that BOA is reducing root growth by disrupting lipid metabolism and protein synthesis as part of its mode of action, protein

denaturation, chlorophyll bleaching, and DNA modifications. Indirect action is also considered to be important in BOA's mode of action: increasing membrane permeability, ion leakage, disrupting proton pump function, altering electron transport by modifications on chloroplast membrane. Simultaneously, ATPase activity is inhibited as a consequence of increased membrane permeability with the subsequent increase on ion leakage (reported in previous experiments; Reigosa et al., 2001) and modifications on membrane structure. A reduction in ATPase activity will increase pH level near the cell wall, and could cause a reduction of (indole-3-acetic acid) IAA-induced "acid growth." It could disturb the function of expansins causing a thicker cell wall. Decreases in cell wall extensibility will reduce turgor cell with the observed decreased Ψ_w and RWC. The strong reduction in transpiration and concomitantly in stomata density is a common mechanism of plants to avoid water stress due to this water imbalance. The decrease in transpiration and the previously detected effect on membrane integrity will be decisive factors in the severely reduced net photosynthetic rate, which will directly affect the C/N ratio and eventually plant growth and development.

Free radicals can act not only as toxic components on plant metabolism but also as signal factors. A plant mitogen-activated protein kinase (MAPK) pathway involved in mitotic regulation can be activated with an increased accumulation of free radicals such as H_2O_2 (reduction of guaiacol peroxidase activity) and $O_2\cdot^-$ (reduction of superoxide dismutase activity), resulting in the induction of stress-inducible genes and also in the inhibition of auxin-inducible genes (Hirt, 2000). The negative control on auxin genes would lead to decreased cell wall extensibility with reduction on cell turgor. It will lead to the previously discussed chain of effects: reduced transpiration, stomatal density, and net photosynthesis, but also diminished elongation, cell division, and lateral root growth. Reductions on lateral root growth and root length are similar to the effects in cucumber root tips following BOA exposure reported by Burgos et al. (2004), corroborating an important effect of this allelochemical on root ultrastructure. The consequences of these interactions will be reflected in decreased plant growth and development. In Perez (1990) hypothesized that hydroxamic acids could act as new auxin-inhibiting substances. We also report here that BOA could act at this level, probably by indirect action (oxidative damage).

In summary, we conclude that BOA affects the oxidative metabolism via multiple modes of action, and it likely interferes with auxin function by transcriptional regulation. It initiates a process of induced senescence with the consequent inhibition of plant growth and development in lettuce plants.

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PHEROMONAL ACTIVITY OF COMPOUNDS IDENTIFIED FROM MALE *Phyllotreta cruciferae*: FIELD TESTS OF RACEMIC MIXTURES, PURE ENANTIOMERS, AND COMBINATIONS WITH ALLYL ISOTHIOCYANATE

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Abstract—Four himachalene sesquiterpenes and (+)-γ-cadinene, previously identified as possible pheromone components from males of a North American population of *Phyllotreta cruciferae* Goeze (Coleoptera, Chrysomelidae), were tested for attractiveness in field trapping experiments in Hungary. A mixture of the four synthetic racemic himachalene derivatives and (+)-γ-cadinene from a botanical source was slightly attractive to beetles, but much more attractive when blended with the known host-plant-derived attractant allyl isothiocyanate. This result was consistent with a previous study in North America. In tests with optically pure synthetic compounds, a blend of the same himachalene enantiomers found from male beetles was equivalent to the corresponding blend of racemic compounds, whereas a blend of the opposite enantiomers was not active. Through subtraction tests, it was found that the single compound, (6*R*,7*S*)-2,2,6,10-tetramethylbicyclo[5.4.0.]-undeca-9,11-diene [compound (+)-**A** in this study], was as active as the whole mixture, suggesting that this compound is the key pheromone component of the European population of *P. cruciferae*. During field trials, several

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congeneric species, including *P. vittula*, *P. nemorum*, *P. nodicornis*, and *P. ochripes*, also were caught, suggesting that the same compound(s) may be relatively widespread as pheromone components in this genus.

Key Words—*Phyllotreta cruciferae*, *Phyllotreta* spp., Coleoptera, Chrysomelidae, field trapping, pheromone, sesquiterpene, himachalene, cadinene, enantiomer, electrophysiology

INTRODUCTION

Flea beetles of the genus *Phyllotreta* (Coleoptera, Chrysomelidae, Halticinae) are important pests of cruciferous crops such as cabbage, rapeseed, and radishes, both in Europe (Jourdheuil, 1966; Säringer, 1998) and in North America (Lamb, 1989). Their importance as pests is aggravated by the fact that several species are known to vector plant pathogens (Markham and Smith, 1949; Campbell and Colt, 1967; Ryden, 1989; Dillard et al., 1998; Glits, 2000).

Effective tools to detect and monitor flea beetles would be of great utility in their control. The secondary plant metabolite allyl isothiocyanate (allyl ITCN) has long been known as a feeding and oviposition stimulant and also as an attractant for *P. cruciferae* Goeze and other *Phyllotreta* spp. (Görnitz, 1956; Feeny et al., 1970; Hicks, 1974; Vincent and Stewart, 1984; Pivnick et al., 1992).

Male *P. cruciferae* produce an aggregation pheromone (Peng and Weiss, 1992; Peng et al., 1999). Analysis of volatiles emitted by males of a North American population of *P. cruciferae* identified six sesquiterpenes (Figure 1) as candidate pheromone components (Bartelt et al., 2001). These were produced only by males, although the major compound (**A**) was readily sensed by the antennae of both sexes (Bartelt et al., 2001). These compounds, or a subset of them, was thought likely to constitute the pheromone. All six compounds are chiral, and only the enantiomers shown in Figure 1 are emitted by the beetles. The beetle-derived enantiomers all have a positive optical rotation in hexane (Bartelt et al., 2001), and signs of rotation used below refer to dilute hexane solutions.

Assignment of the true stereochemistry for **A**, **C**, **E**, and **H** has been complicated. The configurations were initially determined by Bartelt et al. (2001): relative stereochemistry of the two stereogenic centers of **A**, **C**, and **H** was based on NMR and molecular modeling. Assignment of their absolute configurations was based on linking them to **E** through chemical conversions and comparison of the optical rotation of **E** to literature information. Pandy and Dev (1968) had reported an enantiomer of **E** from Himalayan cedar trees and established its absolute configuration as (*S*) by enantioselective synthesis. Beetle-derived **E** had a specific rotation that was of the same sign (+) as that

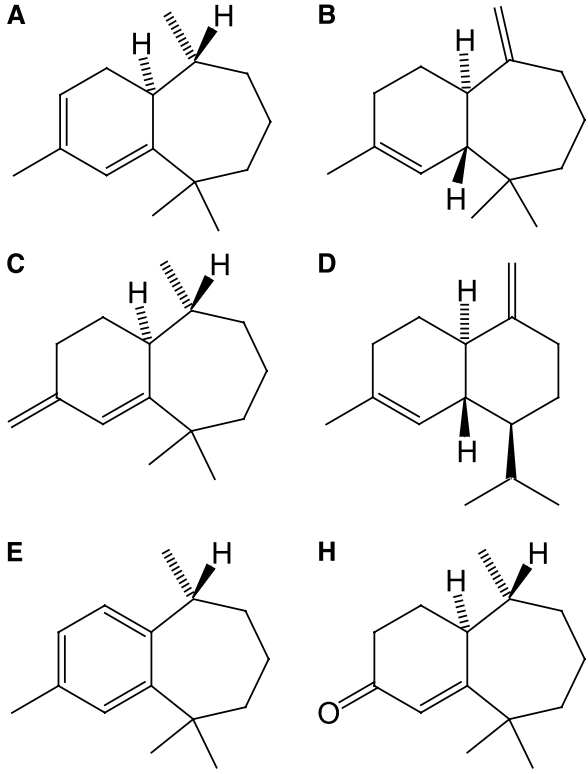

FIG. 1. Male-specific compounds emitted by *P. cruciferae*. Structure lettering corresponds to Bartelt et al. (2001); compounds **F** and **G** in that paper were found in *Aphthona* species but not in *P. cruciferae*. Absolute configurations shown for **A**, **C**, **E**, and **H** were established by chiral synthesis (Muto et al., 2004) and comparison to beetle-derived samples by chiral GC and polarimetry. (**A**) (+)-(6*R*, 7*S*)-2,2,6,10-Tetramethylbicyclo[5.4.0]-undeca-1(11),9-diene; (**B**) (+)-(1*R*,7*R*)-2,2,10-trimethyl-6-methylene-bicyclo[5.4.0]undec-10-ene; (**C**) (+)-(6*R*,7*S*)-2,2,6-trimethyl-10-methylene-bicyclo[5.4.0]-undec-1(11)-ene; (**D**) (+)-γ-cadinene; (**E**) (+)-(*R*)-*ar*-himachalene; (**H**) (+)-(1*S*, 2*R*)-2,6,6-trimethylbicyclo[5.4.0]undec-7-en-9-one.

reported by Pandy and Dev (1968) for the tree compound; thus, both were initially concluded to have the same configuration.

Bartelt et al. (2003) synthesized the racemic forms of the four compounds. Subsequently, Muto et al. (2004) synthesized the individual enantiomers of compounds **A**, **C**, **E**, and **H** using citronellal of known configuration as the chiral starting material. These synthetic studies supported the basic structures and relative stereochemistry reported by Bartelt et al. (2001), but disconcert-

ingly, the absolute configurations determined by Muto et al. (2004) were exactly the reverse of those proposed by Bartelt et al. (2001).

This contradiction was resolved by Mori (2005). The solvent used by Pandy and Dev (1968) to measure the optical rotation of **E** from cedar was chloroform, whereas that used by Bartelt et al. (2001) for beetle-derived **E** was hexane (because several of the beetle-derived compounds encountered in that study deteriorated in chloroform). Mori (2005) discovered, surprisingly, that the optical rotation of **E** in hexane is similar in magnitude—but opposite in sign—to that in chloroform, which explained the previous discrepancy in assigned configurations. Interestingly, compounds **A**, **C**, and **H** do not show a corresponding sign reversal with this solvent change (Bartelt, unpublished data). The structural assignments of Muto et al. (2004), shown in Figure 1, are considered definitive.

Field attractiveness of the sesquiterpenes was first tested by Soroka et al. (2005), using a blend of synthetic racemic **A**, **C**, **E**, and **H** (Bartelt et al., 2003), plus enantiomerically pure **D** (obtained from citronella oil; Soroka et al., 2005). The compounds were formulated on rubber septa so that the emitted ratios of beetle-related enantiomers would be the same as observed in volatile collections from the beetles (Bartelt et al., 2001). Compound **B** was not used in the study because no bulk source was found. Soroka et al. (2005) found that this blend by itself was modestly attractive at two doses, and that the pheromone effect was enhanced when allyl isothiocyanate (allyl ITCN) was present in the trap as well.

In the present paper, we report on the field activity of racemic and enantiomerically pure **A**, **C**, **D**, **E**, and **H** with a European population of *P. cruciferae*, in the presence or absence of allyl ITCN.

METHODS AND MATERIALS

The original objective of this research was to repeat the North American field tests (Soroka et al., 2005) on the European population of *P. cruciferae*. Subsequently, the pure synthetic enantiomers of **A**, **C**, **E**, and **H** became available (Muto et al., 2004), so that it was possible to compare these to the racemic materials. Because the initial field baits also contained **D**, an intermediate field experiment was run to measure its behavioral importance in the initial blend. Then the enantiomers of **A**, **C**, **E**, and **H** were compared directly, all as blends simulating beetle emissions, but lacking **D**. Finally, subtractive tests were conducted to determine whether the components present in minor amounts in the beetle emissions (**C**, **E**, and **H**) were important for attraction. All of the tests included treatments with allyl ITCN.

Electrophysiology. Additional data were acquired during 2003 to evaluate antennal responses of the North American strain of *P. cruciferae* to the six

male-specific compounds. Coupled gas chromatography–electroantennographic detection (GC-EAD) was done as described previously (Bartelt et al., 2001). A solution containing the beetle-derived enantiomers of **A**–**E** and **H** was prepared (ca. 10–20 ng/μl) and tested with antennae of female beetles (about 1 μl per injection, $N = 4$).

Field Sites. Tests in Hungary were conducted at Budakeszi, Pusztazámor (Pest county), and Nadap (Fejér county). Trapping tests were performed in rapeseed or white mustard fields. Traps were set at the soil level in the weedy edge of the fields. Traps were arranged as blocks so that each block contained one replicate of each treatment. Traps within blocks were separated by 8–10 m, and blocks were sited 15–20 m apart.

Capture data were transformed to $(x + 0.5)^{1/2}$ and were analyzed by ANOVA. Treatment means were separated by Games–Howell test or by Bonferroni–Dunn test, as appropriate (see also table and figure legends). All statistical procedures were conducted using the software packages StatView® v4.01 and SuperANOVA® v1.11 (Abacus Concepts, Inc., Berkeley, CA, USA).

Traps. CSALOMON® VARL+ funnel traps (Plant Prot. Inst., HAS, Budapest, Hungary) were used in the tests. These originally were developed for catching moths (Tóth et al., 2000; Subchev et al., 2004) but proved to be applicable also for capturing flea beetles (Tóth et al., 2004). The trap consisted of an opaque plastic funnel (top o.d.: 13 cm; funnel hole diam: 3 cm; height: 16 cm), covered with a 20 × 20 cm flat plastic roof, and connected below to a transparent plastic round catch container (about 1-l capacity; attached by a rubber band). The bait was suspended from the middle of the roof, and the bait dispenser was slightly above the level of the upper edge of the large funnel opening. A small piece (1 × 1 cm) of household pesticidal strip (Chemotox®, Sara Lee, Temana Intl. Ltd., Slough, UK; active ingredient 15% dichlorvos) was placed into the catch container as a killing agent for captured insects.

Chemicals. Racemic pheromone components **A**, **C**, **E**, and **H** were synthesized by described methods (Bartelt et al., 2003). The beetle-specific enantiomer of compound **D** was obtained from citronella oil (Soroka et al., 2005). The individual enantiomers (about 97% enantiomeric purity) of **A**, **C**, **E**, and **H** were available from Muto et al. (2004). Allyl isothiocyanate was purchased from Sigma-Aldrich Kft. (Budapest, Hungary) and was >95% pure as stated by the supplier.

Baits. The allyl ITCN baits for experiments 1 and 2 used 100-mg amounts administered onto a 1-cm piece of dental roll (Celluron®, Paul Hartmann Ag, Heidenheim, Germany), which was placed into a polyethylene bag (about 1.0 × 1.5 cm) made of 0.02-mm-thick polyethylene film. The dispensers were heat-sealed and were attached to 8 × 1 cm plastic handles for easy manipulation when assembling the traps. In later experiments, 2 mg of allyl ITCN was loaded into 0.7-ml polyethylene vials with lid (No. 730, Kartell Co., Italy). These vials

were used with lids closed (so that allyl ITCN penetrated through the walls) in the field tests. Baits were wrapped singly in pieces of aluminum foil and were stored at −65°C until use. In the field, old baits were replaced with new ones at 2- to 3-wk intervals.

Pheromone baits were prepared as described in detail by Soroka et al. (2005). When the standard dose of racemic **A**, **C**, **E**, and **H** was used, the load rates were 500, 34, 56, and 164 μg per septum, respectively. When pure enantiomers of **A**, **C**, **E**, and **H** were used, the weights of the compounds were one half of the racemic amounts (250, 17, 28, and 82 μg per septum, respectively), so that the amount of a given enantiomer was constant. Compound **D**, a single enantiomer, was used at 123 μg per septum. For subtractive blends, the amounts of the components that were included were as above. Low-dose septa were also prepared for experiments 1 and 2, and these had one tenth the amount of material in the standard dose.

Field Experiments. Experiment 1 and 2. These preliminary tests were aimed at studying the activity of the mixture of components **A**, **C**, **E**, and **H** (racemic) and (+)-**D** on its own and the influence of its addition to allyl ITCN. Treatments included traps with components **A**, **C**, **E**, and **H** (racemic) and (+)-**D** at two dose levels, allyl ITCN on its own, its combination with the beetle-related components, and unbaited traps. Polyethylene bag dispensers were used to dispense allyl ITCN. Experiment 1 was run at Nadap, April 1–18, 2003, with five replicate blocks, in the weedy edge of a field that had been planted in rapeseed in 2002. Traps were inspected twice weekly. Experiment 2 was conducted at Budakalász, March 27–May 1, 2003, with five replicate blocks, in the weedy edge of a 2002 white mustard field.

Experiment 3. This test was aimed at confirming results of the preliminary tests on the increase of catches when components **A**, **C**, **E**, **H** (racemic), and (+)-**D** and allyl ITCN were presented together in the same trap, but with a lower dose of allyl ITCN (polyethylene vials, see above). Treatments included traps with components **A**, **C**, **E**, **H** (racemic), and (+)-**D** on their own, their combination with allyl ITCN, allyl ITCN on its own, and unbaited controls. The experimental methods were similar to experiments 1 and 2. The site was Budakalász, August 19–27, 2003, with 10 replicate blocks in a white mustard field (after harvest). Traps were inspected every other day. Allyl ITCN dispensers were replaced with fresh ones on August 23.

Experiments 4 and 5. The objective was to measure the importance of (+)-**D** to the attractiveness of the mixture of beetle-related compounds. Treatments included traps with allyl ITCN on its own (polyethylene vial), its combination with component (+)-**D**, its combination with components **A**, **C**, **E**, and **H** (racemic), or its combination with all components **A**, **C**, **E**, and **H** (racemic) and (+)-**D** and unbaited control traps. These treatments were complemented with traps with a mixture of all components **A**, **C**, **E**, and **H** (racemic) and (+)-**D** on

its own in experiment 5. Two parallel tests were conducted: (1) at Budakalász, August 19–27, 2003, with 10 replicate blocks in a white mustard field (after harvest); allyl ITCN dispensers were replaced by fresh ones on August 23 (experiment 4); and (2) at Pusztazámor, September 10–19, 2003 (experiment 5), with six replicate blocks in the weedy edge of a harvested rapeseed field. Allyl ITCN dispensers were replaced by fresh ones on September 15.

Experiment 6. This test was aimed at studying the activity of pure enantiomers of components **A**, **C**, **E**, and **H**. Treatments included traps with allyl ITCN on its own (polyethylene vial), its combination with pure (+) or pure (−) enantiomers of **A**, **C**, **E**, and **H**, or with their racemic mixture, and unbaited control. The test was conducted at Budakalász, September 10–19, 2003, with 10 replicate blocks in a white mustard field (after harvest). Traps were inspected every other day. Allyl ITCN dispensers were replaced by fresh ones on September 15.

Experiment 7. This test measured whether components **C**, **E**, and **H** could be omitted from the test mixture without apparent loss of activity. A secondary objective was to confirm results in experiment 6 on the activity of pure enantiomers. Treatments included traps with allyl ITCN on its own (polyethylene vial) and traps with allyl ITCN in combination with pure (+), with pure (−), and with racemic **A**, **C**, **E**, and **H**, with only components **A** and **C** (racemic), and with only component **A** (racemic). Traps without bait were also set out as a control. The test was run at Pusztazámor, April 4–19, 2004, with five replicate blocks in the weedy edge of a white mustard field. Allyl ITCN dispensers were replaced by fresh ones on April 13.

RESULTS

Electrophysiology. In all of the GC-EAD analyses with *P. cruciferae* (example shown in Figure 2), compound **A** was the most strongly detected. Weaker responses were always elicited by **C** and **H** as well, but components **B**, **D**, and **E** elicited no antennal responses.

Field Experiments. Experiments 1, 2, and 3, activity of mixture of components **A**, **C**, **E**, and **H** (racemic) and (+)-**D** on its own, and the influence of its addition to allyl ITCN. In the test at Nadap (Figure 3A), few *P. cruciferae* were caught in traps containing only racemic **A**, **C**, **E**, and **H** and (+)-**D** (referred to below as **ACDEH**), although traps with the larger dose caught more beetles than unbaited traps. More beetles were found in traps with allyl ITCN than in unbaited traps. Catches increased further when dispensers with **ACDEH** were added to traps with allyl ITCN, the difference from addition of allyl ITCN being significant with the larger dose. Catches showed the same trend in the parallel

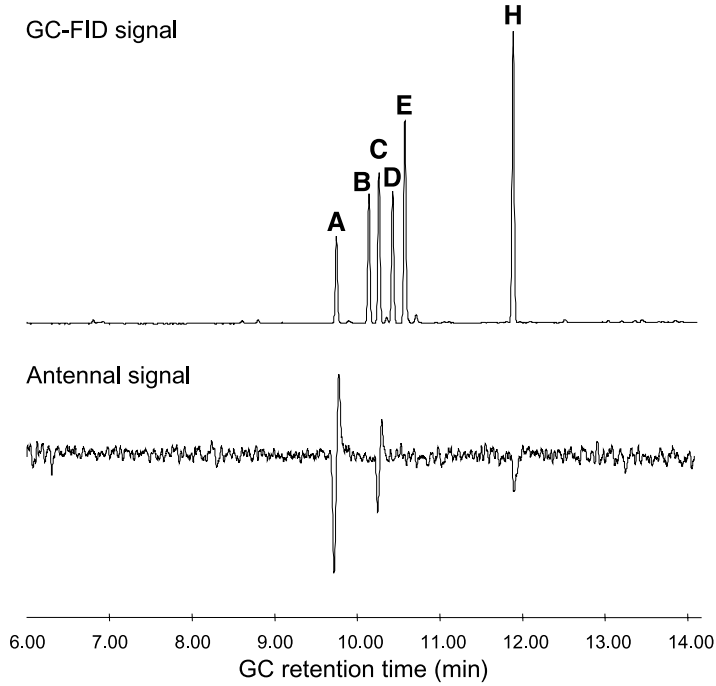

FIG. 2. Coupled GC-EAD response of an antenna from female *P. cruciferae* to a mixture of compounds **A**–**E** and **H**. Responses were noted to compounds **A**, **C**, and **H**.

test at Budakalász (Figure 3B), although there was no difference between traps with allyl ITCN on its own and those containing **ACDEH** + allyl ITCN.

Large numbers of the closely related *P. vittula* (Redtenbacher) were also caught. Traps with the combination of a high dose of allyl ITCN and **ACDEH** caught more than allyl ITCN alone in both tests (Figure 3A, B). Other treatments caught negligible numbers of beetles.

The catches of *P. nodicornis* (Marsham) and *P. ochripes* (Curtis) at Nadap (Figure 3A) and those of *P. nemorum* (Linnaeus) at Budakalász (Figure 3B) also showed similar trends.

In the ensuing tests (experiments 3–7), the dose of allyl ITCN was decreased in the hope that effects of the **ACDEH** mixture would be easier to detect. In experiment 3, traps with allyl ITCN plus **ACDEH** caught more *P. cruciferae* than allyl ITCN on its own (Figure 4). Both allyl ITCN and the **ACDEH** blend attracted more beetles than unbaited traps, and allyl ITCN on its own attracted more than the **ACDEH** blend. No other flea beetle species were caught in significant numbers in this test.

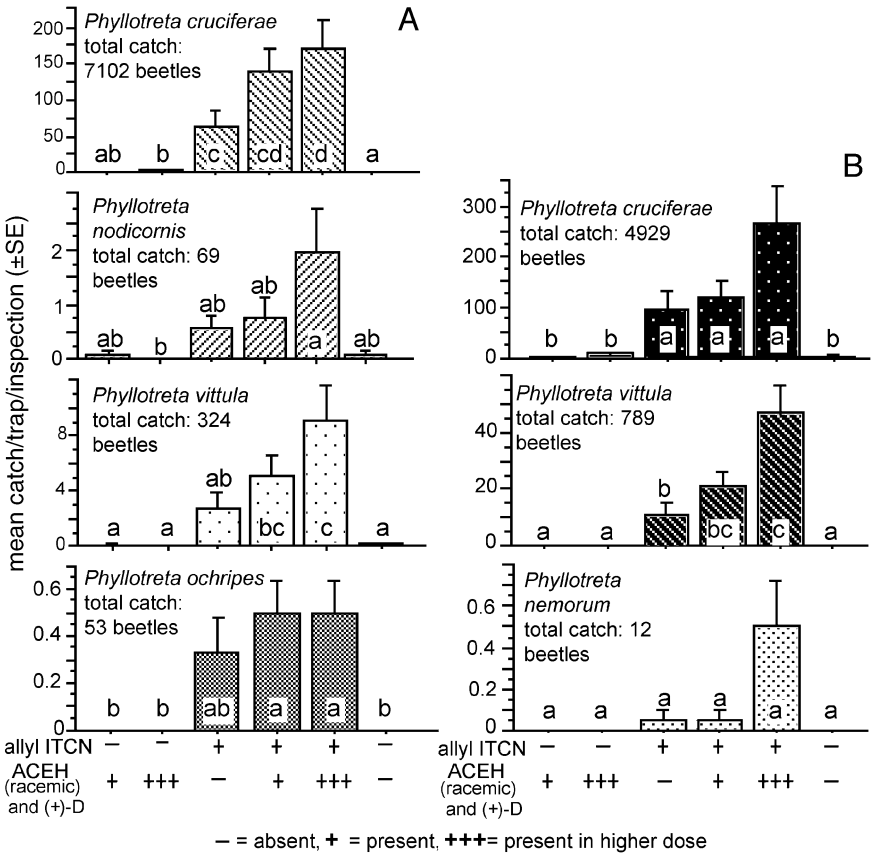

FIG. 3. Catches of flea beetles in traps baited with allyl ITCN, a mixture of racemic **ACEH** and (+)-**D** (at two doses), beetle compounds and allyl ITCN together, or controls. (A) Nadap, April 1–18, 2003 (experiment 1). (B) Budakalász, March 27–May 5, 2003 (experiment 2). Significance: within one diagram, bars with the same letters are not significantly different ($P > 0.05$, ANOVA then Games–Howell). In cases where one or more of the treatments caught no beetles, significant differences from zero catch were checked by Bonferroni–Dunn ($P > 0.05$).

Experiments 4 and 5, omission of component **D** from the mixture of **A**, **C**, **D**, **E**, and **H**. The largest catches of *P. cruciferae* were recorded in traps with allyl ITCN plus **ACDEH** or **ACEH** [component (+)-**D** subtracted from **ACDEH**], which differed significantly from catches of all other treatments (Figure 5A). Allyl ITCN attracted more beetles than unbaited traps, and allyl ITCN plus component (+)-**D** attracted more beetles than allyl ITCN alone,

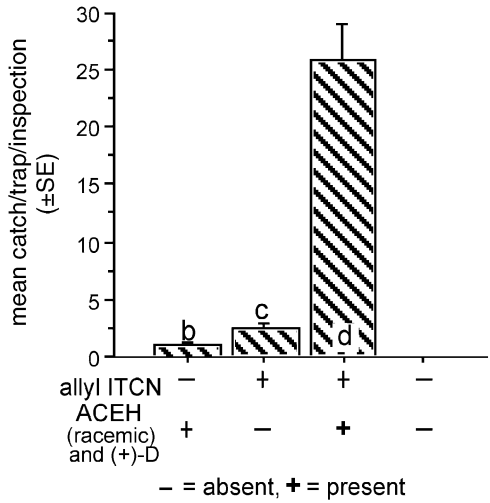

FIG. 4. Catches of *P. cruciferae* in traps baited with allyl ITCN as a single component, a mixture of racemic **ACEH** and (+)-**D**, both baits together, or controls (experiment 3). Budakalász, August 19–27, 2003. For significance levels, see Figure 3.

suggesting some activity for component (+)-**D**. In this test, *P. vittula* were caught in low numbers, the only treatment catching more beetles than unbaited controls being allyl ITCN plus **ACEH**.

In a supplementary test at Pusztazámor, *P. cruciferae* catches showed similar patterns (Figure 5B), with the exception that traps with the combination of allyl ITCN plus (+)-**D** were no better than allyl ITCN. The mixture of **ACDEH** without allyl ITCN (which was not tested in the previous test in Figure 5B) again showed minimal activity.

In this test, captures of *P. vittula* were best in traps with allyl ITCN plus **ACDEH** or **ACEH** (Figure 5B). Lower catches were observed in traps with allyl ITCN on its own. Unbaited traps or traps with **ACDEH** did not catch beetles.

Similar trends also were observed with *P. nigripes* (Fabricius) (Figure 5B), although no significant difference was found among traps containing allyl ITCN on its own or in combination with other components. Unbaited traps or traps with **ACDEH** alone caught fewer beetles than other treatments.

Experiment 6, activity of pure enantiomers of components **A**, **C**, **E**, and **H**. Blends of the pure enantiomers of **ACEH** with allyl ITCN and racemic **ACEH** with allyl ITCN were equally attractive to *P. cruciferae* (Figure 6). The blend of allyl ITCN with the (−) enantiomers of **ACEH** was no different than allyl ITCN alone.

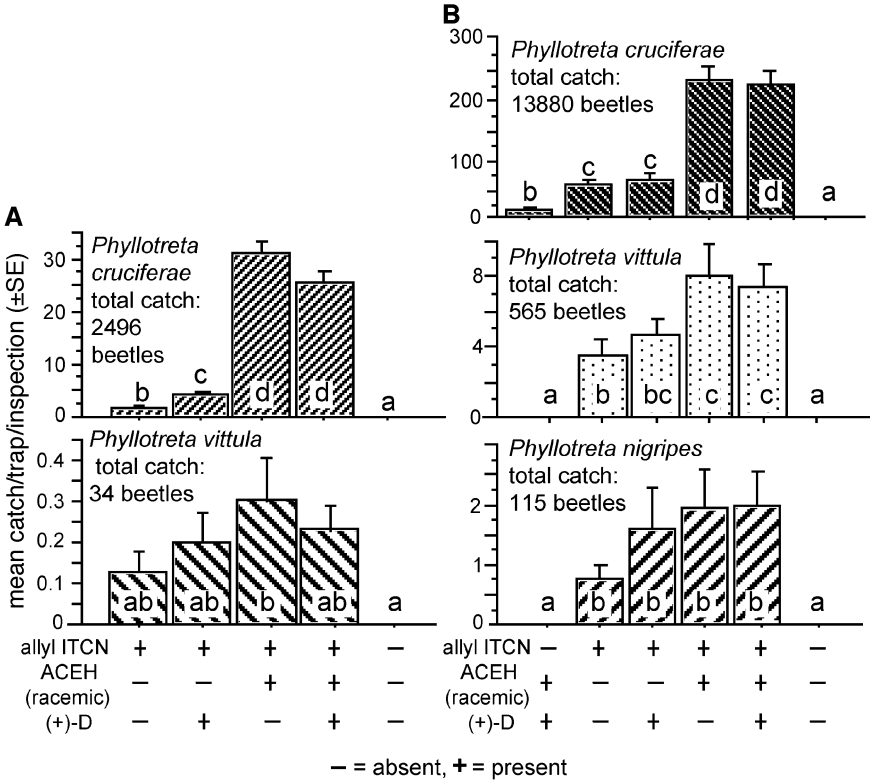

FIG. 5. Catches of *P. cruciferae* in traps baited with allyl ITCN as a single component, or in combination with component (+)-**D**, with racemic mixture **ACEH**, or with all five compounds. (A) Budakalász, August 19–27, 2003 (experiment 4); (B) Pusztazámor, September 10–19, 2003 (experiment 5). For significance levels, see Figure 3.

Several other species were caught [*P. vittula*, *P. procera* (Redtenbacher), *P. nigripes*, and *P. undulata* (Kutschera)] in low numbers, but, in general, no treatment was better than allyl ITCN (Figure 6).

Experiment 7, subtraction test of activity of pure enantiomers of components **A**, **C**, **E**, and **H**. More *P. cruciferae* were caught in traps baited with the (+) enantiomers or racemic **ACEH** plus allyl ITCN than in traps with the minus (−) enantiomers or with allyl ITCN on its own (Figure 7). Activity did not decrease when components **E**, **H**, and **C** were removed from the blends, indicating minimal or no role of **E**, **H**, or **C** as pheromone components.

Catches of *P. vittula* showed the same tendency, but the only treatment attracting more than allyl ITCN on its own was the racemic **ACEH** plus allyl ITCN combination (Figure 7).

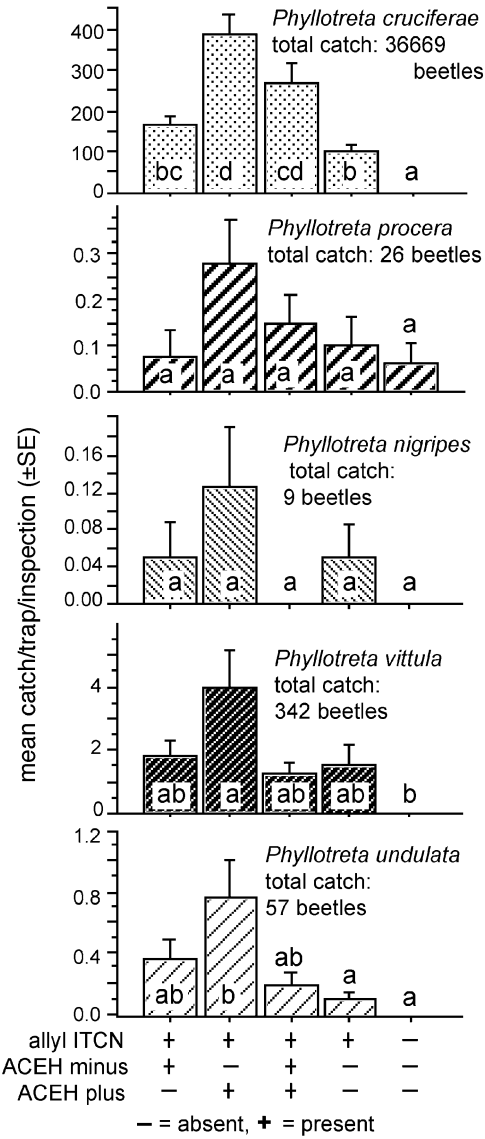

FIG. 6. Catches of *Phyllotreta* species in traps baited with allyl ITCN or its combination with (+) or (−) enantiomers of **ACEH**, or racemic **ACEH**. Budakalász, September 10–19, 2003 (experiment 6). For significance levels, see Figure 3.

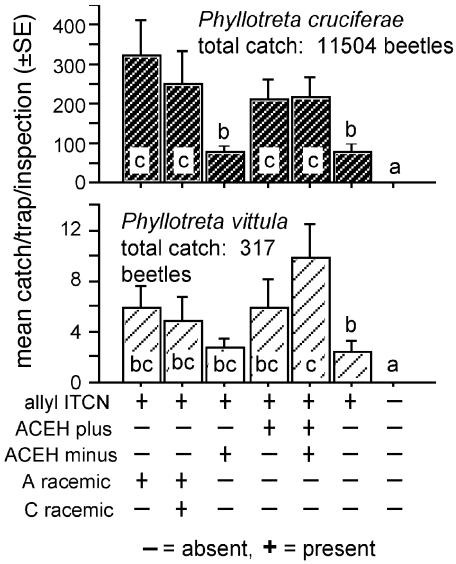

FIG. 7. Catches of *Phyllotreta* species in traps baited with allyl ITCN or its combination with (+) or (−) enantiomers of **ACEH** or with racemic **ACEH**, **AC**, or **A**. Pusztazámor, April 4–19, 2004 (experiment 7). For significance levels, see Figure 3.

DISCUSSION

In the present study, a mixture of components **A**, **C**, **D**, **E**, and **H**, although marginally attractive on its own, increased catches of *P. cruciferae* when presented together with the host-plant-derived attractant allyl ITCN. This is consistent with the results from North America (Soroka et al., 2005), suggesting that the pheromone compositions of the European and North American populations of *P. cruciferae* are similar.

There are a number of examples known of insect pheromones being synergized by host volatiles. In the well-documented case of *Melolontha* scarab beetles (Coleoptera, Scarabaeidae), males orient towards green leaf volatiles originating from damaged leaves from feeding female beetles (Ruther et al., 2000, 2002). Green leaf volatiles are somewhat attractive (Imrei and Tóth, 2002; Reinecke et al., 2002b), but many more beetles are attracted to blends of the green leaf volatiles with the pheromone components benzoquinone or toluquinone (Reinecke et al., 2002a; Ruther and Hilker, 2003). The quinones on their own show no activity. Similarly, with the scarab *Oryctes elegans* Prell. (Coleoptera, Scarabaeidae), the main aggregation pheromone component alone

was minimally attractive but, when presented together with host plant odor, it was clearly synergistic (Rochat et al., 2004).

Similar cases of strong synergism between pheromone components and host volatiles have been described for several species of *Rhynchophorus* weevils (Coleoptera, Curculionidae, Jaffé et al., 1993; Giblin-Davis et al., 1994; Oehlschlager et al., 1995; Rochat et al., 1995) and *Carpophilus* spp. sap beetles (Coleoptera, Nitidulidae; reviewed by Bartelt, 1999).

In the present study, beetles of both sexes were caught, but no special effort was made to study possible sex-specific differences in responses of *P. cruciferae*. In any case, effects would have been obscured by the activity of allyl ITCN, which attracts both sexes. It remains to be determined whether the communication strategy of *P. cruciferae* is sex-related, as in *Melolontha* scarabs, or is of the aggregation type as in the other examples above.

The biological activity of the insect-produced compounds was clearly connected with chirality. Because the (−) enantiomers were not inhibitory, racemic samples could be used as bait components in trapping studies. Furthermore, compounds **C**, **D**, **E**, and **H** had no obvious biological activity. Compound (+)-**A** [(6*R*,7*S*)-2,2,6,10-tetramethylbicyclo[5.4.0.]undeca-9,11-diene] was the only male-specific compound for which pheromonal activity could be clearly established for *P. cruciferae*. The closely related *P. vittula* responded in similar fashion to *P. cruciferae* with respect to the component mixtures and pure enantiomers tested. This suggests that compound **A** plays a role in pheromonal communication of this species as well. It remains to be seen whether *P. vittula* males produce this compound. *P. vittula* is among the economically most important pest flea beetles in Europe (Kaszab, 1962; Jourdheuil, 1966; Vig, 1996). Other *Phyllotreta* species were caught in low numbers, and the general trends resembled those seen with *P. cruciferae* and *P. vittula*. This suggests that the compounds tested may occur widely within the genus. Previously, compound **A** was found to be a common major component in the emissions collected from males of several North American flea beetle species (Bartelt et al., 2001) but at present, field activity data are available only for *P. cruciferae*.

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IDENTIFICATION OF A SEX PHEROMONE FROM MALE YELLOW MEALWORM BEETLES, *Tenebrio molitor*

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Abstract—The sex pheromone released by the adult female *Tenebrio molitor*, 4-methyl-1-nonanol, is well known. In addition, there is evidence that adult males release a pheromone that attracts females. The purpose of the present study was to isolate and identify male-released pheromone(s). Emissions from virgin adult males and females were collected on filter paper and extracted with pentane. Extracts were analyzed by gas chromatography-mass spectrometry. One male-specific compound was detected and identified as (*Z*)-3-dodecenyl acetate (Z3-12:Ac). In arena bioassays, *E*3-12:Ac was attractive to females only, at 1 and 10 μg doses. E3-12:Ac was also attractive to females at a 10-μg dose. The presence of both male and female pheromones, each attracting the opposite sex, may contribute to maintaining a high-density population of both sexes.

Key Words—4-methyl-1-nonanol, bioassay, Coleoptera, (*E*)-3-dodecenyl acetate, electroantennography, male sex pheromone, *Tenebrio molitor*, (*Z*)-3-dodecenyl acetate.

INTRODUCTION

Tenebrio molitor L. (Coleoptera: Tenebrionidae) is a cosmopolitan secondary pest and scavenger of many stored cereal products. *T. molitor* are also found in chicken and other bird houses where feathers, food, and excrement are mixed (Cotton, 1956). The larvae of *T. molitor* are widely available as pet food for birds, fish, and reptiles (Rees, 2004).

Adult male and female mealworms both produce sex pheromone(s) that elicit responses from the opposite sex (Happ and Wheeler, 1969; Smart et al.,

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1980). The sex pheromone released by the adult female, 4-methyl-1-nonanol, is well known (Tanaka et al., 1986).

Adult males release a pheromone that attracts females (Valentine, 1931; Tschinkel et al., 1967; Happ, 1969; August, 1971; Tanaka et al., 1986), and possibly also an antiaphrodisiac that inhibits the response of other males to the female scent (Happ, 1969). August (1971) reported that locomotion of females decreased in close proximity to males in response to a male-produced pheromone, resulting in females aggregating near live males. If the live male was moved to another chamber of the olfactometer, females reaggregated in this chamber within an hour. Live males or extracts thereof did not cause aggregation of males. The purpose of the present study was to identify the volatile pheromone(s) produced by male *T. molitor* that elicited this aggregation behavior.

METHODS AND MATERIALS

Insects. Tenebrio molitor, a laboratory strain kept in culture since 1958, was cultured on a 5:5:1 mixture by weight of wheatfeed, rolled oats, and yeast at 25°C, 70% RH, with a 16L:8D photoperiod. Insects were sexed at the pupal stage (Bhattacharya et al., 1970), kept separately, and tested as adults between 8 and 25 d after pupal eclosion.

Chemicals. 4-Methyl-1-nonanol was synthesized by $LiAlH_4$ reduction of 4-methylnonanoic acid (purity 99%; Aldrich Flavors and Fragrances, UK). (*Z*)-3-Dodecenyl acetate (Z3-12:Ac; isomeric purity 99%) and (*E*)-3-dodecenyl acetate (E3-12:Ac; isomeric purity 99%) were obtained from Pherobank, Research Institute for Plant Protection, The Netherlands. Pentane (glass distilled grade) was from Rathburns (UK). Cylclohexane (purity 99.99%) was from Aldrich (UK).

Preparation and Analysis of Extracts. Two sexed groups of five to six virgin male and female adults (8–25 d after pupal eclosion) were kept in glass jars (50 × 50 mm) for 24 hr at 20°C in a 16L:8D photoperiod with one 50-mm-diam disk of filter paper (Whatman qualitative No.1) placed in the lid of each jar. The filter papers were then placed into separate 5-ml Supelco glass microreaction vessels and extracted with 3 ml of pentane for 30 min, and the extracts concentrated to 50 μl by using a stream of nitrogen. A group of virgin males ($N = 11$) was also directly extracted with 5 ml of pentane for 1 hr and the extract was concentrated to 100 μl with a stream of nitrogen. This extract was used for ozonolysis of the male extract, and determination the stereochemistry of the male pheromone.

Extracts were analyzed on a Hewlett-Packard 5890 series II coupled to a VG Trio-1 mass spectrometer (MS). Aliquots of the extracts (3 μl) were injected in splitless mode (purge on after 1 min) at 250°C using helium as a carrier gas in

constant flow mode. A 50-m Chrompack CP-Sil 19CB column (0.25 mm i.d., film thickness 0.2 μm) was used except for the determination of the stereochemistry of the pheromone, where a CP-Wax 52CB column was used (0.25 mm i.d., film thickness 0.2 μm). The GC was programmed from 50°C 1 min to 270°C at 10°C/min, and maintained for 20 min. The MS was operated in electron impact ionization mode (EI, 70 eV) and scanned from 33 to 650 amu once every second. The MS and interface temperatures were 200 and 275°C, respectively.

Ozone was bubbled into a 5-ml glass microreaction vessel containing an extract of males in pentane for 1 min. An aliquot (3 μl) of the resulting extract was then analyzed by GC-MS on a CP-Sil 19CB column. The extract was repeatedly ozonolyzed until the male produced GC peak completely disappeared.

Authentic standards of Z3-12:Ac and E3-12:Ac were analyzed by GC-MS using the CP-Wax 52CB column. The oven was programmed from 50°C 1 min to 120°C at 8°C/min, hold for 27 min, then to 250°C at 10°C/min. An aliquot of an extract of males was analyzed under the same conditions.

Electroantennogram Studies. Prior to testing Z3-12:Ac in behavioral bioassays, the electroantennogram (EAG) response of both male and female *T. molitor* to Z3-12:Ac and E3-12:Ac was examined. Whole *T. molitor* proved difficult to restrain so head preparations were used, similar to EAG studies with other coleopteran species (e.g., Okada et al., 1992; Scholz et al., 1998).

Five male and five female adult *T. molitor* (less than 3 wk post-eclosion) were tested. Insects were removed from a culture approximately 1 hr prior to testing. The head was removed and secured using double-sided adhesive tape. The recording and indifferent electrodes were positioned in the terminal antennal segment and in the neck cavity, respectively. Electrodes were filled with saline (Roelofs, 1984) and connected, using chloridized silver wires, to a Grass P16 preamplifier and a chart recorder.

The odor delivery system was as described by White and Birch (1987). Z3-12:Ac and E3-12:Ac were presented at five doses ranging from 1 ng to 10 μg in 10 μl of cyclohexane. The test materials were presented at ca. 2-min intervals in ascending concentration and at each concentration the order of presentation was random. To account for variations due to fatigue, a standard of carob extract prepared from Porapak-Q collected volatiles from kibbled carob pods, was used (Wakefield et al., 2005). The standard was tested at the start and the end of each test and after presentation of each log dose of the two isomers. The absolute net EAG response to a test material or the standard was determined by first subtracting the mean response to a solvent control stimulation at the start and the end of each test from the response to the sample. Results were then expressed as a percentage of the response to the standard by:

$$\text{Relative net EAG}_x = \frac{2 \times \text{absolute net EAG}_x}{\text{absolute net standard}_x + \text{standard}_{x+1}} \times 100$$

Responses of males and females were compared using Student's *t* test. The perception threshold, defined as the lowest amount at which the absolute EAG response to the test stimulus was significantly greater than the mean of the control responses, was also determined.

Behavioral Bioassays. Pupae were removed from the culture and kept at 22°C and 16L:8D photoperiod, in individual glass tubes (75 × 25 mm) with a perforated plastic lid for ventilation and a folded filter paper as a crawling surface. The bioassay was adapted from the method described by Finnegan and Chambers (1993). Individual insects were placed in an arena comprised a 20-cm-diam metal ring on filter paper circles (27 cm diam, Whatman No. 1) and covered with a glass plate. The rings were 40 mm high and narrowed toward the top, making a considerable overhang for the insects to climb, thus preventing escape. Insects were allowed to settle for 30 min. One insect was introduced into the center of each arena. With the beetle at the perimeter of the arena, one 20-mm-diam filter paper disk (Whatman No.1) treated with test material in 10 μl of pentane and a control disk treated with 10 μl of pentane were placed 25 mm apart, equidistant from the center of the arena along a diameter perpendicular to the insect. The solvent was allowed to evaporate from the disks for 30 sec before they were placed in the arenas. The disk which was touched first was recorded (= a behavioral response). Bioassays were run for 10 min. Except where stated otherwise, each dose of each test material was tested against 40 adults of each sex.

Doses of 100 ng, 1 μg, or 10 μg of Z3-12:Ac in 10 μl of pentane were applied to test filter papers and tested against both males and females. E3-12:Ac was tested against females only at (10 μg), so that its biological activity could be compared with that of Z3:12:Ac, even though E3-12:Ac was not found in male extracts. The responses of males and females to each dose of Z3-12:Ac, and E3-12:Ac *versus* the controls were analyzed using χ^2 test with Yates' correction for 1 degree of freedom.

RESULTS

Identification of Material Produced by Males. The extract of filter papers exposed to females contained 4-methyl-1-nonanol, previously identified as a sex pheromone released by females (Tanaka et al., 1986). Its identity was confirmed by matching its mass spectrum and retention time on the CP-Sil 19CB column with those of an authentic standard.

Extracts of filter papers exposed to males (Figure 1C) contained a compound (peak 2) that was not detected in extracts of blank filter papers (Figure 1A), or filter papers exposed to females (Figure 1B). This compound gave a fragmentation pattern (Figure 2A) with ions at *m*/*z* 61, 96, 109, 124, 138,

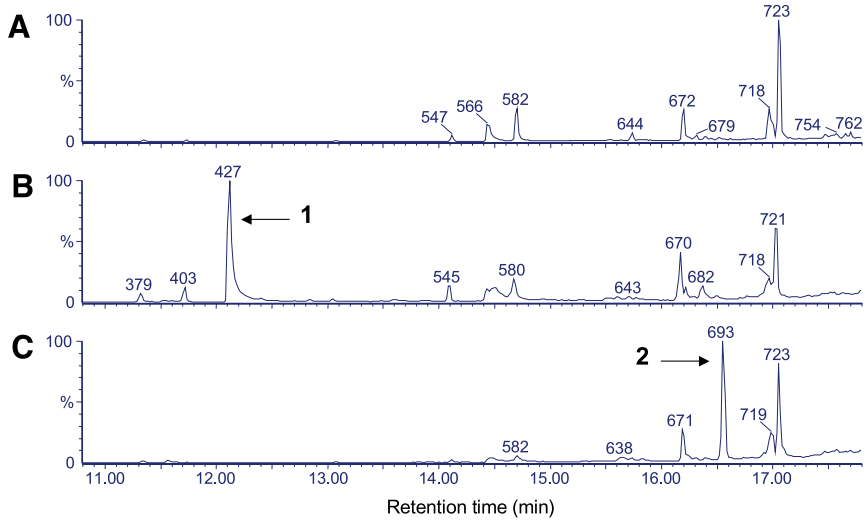

FIG. 1. Total ion chromatograms of extracts of filter papers which were (A) blank, (B) exposed to five females, and (C) exposed to five males. Identifications of peaks: (**1**) 4-methyl-1-nonanol, (**2**) Z3-12:Ac. CP-Sil 19CB column.

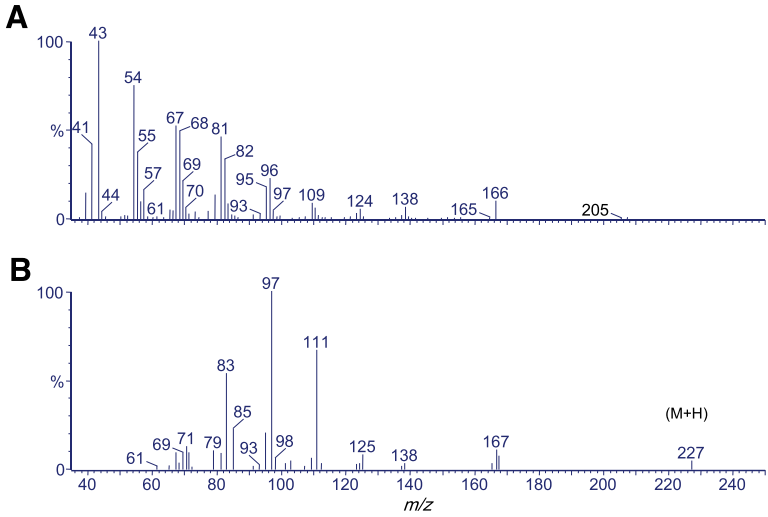

FIG. 2. (A) EI and (B) CI mass spectra of male produced compound.

TABLE 1. MEAN EAG RESPONSES (± SE) IN MICROVOLTS TO Z3-12:AC AS A PERCENTAGE OF THE RESPONSE TO A CAROB EXTRACT STANDARD (N = 5)

Dose	Female	Male
1 ng	−4.5 ± 2.1	0.5 ± 3.7
10 ng	3.0 ± 3.4	0.8 ± 3.9
100 ng	23.6 ± 8.4	6.2 ± 6.0
1 μg	7.8 ± 8.7	35.4 ± 13.8
10 μg	40.5 ± 4.1*	21.9 ± 4.0*

*Significant difference (t = 3.28, df = 8, $P < 0.05$) between the means for males and females.

and 166, consistent with a dodecenyl acetate. The CI (isobutane) spectrum showed an ion at m/z 227 $(M + H)^+$, confirming the molecular weight as 226 (Figure 2B). After ozonolysis of the extract, peak 2 disappeared, confirming that the compound was unsaturated. A new peak appeared at scan 299 and was identified as nonanal from diagnostic fragment ions at m/z 142 (M +, 0.8), 124 (3), 114 (8), 98 (35), 82, (31), 70 (41), 57 (85), 55 (72), 43 (79), and 41 (100%). This indicated that the double bond was in the 3-position in the starting material. Authentic Z3-12:Ac and E3-12:Ac eluted at scans 2071 and 2056 respectively, on the CPWAX-52CB column. The compound of interest in the extract from males eluted at scan 2070, confirming the material as Z3-12:Ac. No E3-12:Ac was detected in the male extract.

Electroantennography. The mean responses to the carob standard based on the first presentation of the standard were 305 ± 45.5 and 183.5 ± 40.8 μV for females and males, respectively (N = 5). Based on the final presentation of the carob standard, the mean responses to the standard were 430 ± 79.6 and 257.5 ± 71.9 μV for females and males, respectively (N = 5). There was no significant difference (ANOVA, F = 2.81, $P > 0.05$, df = 19) between the first response and the final response to the carob standard or for the response by males and females at either point.

TABLE 2. MEAN EAG RESPONSES (± SE) IN MICROVOLTS TO E3-12:AC AS A PERCENTAGE OF THE RESPONSE TO A CAROB EXTRACT STANDARD (N = 5)

Dose	Female	Male
1 ng	−3.1 ± 2.5	0.1 ± 2.2
10 ng	2.1 ± 3.5	1.6 ± 4.3
100 ng	2.0 ± 5.8	32.3 ± 15.5
1 μg	12.8 ± 8.8	1.5 ± 4.3
10 μg	22.6 ± 8.0	39.2 ± 22.3

TABLE 3. TWO-CHOICE BIOASSAY RESPONSES OF MALE AND FEMALE *T. molitor* TO Z3-12:AC ($N = 40$)

Dose	Percent response[a]		
	Z3-12:Ac	Control	No response
Males			
100 ng	37.5	25	37.5
1 μg	55	40	5
10 μg	55	35	10
Females			
100 ng	50	35	15
1 μg	65*	20	15
10 μg	75**	20	5

[a] χ^2 test: $\chi^2 = 9.53$, $df = 1$, $P = 0.002$ (*); $\chi^2 = 12.74$, $df = 1$, $P < 0.001$ (**).

The mean EAG responses of female and male *T. molitor* to Z3-12:Ac and E3-12:Ac as a percentage of the response to the standard are shown in Tables 1 and 2, respectively. There was no difference in response by males and females except for 10 μg of Z3-12:Ac, where the response by females was greater than the response by males ($t = 3.28$, $P < 0.05$, $df = 8$). The perception threshold for both Z3-12:Ac and E3-12:Ac by females was 10 μg ($P < 0.05$, $df = 4$). In males, the response to Z3-12:Ac and E3-12:Ac did not differ significantly from the control response at the quantities tested ($P > 0.05$, $df = 4$).

Behavioral Bioassay Tests. Z3-12:Ac was not attractive to males at any dose tested (χ^2 test, $P > 0.05$) (Table 3). However, at 1 and 10 μg, Z3-12:Ac was attractive to females ($P = 0.002$ and $P < 0.001$, respectively) (Table 3). E3-12:Ac was also attractive to females at 10 μg (62.5% of females responded to E3-12:Ac and 22.5% to the control; $\chi^2 = 7.53$, $df = 1$, $P = 0.006$).

DISCUSSION

Males of many beetle species that infest stored products produce aggregation pheromones (Chambers, 1991). In contrast, Z3-12:Ac is a male-produced sex pheromone that elicits a response only from females. August (1971) tested live males and extracts of males over a range of doses in a choice test and found that one male equivalent of male extract caused attraction of females into a central chamber and the response was dose-dependent. In our study, in the highest dose of Z3-12:Ac tested (10 μg), approximately five male equivalents was highly attractive to females. August (1971) found that male extracts were not attractive to males. Similarly, we found that Z3-12:Ac was not attractive to males at any dose tested.

August (1971) also found that the response of females to live males (where the males were not visible to the females) was greater than to the optimally attractive dose of male extract (5 male equivalents). He suggested that this could be due to other semiochemicals present in the male extracts, which might be repellent to females at high concentrations. Happ and Wheeler (1969) found that males release an antiaphrodisiac after they are exposed to female scent, which reduces the attractancy of the female scent to subsequent males. When they tested a mixture of male and female scent against males, it was considerably much less attractive than the female scent alone. They also found that females often extruded their ovipositors on exposure to male sex attractant, and suggested that, together with the antiaphrodisiac component, males increase the probability that females will utilize their sperm before another male has a chance to mate. The male semiochemical(s) suggested by August (1971) and the antiaphrodisiac reported by Happ and Wheeler (1969) could be the same compound(s). Here, we found evidence for the production by males of 2,5-dimethylpyrazine, but the amounts were very small and we did not determine whether this compound has any biological activity in *T. molitor*.

E3-12:Ac (98%) and Z3-12:Ac (2%) are the two components of the sex pheromone of the potato tuber moth, *Scrobiphalpopsis solanivora* Povolny (Nesbitt et al., 1985). E3-12:Ac has also been identified as the sex pheromone of *Scrobipalpa ocellatella* Boyd (Renou et al., 1980). These are the only other species of insects reported to use E3-12:Ac or Z3-12:Ac as semiochemicals. Many species of moths are reported to use one or more isomers of dodecenyl acetate as components of their female sex pheromones, but to our knowledge, this is the first report of a dodecenyl acetate as a beetle pheromone.

It is unusual for both sexes of a species to release a pheromone that attracts the other sex. Both sexes of the Azuki bean weevil (*Callosobruchus chinensis* L.) release a nonvolatile, copulatory release pheromone, which affects only males and is distinct from the female-released sex pheromone (Tanaka et al., 1981). Male and female *Tribolium confusum* (the confused flour beetle) release three hydrocarbons which increase the frequency of copulation, but which affect only males (Keville and Kannowski, 1975). In contrast, the pheromone identified in our study acts as a sex attractant for females, as opposed to a contact copulatory release pheromone.

Studies of *T. molitor* mating behavior by Obata and Hidaka (1982) found a low rate of successful copulation due to relatively random mounting behavior by males, and suggested that a "critical" high-density population is needed to overcome this. A high-density "crowded state" was reported to accelerate oocyte development, pheromone emission, and mating success (Mordue, 1965; Happ and Wheeler, 1969; Gerber, 1973). As females do not respond to their own pheromone, a female pheromone would only lead to an increase in the density of males, and not of both sexes. The presence of both male and female

pheromones, each attracting the opposite sex may, therefore, contribute to maintaining a high-density population of both sexes.

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PHEROMONE COMMUNICATION IN THE HONEYBEE (*Apis mellifera* L.)

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Abstract—Recent studies have demonstrated a remarkable and unexpected complexity in social insect pheromone communication, particularly for honeybees (*Apis mellifera* L.). The intricate interactions characteristic of social insects demand a complex language, based on specialized chemical signals that provide a syntax that is deeper in complexity and richer in nuance than previously imagined. Here, we discuss this rapidly evolving field for honeybees, the only social insect for which any primer pheromones have been identified. Novel research has demonstrated the importance of complexity, synergy, context, and dose, mediated through spatial and temporal pheromone distribution, and has revealed an unprecedented wealth of identified semiochemicals and functions. These new results demand fresh terminology, and we propose adding "colony pheromone" and "passenger pheromone" to the current terms sociochemical, releaser, and primer pheromone to better encompass our growing understanding of chemical communication in social insects.

Key Words—*Apis mellifera*, honeybee, social insect, chemical communication, pheromone, chemoecology.

INTRODUCTION

In their preface to *Chemical Ecology of Insects* published in 1984, Bell and Cardé stated "Evolution of sociality seemed to spawn a chemical language that is equivalent to the visual and auditory repertoire of higher vertebrates." This is now revealed to be a prophetic insight (Bell and Cardé, 1984). At that time, the chemical communication systems utilized by social insects had been recognized

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as complex but seemed to conveniently compartmentalize into two pheromone categories, releaser or primer. A releaser pheromone initiates an immediate behavioral response upon reception, whereas a primer pheromone alters more long-term endocrine and reproductive systems in the recipient (Wilson and Bossert, 1963). Recent studies of honeybee pheromones suggest that chemical communication in social insects is deeper and richer than had been imagined by earlier generations of chemical ecologists. Pheromone signals in honeybees and likely other social insects are often enhanced by complexity, synergy, and the context in which they are deployed, mediated through both temporal and spatial distribution. Even our current limited view of social insect pheromones exceeds the boundaries of present terminology and requires a fresh appraisal. Our intention is not to review all the literature concerning honeybee pheromones as performed before (Free, 1987), but rather to suggest a new template with which to approach sociochemistry.

HONEYBEE SOCIOCHEMISTRY

Our understanding of the chemical language expressed by social insects is best provided through the pharmacopoeia of *Apis mellifera* L., the honeybee. The social organization of honeybee colonies is determined primarily by chemical signals that are actively produced and transmitted by the queen, the adult workers at various tasks and lifestages, and the brood. The honeybee dance language used to recruit foragers to food, water, and nest sites is better known (von Frisch, 1967), but constitutes only one fraction of the communication systems that operate in a functioning colony.

These chemical signals are made remarkably specific in function by temporal fluctuations timed to release specific behaviors or to prime key physiological characteristics of signal recipients, based on both context and synergistic interactions between components. Like a fire alarm, a constant chemical signal would be of no value; time and place are of the essence. The wealth of identified semiochemicals utilized by honeybees and their functions is unprecedented in the insect literature, possibly as a result of the extensive investigations that this agriculturally beneficial insect has stimulated, but also because of its inherent social complexity. Even a cursory review of the honeybee's chemical communication repertoire reveals a rich language.

DEFENSE: ALARM PHEROMONE

For the beekeeper, the banana smell of isopentyl acetate warns that the colony is aroused and that further stinging is imminent. A mixture of substances

is released from a honeybee worker sting gland in the act of stinging, thus recruiting other workers (Breed et al., 2004). Free (1987) lists isopentyl acetate and 24 other substances that are found in the sting gland and thought to be involved in the alarm reaction to colony attack. More recently, Hunt et al., (2003) discovered 3-methyl-2-buten-1-yl acetate as a new alarm component in the sting apparatus of Africanized honeybees. The necessity for chemical complexity of the alarm signal is not obvious. Some specific behavioral attributes have been ascribed to individual components, but no clear theme emerges (Winston, 1987). Many of the components are chemically quite distinct, constituting both aromatic and aliphatic molecules, ruling out complexity as a byproduct of closely related biosynthetic pathways.

ATTRACTION: NASANOV PHEROMONE

The incredible phenomenon of a cohesive honeybee swarm is partially mediated by a complex of the seven-component Nasanov pheromone (Pickett et al., 1980). The signal is released from the dorsal surface of the worker honeybee abdomen to attract her sister workers in a peaceful and organized manner during swarming or at colony entrances (Free, 1987; Winston, 1987). The lemon-grass fragrance we attribute to this bouquet is mostly because of our ready perception of three principal volatile components in this mixture: geraniol, geranial, and neral. The swarm cluster is initiated by alighting workers releasing Nasanov pheromone and then enhanced by the presence of the queen and her attractive signals (Avitabile et al., 1975). Many of the components of the Nasanov pheromone are biosynthetically related, so the complexity of this signal may be partially a function of their production.

ATTRACTION TO THE QUEEN: RETINUE PHEROMONE

A mated, laying queen is essential to the well being and proper functioning of the colony. She communicates her presence and manifests her influence by releasing a mixture of substances that is attractive to workers, enticing them to lick and antennate her to gather a small sample of her attractive blend. It is this retinue behavior of workers to the queen that was recognized by the earliest students of honeybee biology and distinguished her as "queen" of the colony. This "queen substance" was originally considered to be a single material, 9-oxo-(*E*)-2-decenoic acid (ODA) (Barbier and Lederer, 1960; Butler et al., 1961; Pain, 1961), and has been shown to be an essential component of the honeybee sex pheromone, attracting drones to the queen during her mating flight (Gary, 1962). However, queen-like amounts of ODA were neither

attractive to workers nor substituted for her in her absence. Even addition of the two enantiomers of ODA's biosynthetic precursor, (*R*)- and (*S*)-9-hydroxy-(*E*)-2-decenoic acid (HDA), failed to constitute an attractive blend for workers, although (*R*)-HDA is involved in the swarm-settling queen signal (Slessor et al., 1988). Two further components, methyl *p*-hydroxybenzoate (HOB) and 4-hydroxy-3-methyoxyphenylethanol (HVA), were finally recognized and formulated with the decenoic acids to provide a source as attractive as an equivalent extract from the mandibular glands in which the five compounds are produced (Slessor et al., 1988).

Individual components and even subsets of components are not attractive. Only when all five components of the queen mandibular pheromone (QMP) are combined does the blend elicit the full retinue response, a striking example of semiochemical synergy (Slessor et al., 1988). Another important aspect of QMP that distinguishes it from many insect pheromones is that the components are on the borderline of volatility, apparently requiring close proximity between the queen and the recipient worker.

Early in our studies of QMP, it became evident that some strains of honeybees did not find this blend at all attractive, yet they tended their queen and otherwise functioned normally in colonies, implying as yet unidentified, additional substances involved in the retinue response. After 15 years, four further synergistic substances were identified in this "simple" retinue response (Keeling et al., 2003). Disruptive selection for high and low QMP retinue responding workers using closed mating demonstrated this pheromone response to have a strong genetic component (Pankiw et al., 2000). Two-way selection of colony phenotypes for low QMP and high queen extract responders provided over 380,000 worker test subjects for bioassays and led to the elucidation of the nine-component retinue pheromone (QRP = QMP + methyl oleate, coniferyl alcohol, palmityl alcohol, and linolenic acid). The terminology shift to QRP was necessary because the three new fatty-acid-derived constituents are not of mandibular gland origin (Keeling et al., 2003). The complete identity of QRP is still not fully defined because a very small difference in response of specially bred workers to synthetic QRP and multiple queen extract is evident. To identify the material(s) responsible for this small difference would necessitate a new and more extensive bee breeding and chemical isolation program.

The queen signals her presence to the colony through the attractive 9+ component releaser pheromone QRP. Absence of the signal for a few hours results in the workers choosing a few freshly laid eggs to rear into new queens, and long-term absence results in some workers developing ovaries (de Groot and Voogd, 1954; Butler and Fairey, 1963). Thus, colony reproduction and worker physiology are partially under the control of QRP (Pankiw et al., 1998), making it a primer as well as a releaser pheromone. The retinue facilitates the

distribution of QMP that inhibits the development of worker ovaries (Hoover et al., 2003) and orients the comb building (Ledoux et al., 2001). It also facilitates distribution of a known primer component, ODA, that modulates the biosynthesis of juvenile hormone in recipient workers (Kaatz et al., 1992), regulating their age-related tasks (Robinson et al., 1989). Clearly, the synergistic releaser QRP embodies primer signals.

LARVAL SIGNALS: ESTER "BROOD PHEROMONE"

In the early 1990s, we identified a mixture of 10 ethyl and methyl esters of the common fatty acids palmitic, linoleic, linolenic, stearic, and oleic acids from larvae, and 4 of these methyl esters were originally considered to be a signal from larvae to adults to cap the brood cells prior to their pupation (Le Conte et al., 1990). The methyl esters of palmitic and oleic acid were most effective in recruiting workers to this role. In addition, differences in the proportions of esters provide a chemical signature of larval age (Le Conte et al., 1994, 1994/1995). Further studies demonstrated that individual esters and subsets of esters provide context-specific signals to bring about other behaviors. In the presence of a fully functioning queen, the workers will tear down any freshly constructed queen cells, but the presence of methyl oleate in or about these cells results in a greater acceptance of new queen cells by workers (Le Conte et al., 1994/1995). The action of methyl linolenate results in enhanced provisioning of these new queen cells with more royal jelly, facilitating the healthy development of new queen larvae (Le Conte et al., 1995). Each individual ester or ester subset provides a message to the worker recipient, indicating an action to be carried out.

In the 1990s, an even more important role for the esters began to emerge. Nurse honeybees feed larvae a mixture of nutrients that contains a proteinaceous gland exudate. When nurse bees are treated with a blend of methyl palmitate and ethyl oleate, protein levels in the gland are elevated, probably through stimulation of the gland's biosynthetic capacity (Mohammedi et al., 1996). This finding has recently been confirmed (Pankiw et al., 2004). In the prolonged absence of a queen and larvae, the ovaries of worker honeybees mature and in extreme cases become drone-laying workers (Winston, 1987). Two of the "brood" esters with demonstrated releaser effects, ethyl palmitate and methyl linolenate, partially inhibit the ovarian development of worker bees when they are isolated from a queen and brood (Arnold et al., 1994; Mohammedi et al., 1998). The inhibitory effects of these esters on the physiological development of the recipient workers' ovaries constitute a primer pheromone effect.

WORKER ESTERS

The tasks of adult worker honeybees change as they age, from in-colony duties, such as nursing larvae, to pollen and nectar processing, followed by outside responsibilities of guarding and foraging (Lindauer, 1953). Plasticity of ontogeny in behavioral development allows the colony to cope effectively with changing environments, including internal requirements such as the production of new workers, or external opportunities such as increased nectar flow. This organizational shift and its plasticity are well recognized, but its underlying control has been a mystery, likely involving hormonal and pheromonal components (Robinson, 1992). A major influence in a worker's developmental rate is provided by her older sister foragers whose presence slows the nurse's progression through the age-related tasks (Robinson, 1992; Huang and Robinson, 1996). Recently, the feeding of larval esters to experimental colonies has demonstrated inhibition in age to first foraging (Le Conte et al., 2001). Ethyl oleate is present at significantly higher levels in foragers than in nurses, and the presence of this ester accounts for the ontogenetic inhibition and task mediation in honeybee colonies (Leoncini, 2002; Leoncini et al., 2004).

QUEEN ESTERS

Chemical analysis of queens demonstrated the presence of seven esters including methyl oleate (Wossler and Crewe, 1999a), the palmitates, oleates, ethyl stearate, and two new esters, ethyl and methyl palmitoleate, with methyl oleate a synergistic component of queen retinue formation (Keeling, 2001). In the dispersal of QRP throughout the colony, queen esters are distributed as passive chemical passengers in the queen bouquet because they possess no attractive capability alone or in the QRP blend. For example, ethyl palmitate derived from the queen may well be the active agent in her ability to inhibit worker ovarian development as ethyl palmitate also was demonstrated to be produced by the larvae and to inhibit the ovary development of workers (Mohammedi et al., 1998). Thus, both the larvae and the queen manipulate the ovary development of workers to their advantage.

In addition, the major ester in the queen, ethyl oleate (Keeling, 2001), which is involved in regulating the ontogeny of the young workers who attend and feed the queen (Mohammedi et al., 1996), is a passive chemical passenger. Such a substance as ethyl oleate in the queen's effluvia, although not involved in the attraction process, but critically important in her pheromonal capabilities, can be regarded as a "passenger pheromone." Esters that play no role in the queen's releaser retinue response and yet are delivered as an integral part of that mixture are central to her impact on workers, functioning

as "primer pheromones." We propose that these be called "passenger pheromones" (Table 1).

COLONY ESTERS

Just as we had to revise the nomenclature of queen mandibular pheromone (QMP) to queen retinue pheromone (QRP) to properly reflect its multiglandular source, these fatty acid esters might now be properly viewed as "colony pheromones," defined as originating in multiple castes and developmental stages and having diverse releaser and primer roles. Palmitate and oleate ethyl are good examples of a colony pheromone (Table 1). Remarkably, the queen and larvae work in concert to inhibit the ovarian development of young adult worker bees (Jay, 1968, 1972; Free, 1987). Ethyl palmitate inhibits ovary development of workers (Mohammedi et al., 1998). The flux of ethyl palmitate from the two sources is unknown at present, although drone larvae ready for capping have ~90 ng (Le Conte et al., 1989), worker larvae somewhat less (Le Conte et al., 1990), and a mated laying queen contains ~300 ng (Keeling, 2001). Older foragers, the larvae, and the queen exhibit primer effects by influencing the developmental rate of their younger sisters and offspring through the release of ethyl oleate. Foragers contain roughly 45 ng, larvae 50 ng (Le Conte et al., 1989; Leoncini et al., 2004), and queens 6300 ng (Keeling, 2001) of ethyl oleate on average, although release rates remain unknown. Moreover, oleate and palmitate ethyl are known to be involved in larval recognition by workers (Le Conte et al., 1994). In addition to influencing the ontogeny of their sisters, the esters stimulate and release pollen foraging behavior and may prime bees for a particular foraging role at a young age (Pankiw and Page, 2001).

COMPLEXITY: WHY SO MANY?

The identified queen, worker, and brood substances essential for the functioning of this highly evolved social organism now number nearly 50 in all, and still are incompletely characterized. In human society, language with its vast oral and written vocabulary is the commerce of communication. In honeybee society, the vocabulary is an array of specific chemicals or blends of chemicals. A few of the substances now thought to be honeybee signal chemicals eventually may be recognized as inactive biosynthetic precursors and degradation products, the noise of this chemical language. However, even with the noise filtered out, honeybee language is undoubtedly more complicated than previously recognized (Slessor, 2003). The chemical complexity of the queen's tergal (Wossler and Crewe, 1999a,b), labial (Katzav-Gozansky et al., 2001a),

TABLE 1. MULTISOURCES AND TARGETS OF FATTY ACID ESTERS IN THE HONEY BEE

	Source	Target	PM	PE	SM	SE	OM	OE	LM	LE	LNM	LNE	10 esters
Releaser pheromone	Larvae	Crapping of the cell	++				+++		+++		+++		
		Larval age recognition	++	++		++		+	++	++		+	
		Queen cell recognition					+++		+++		+++		
		Royal jelly feeding	+++		+				+++				
		Pollen foraging											+++
		Acceptance of queen cells	+		+++				++				
	Queen	Queen retinue behavior					++						
Primer pheromone	Larvae	Worker ovary development		+++							+++		
		Hypopharyngeal glands	++					+++					
		Behavioral development						+++					+++
	Forager	Behavioral development						+++					
	Queen	Behavioral development						+++					
		Hypopharyngeal glands	++					+++					
		Worker ovary development		+++									

P, Palmitate; S, Stearate; O, Oleate; L, Linoleate; LN, Linolenate; M, Methyl; E, Ethyl; +, weak effect; ++, significant effect; +++, very significant effect; , colony pheromone; , passenger pheromone.

and Dufour's gland (Katzav-Gozansky et al., 1997, 2001b) implicates these and other worker, brood, and queen glands as informational sources. No components responsible for their effects, however, have yet been fully identified. Queen recognition, the ability of a colony to recognize its own queen (reviewed in Winston, 1987), worker policing of nonqueen laid eggs (Ratnieks and Visscher, 1989; Ratnieks, 1995), and colony/nestmate recognition (Breed et al., 1992, 1995) are examples of interactions that require further communication channels and suggest additional signal chemicals. Complex social interactions demand an intricate language. In the honeybee and likely in other social insects, the use of many highly specialized chemical signals provides that syntax.

CONTEXT, COMPLEXITY, AND SYNERGY

The majority of known insect pheromones are perceived as volatile materials diluted and carried by air currents, received, and processed through antennal reception. Honeybees utilize this dispersal mechanism for alarm and Nasanov pheromones, and, similarly, components of queen pheromone attract drones for mating and workers to swarm clusters (see Winston, 1987). However, a distinguishing feature of QRP and colony esters is that they are passed primarily by contact. Partially volatile components appear to be quickly absorbed into the wax matrix of the colony (Naumann et al., 1991), effectively removing them and ensuring their context dependence. Alternatively, the transmission of chemical components from wax can provide discrimination and nestmate recognition cues to naive individuals (Breed et al., 1995, 1998), underlining the integral role that wax plays. In the retinue, the young workers attend the queen, licking and antennating her, removing QRP from her body, and often obtaining sufficient QRP that they become attractive to other workers after they have left the queen (Seeley, 1979; Naumann et al., 1991). These messenger bees contribute to the dispersal of QRP, but when the QRP signal fails to be transmitted throughout the colony because of congestion and population growth (Naumann et al., 1993), workers consider themselves queenless and begin to build queen cells, the precursor to colony reproduction by swarming (Winston et al., 1991). If these signals were volatile and/or not removed by wax, they would be distributed virtually homogeneously throughout the colony by the continuous circulation of air maintained by the workers. The relative involatility of QRP and the absorptive nature of wax, thus, have important repercussions enabling synergy for multicomponent pheromones. Inactive on their own, QRP components are highly synergistic, the whole blend being required for a complete signal. Only if all components are perceived as a unit does the signal transmit the important message "the queen is here and functioning appropriately."

Synergy provides a further bonus for the organization because all chemical constituents need not be unique to the emitter. A single unique component is sufficient to render a distinctive identity and response when carried along with other ubiquitous and active ingredients. This phenomenon is particularly important where individuals are nearly all biosynthetically equivalent, as in a caste-based social organization (Plettner et al., 1996; Martin and Jones, 2004). In the honeybee colony, the queen must uniquely produce only HVA and coniferyl alcohol (and possibly HOB) to ensure that she transmits a blend with both releaser and multiprimer effects.

Context is less well documented and understood, but clearly plays an important role. Methyl oleate is perceived as a capping signal when emitted by larvae (Le Conte et al., 1990), but also is a synergistic component of QRP (Keeling et al., 2003; Table 1). Workers have reduced capacity to produce queen-like QRP components, and yet clearly respond to its primer effects. Young workers contain esters including ethyl oleate (Leoncini, 2002; Leoncini et al., 2004), which inhibits their ontogenetic development when received from the queen or their older sisters. Could it be that the receipt of primer pheromone through direct contact with antennae or other receptive sensory organs is the principal initiating mechanism, or is a threshold dosage the activating mechanism? At very dilute concentrations, QMP stimulates foragers to perceive a nectar source as a richer resource than the sugar concentration suggests (Higo et al., 1992). What underlying effects contribute to this contextual and dose misrepresentation of the queen signal?

Complexity of a signal is greatly facilitated by synergy, which, in turn, contributes to contextual uniqueness. These properties combined—complexity, synergy, context, and dose—appear to be hallmarks of social insect pheromones and distinguish them from the typical pheromones of nonsocial insects. Pankiw (2004) has recently described honeybee chemical communication as an emergent property of a complex system with dynamic properties requiring a complex systems approach for their analysis.

ISOLATION AND IDENTIFICATION: BIOASSAYS

Contextual dependence, synergy, and complexity make sociochemical identification extremely difficult. Chemical comparisons of interracial differences and similarities can provide clues to the involvement of specific substances of behavioral importance (Wossler and Crewe, 1999b; Brillet et al., 2002). However, no insect primer has yet been isolated and identified by bioassay. Substances have been designated with primer status only after their presence has been established through a releaser-based bioassay and then demonstrated in a primer bioassay. An effective bioassay must incorporate

transfer of the bioactive mixture in an appropriate manner and dose and be followed by some quantitative measure of the resulting effect. The process must be elegant and simple because it may have to be replicated many thousands of times, not only to show the involvement of an active ingredient but also to provide satisfactory proof that an inactive ingredient has no effect on the response being measured; a benign effect is considerably more difficult to demonstrate than a positive one. Dose dependency must be tested to ensure that the applied dose is biologically relevant, similar to that encountered by an individual in a normal situation. Highly replicated bioassays may require hundreds of thousands of animals at a specific point in their life or of a particular strain. Primer bioassays provide an even greater challenge, necessitating a time period for the physiological change to become apparent and the time and effort involved in measurement of the change, e.g., dissection and evaluation of ovarian development. Efficient primer isolation and identification requires new methodology, with molecular biology the most promising tool. Initiation of physiological changes may be monitored at the RNA or protein level rather than the behavioral or physiological level. Differential or microarray displays (Whitfield et al., 2002; Grozinger et al., 2003) offer tremendous advantages over multitudinous bioassays necessary for the isolation and identification of primer pheromones. Nevertheless, the primer bioassay remains essential to prove the function of the putative primer pheromone rather than directing its isolation.

SOCIOCHEMICALS: REFINING TERMS

The pheromone complexity of the honeybee suggests that the evolution of insect sociality created a large number of complex chemical languages. The term "sociochemical" refers to any chemical or defined mixture of chemicals that mediates social behavior (Bell and Cardé, 1984). Within this framework, honeybees utilize releasers, some apparently monofunctional, such as Nasanov pheromone, and others clearly multifunctional, such as "colony pheromone" (defined above as originating in multiple castes and developmental stages and having diverse releaser and primer roles, e.g., esters in honeybees). The latter, in addition to its releaser roles under appropriate context, functions in subsets as both stimulatory and inhibitory primers. Foragers modulate their sisters' physiological development with an inhibitory primer that may be transferred during nectar and pollen delivery.

The queen, by means of an attractive releaser (QRP), has at least one component (ODA) that mediates a worker's endocrine function. In addition, this attractive material carries with it several other passenger substances (ethyl oleate and palmitate) that are transferred passively during the retinue response. The ester blend released by larvae contains both context-dependent releasers

and primers with some apparently in passenger mode. This new concept of "passenger pheromones," substances that are transmitted in a pheromone complex, which are inactive in the transmission process, and yet which mediate some primer or releaser function upon reception, appears to be central to the functioning and understanding of the complex chemical language of social insects.

Extrapolation from the limited understanding of one species to the many social insect societies is fraught with uncertainty. What is clear after four decades of investigation is that Bell and Cardé (1984) were correct, and that chemical communication might equal or exceed the complexity of the better-known auditory and visual systems of vertebrates.

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RAPID COMMUNICATION

SEXUAL ISOLATION AND CUTICULAR HYDROCARBON DIFFERENCES BETWEEN *Drosophila santomea* AND *Drosophila yakuba*

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Abstract—*Drosophila santomea* and *Drosophila yakuba* are two sister species inhabiting Saõ Tomé island. Previous studies showed that both species display strong reproductive isolation, although they can produce a few viable hybrids. Our study tried to understand the mechanism of this ethological isolation between two allopatric strains. A strong sexual isolation was confirmed, with a marked asymmetry. Comparisons of latency times to either courtship or copulation suggest that males do not discriminate females, whereas *D. yakuba* females, but not *D. santomea* females, accept their homospecifics more quickly. Cuticular hydrocarbon compositions of both species and sexes were also established with gas chromatography (GC) and GC/mass spectrometry analysis. All have (*Z*)-7-tricosene as their major compound. There are several quantitative differences between species for few minor compounds. The largest difference concerns *n*-heneicosane, which is more abundant in *D. santomea* than in *D. yakuba* flies (up to seven times more between males). A similar quantitative difference was also found in a pair of sympatric strains. Furthermore, *D. yakuba* males artificially perfumed with *n*-heneicosane were discriminated negatively by *D. yakuba* females, suggesting a role for this compound in the sexual isolation between these two species.

Key Words—*Drosophila santomea*, *Drosophila yakuba*, reproductive isolation, courtship, hydrocarbon pheromone.

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INTRODUCTION

Mate selection consists of both choosing a conspecific and identifying the opposite sex. For that purpose, the selection is made on the basis of courtship traits that are characteristic of each species. *Drosophila*'s courtship corresponds to a stereotypic sequence of behaviors involving visual, tactile, acoustic, and chemical stimuli (Ewing, 1983). Specifically, cuticular pheromones are involved in mate stimulation and discrimination (Jallon, 1984; Cobb and Jallon, 1990; Ferveur, 2005). These cuticular hydrocarbons (CHCs) are not very volatile compounds that are perceived only at short distances and may stimulate or inhibit potential partners. In the melanogaster subgroup, they may differ markedly between sexes as in *Drosophila melanogaster*, or be similar as in *Drosophila simulans* (Jallon, 1984; Jallon and David, 1987).

Drosophila santomea and *Drosophila yakuba* are two sister species living on the island of Saô Tomé (Lachaise et al., 2000). Coyne et al. (2002) showed a strong reproductive isolation between them, with similar amplitude for sympatric and allopatric strains. Nevertheless, both species can mate with each other, and hybrids are viable. It was concluded that the two species were recently isolated and that reproductive isolation resulted from a prezygotic barrier, possibly an ethological one (Llopart et al., 2002). *D. santomea* flies lack the abdominal dark pigmentation present in *D. yakuba* flies, but Llopart et al. (2002) showed that this visual cue is not important for interspecific mate discrimination.

In the present study, we first compared the possible behavioral temporal parameters in homospecific and heterospecific matings and then analyzed CHCs, which could be potentially involved in sexual selection between the two species.

METHODS AND MATERIALS

D. santomea flies originated from strain Car 1490-1, collected above the hybrid zone, at an altitude of 1,490 m on Saô Tomé. *D. yakuba* flies are issued from strain SJ 14, collected below the hybrid zone at low altitude. Two other strains collected in the hybrid zone were also used for chemical analysis: strain STO5 for *D. santomea* and SA1 for *D. yakuba*.

Both species were reared on the usual cornmeal–malt medium at 25°C under 12:12 light/dark conditions. All flies were sexed, isolated under carbon dioxide anesthesia within 12 hr after imaginal emergence, and maintained in glass vials (25 ml). Flies were 4-d-old when used for experiments. All tests were run in the morning, between 0900 and 1300 hours, under dim electric light in a temperature-controlled room.

Mating tests were set up in a chamber (2.15 cm diam × 4 mm height) with a glass roof, which could be divided into two parts by a removable separation; thus, each sex could be introduced on either side, and flies could habituate before removing the separation, at time 0. In each test, three females and three males were put together and observed during 45 min. In addition to the percentage of first copulations succeeding the first courtship (percentage of mating success), two temporal parameters were recorded: latency time to the first courtship for males that initiate courtship and latency time to the first copulation for females that accept or do not accept the males.

To analyze fly CHCs for each strain of each species and each sex, ten 4-d-old virgin flies were anesthetized, bathed individually in heptane, and their extracts analyzed in a PerkinElmer AutoSystem GC chromatograph or a Fisons MD800 (under electron impact at 70 eV) gas chromatography/mass spectrometry (GC/MS) apparatus, both equipped with a BP1 SGE capillary column (length 25 m, internal diam 0.22 mm, film thickness 0.1 μm) and at the same temperature program (180–270°C, 3°C/min) (Rouault et al., 2004). The relative proportions of the areas of the various peaks were calculated (in percent) and compared. An external standard, *n*-hexacosane, which is naturally absent in fly cuticle, was used in a given amount, and the area under each peak was compared to that of the standard to determine its absolute amount.

To perfume *D. yakuba* males or females, 3-d-old individuals were anesthetized under carbon dioxide and 0.2 μl of an acetonic solution containing 2 mg/ml *n*-heneicosane were applied topically on each fly, corresponding to more than twice the dose present on *D. santomea* flies. Perfumed flies stood 24 hr before experiments started. Control individuals received acetone alone, which evaporates quickly. Experiments with perfumed flies were done under the same conditions as previously described. Mating success was recorded over a 45-min period. After the experiment, all flies were chemically analyzed by GC.

For statistical comparisons, a Mann–Withney *U* test was applied to compare temporal parameters and a chi-square test for mating success.

RESULTS AND DISCUSSION

Table 1 presents the results of mating experiments within or between two strains of either species, *D. santomea* (Car 1490-1) and *D. yakuba* (SJ 14). They show that homospecific matings are preferred to heterospecific ones: 54% homospecific matings for *D. santomea* and 48% for *D. yakuba*. This confirms the existence of a behavioral reproductive barrier between the two sister species. Second, when comparing heterospecific matings, there is an asymmetrical preference for copulations of *D. yakuba* females with *D. santomea* males

TABLE 1. DIFFERENT PARAMETERS RECORDED DURING 45 MINUTES FOR INTRASPECIFIC AND INTERSPECIFIC MATING EXPERIMENTS

Crossing	Number of Tests	Mating success (%)	Time in minutes ± SD: Latency to first Courtship	Time in minutes ± SD: Latency to first Copulation
FM san	63	54	2.2 ± 0.4	13.4 ± 2.7
FM yak	105	48	1.2 ± 0.2	14.5 ± 1.8
Fsan-Myak	114	4	1.8 ± 0.6	9.7 ± 4.2
Fyak-Msan	99	11	2.2 ± 0.4	24.0 ± 3.3

F, Female; M, male; san, *D. santomea*; yak, *D. yakuba*.
Three males and three females are present in the mating chamber.

(Fyak–Msan, 11%) compared to 4% for *D. santomea* female–*D. yakuba* male pairs.

The courtship latency times of each type of male are not significantly different in the presence of either female, neither for *D. santomea* males ($P = 0.69$) nor for *D. yakuba* males ($P = 0.45$). This suggests that males are equally attracted by the two types of females and, therefore, do not discriminate between them.

When latency times to copulations are compared between females, there are differences. *D. yakuba* females accept homospecific males more quickly than *D. santomea* males ($P = 0.012$). The difference with *D. santomea* females is not significant, however, between the two types of males ($P = 0.49$). Thus, the temporal differences of male courtship and female acceptance suggest that females are the choosy sex and discriminate between the males.

Figure 1 presents the CHC composition of each sex within each species. We observed no qualitative differences between the two species; they all presented similar GC profiles, consisting of 15 peaks. GC/MS analysis led to the identification of long-chain hydrocarbons shown in Figure 1. Peaks 1, 2, 12, and 15 were reported neither in males nor females of the *D. yakuba* continental strain (from Cameroon) described by Jallon and David (1987). Their mass spectra show molecular peaks with *m*/*z* respective values of 296, 310, 380, and 408; moreover, compounds 7, 11, and 14 display mass spectra with M-43 peaks, which suggests a methyl branching on carbon 2. The repeated comigrations of tricosene peaks 3 and 4 and pentacosene peaks 8 and 9 with synthetic (*Z*)-9-tricosene, (*Z*)-7-tricosene, (*Z*)-9-pentacosene, and (*Z*)-7 pentacosene, respectively, suggest (*Z*)-9- and (*Z*)-7- for double bond positions A and B, respectively. Thus, (*Z*)-7-tricosene is the most abundant CHC, whichever sex and species, as in all "monomorphic" species of the melanogaster subgroup (Jallon and David, 1987). There is no significant difference in the absolute

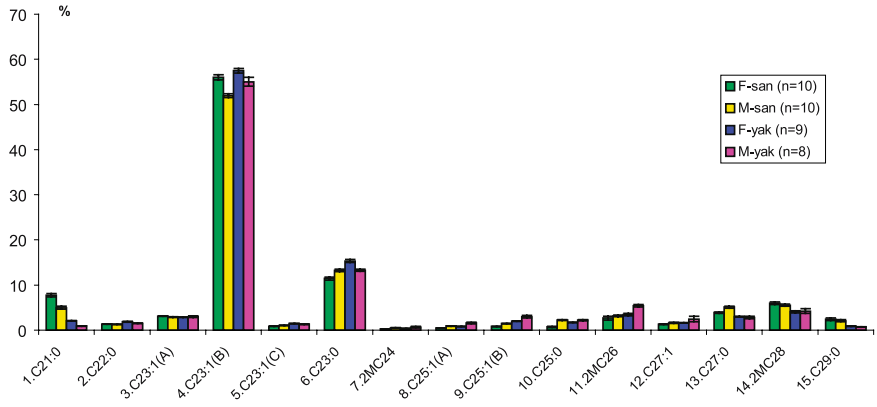

FIG. 1. Cuticular hydrocarbon profiles of 4-d-old virgin females and males of each species, *D. santomea* (strain Car 1490-1) and *D. yakuba* (strain SJ 14). Each peak has been associated with its area percentage over the sum of all CHC peak areas and is presented, with its GC/MS identification, in the order of increasing retention times.

amounts of this compound in the cuticle of either type of female (on average 1265 ± 68 ng per *D. santomea* female and 1281 ± 61 ng per *D. yakuba* female), and these females are not discriminated by either type of males. Thus, it is suggested that in *D. santomea*, (*Z*)-7-tricosene might also play an important role in female sex appeal (Cobb and Jallon, 1990).

There is also no significant difference in the total amounts of CHC in either type of female (2.2 μg/fly) but *D. santomea* males have more CHC (1.6 μg/fly) than *D. yakuba* ones (1.3 μg/fly). If one considers both sexes, there are significant quantitative differences between species for five compounds: *n*-heneicosane (peak 1), (*Z*)-9- and (*Z*)-7-pentacosene (peaks 8 and 9), *n*-heptacosane (peak 13), and *n*-nonacosane (peak 15). The largest difference, however, concerns *n*-heneicosane, which is more abundant in *D. santomea* flies, 3.9 times among females and 6.9 times among males. Analysis of the CHCs of the two other strains, *D. santomea* STO5 and *D. yakuba* SA1 collected in the sympatric zone, shows similar profiles. *n*-Heneicosane is also more abundant in both sexes of this second *D. santomea* strain, although the quantitative difference is not very different from that of the allopatric strains (4.7 times more for females, 5.7 for males).

This raises the possibility that this compound might be used to discriminate between heterospecific flies. To test this hypothesis, *D. yakuba* flies of both sexes were perfumed—or not—with a dose of *n*-heneicosane (400 ng) and presented to (nonperfumed) homospecific flies of the other sex. No significant differences

in mating success were observed between perfumed females and controls crossed with *D. yakuba* males (39 and 34%, respectively). On the other hand, whether perfumed or not, when *D. yakuba* males were crossed with *D. yakuba* females, fewer perfumed males mated with homospecific females: 30.0% compared to 55.5% with nonperfumed ($\chi^2 = 6.251$, $df = 1$, $P = 0.02$). This supports the hypothesis that *D. yakuba* females might discriminate negatively modified *D. yakuba* males that smell like *D. santomea* males.

In summary, our experiments support the classical role of females as the choosy sex in mate selection and suggest that this selection could result from a specific CHC. In *D. melanogaster*, Scott (1994) also showed an intraspecific female choice between males that bear in their cuticle more or less of the major CHC that has evolved among populations (Rouault et al., 2004). Here, females discriminate between heterospecific males using a less abundant compound.

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RAPID COMMUNICATION

JASMONATE IN LEPIDOPTERAN EGGS AND NEONATES

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Abstract—Jasmonic acid (JA) is a key molecule initiating plant defensive responses to attack by pathogens and herbivores. This phytohormone is produced at sites of insect damage and is ingested by feeding insects, but its subsequent occurrence in insect tissues remains to be studied. We report the presence of JA in eggs and neonates of all nine lepidopteran species that we screened, representing four superfamilies and five families of Lepidoptera. Concentrations of JA in some lepidopteran species far exceeded those found in most plant species. Levels of JA varied significantly among species and between eggs and neonates of the same species. In some cases, eggs contained significantly more JA than neonates, but for at least one species (*Lymantria dispar*) neonates had more JA than their eggs despite lacking food upon emergence. The presence of JA in eggs and neonates across a wide taxonomic range may indicate that JA has an undescribed function in insects.

Key Words—Jasmonic acid, octodecanoid pathway, phytohormone.

INTRODUCTION

The phytohormone jasmonic acid (JA) is an important molecule in plants that has been implicated as a key signal initiating plant defensive responses to attack by herbivores and pathogens (Walling, 2000). Jasmonate is derived from linolenic acid via the octodecanoid pathway and activates defensive genes that initiate "induced systemic resistance'' against insect herbivores and the release of volatile compounds that attract natural enemies to herbivore-infested plants (Walling, 2000).

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While conducting another research project, we detected JA in eggs and neonates of the noctuid *Heliothis virescens* (Fabricius), prompting us to ask whether JA was present in eggs and neonates of other lepidopteran species. We are unaware of any previous studies that have examined levels of JA in insects; however, JA is produced by plants at sites of herbivory and is ingested by feeding lepidopterans (Walling, 2000; Li et al., 2002). In fact, JA activates detoxification genes in *Helicoverpa zea* (Boddie), apparently providing "early-warning'' signals that indicate imminent production by plants of toxic defensive compounds (Li et al., 2002). Here, we present evidence that this plant hormone is present in eggs and neonates of all lepidopteran species we screened, possibly indicating that JA plays an unknown functional role in insects.

METHODS AND MATERIALS

Insect Samples. We obtained eggs of nine moth species representing four superfamilies and five families of Lepidoptera (Table 1). Eight species were reared on artificial diets, and one was field-collected. After oviposition, we weighed eggs and either collected them into FastPrep® tubes (Qbiogene, Carlsbad, CA, USA) containing 1 g of Zirmil beads (1.1 mm; Saint-Gobain ZirPro, Mountainside, NJ, USA), freezing them at −80°C until processing (see below), or kept them in an incubator (25°C; 14:10 L/D; 60% RH) until hatching. Upon emergence, we collected neonates into FastPrep tubes with 1 g of Zirmil beads, weighing and freezing them at −80°C until processing. We observed no evidence of larval cannibalism. Because of the low mass of eggs and neonates (<0.1 mg), we combined multiple eggs or larvae into single samples (10–40 eggs per sample depending on mass; 5–10 neonates), processing five samples of both eggs and neonates for each lepidopteran species (total number of eggs analyzed per species: 50–200; total neonates per species: 25–50). We also collected 10 samples (~0.5 mg each) of the setae, which form the matrix of *Lymantria dispar* egg masses; these setae originate from the abdomen of the ovipositing mother. Finally, we obtained from the rearing facilities (Table 1) samples of the artificial diets used to rear these species, collecting and freezing five samples (~100 mg each) of each diet for processing.

Extraction and Quantification of JA and SA. To extract and detect JA, we modified a previously described protocol (Schmelz et al., 2003, 2004) that derivatized carboxylic acids to methyl esters, which were isolated by using vapor phase extraction and analyzed by gas chromatography-mass spectrometry (GC-MS) with isobutane chemical ionization with selected-ion monitoring. Our method deviated from that of the previous authors in that we quantified amounts of methyl jasmonate (meJA) by using standard curves made with the pure compound (Sigma-Aldrich, St. Louis, MO, USA), relying on internal standards

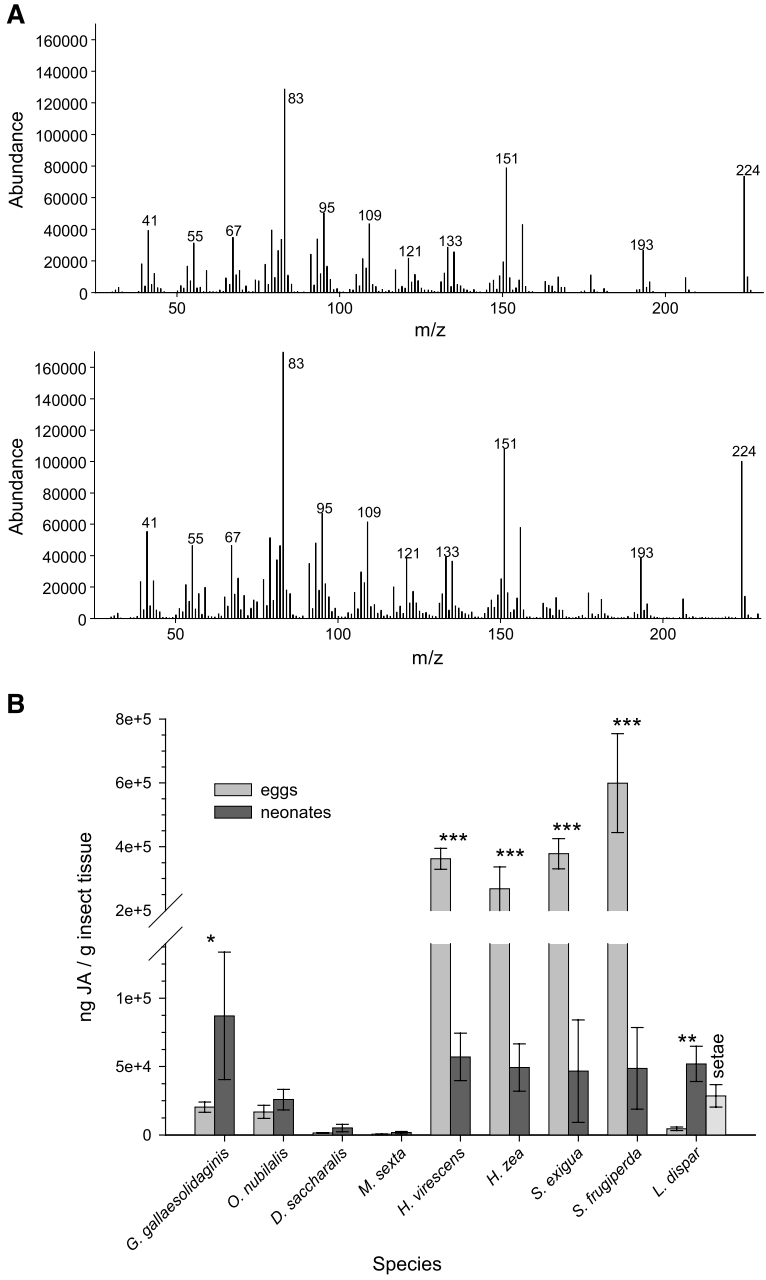
TABLE 1. LEPIDOPTERAN SPECIES WHOSE EGGS AND NEONATES WERE ANALYZED FOR JASMONIC ACID (JA)

Lepidopteran superfamily, family, and species	Common name	Source	Diet	ng JA/g diet (mean ± SE)
Gelechioidea				
Gelechiidae				
Gnorimoschema gallaesolidaginis (Riley)	–	Centre Co., PA, USA	*Solidago altissima* stems	600.1 ± 148.7[a]
Pyraloidea				
Crambidae				
Diatraea saccharalis (Fabricius)	Sugarcane borer	Univ. of Illinois	Wheat germ diet[b]	42.4 ± 23.9
Ostrinia nubilalis Hübner	European corn borer	USDA[c]	Corn-based diet	62.9 ± 30.1
Sphingoidea				
Sphingidae				
Manduca sexta L.	Tobacco hornworm	NC State Univ.	Wheat germ diet[b]	36.9 ± 10.1
Noctuoidea				
Noctuidae				
Heliothis virescens (Fabricius)	Tobacco budworm	USDA[d]	Pinto bean diet	202.7 ± 132.1
Helicoverpa zea (Boddie)	Corn earworm	USDA[d]	Pinto bean diet	202.7 ± 132.1
Spodoptera exigua (Hübner)	Beet armyworm	USDA[d]	Pinto bean diet	202.7 ± 132.1
Spodoptera frugiperda (J.E. Smith)	Fall armyworm	USDA[d]	Pinto bean diet	202.7 ± 132.1
Lymantridae				
Lymantria dispar L.	Gypsy moth	USDA[e]	Wheat germ diet[b]	8.5 ± 3.8

[a] Field-collected stems newly infested by *G. gallaesolidaginis*; unpublished data.
[b] Each wheat germ diet is a species-specific formulation.
[c] Ames, IA, USA.
[d] Tifton, GA, USA.
[e] Otis, MA, USA.

to confirm derivatization and recovery. We also processed samples without the derivatization agent to verify that the meJA we recovered was not itself present in eggs and neonates, but was derived from JA. To confirm the identity of meJA in our samples, we analyzed extracts by GC-MS with electron ionization, comparing retention times and spectra with that of the pure compound.

To normalize the data and stabilize variance, we square-root transformed our data and compared mean amounts of JA among samples by ANOVA


A
Abundance
m/z
83
151
224
95
109
41
55
67
121
133
193
B
ng JA / g insect tissue
eggs
neonates
setae
Species
G. gallaesolidaginis
O. nubilalis
D. saccharalis
M. sexta
H. virescens
H. zea
S. exigua
S. frugiperda
L. dispar

(Statistix, 2003), testing differences between eggs and neonates of each species as planned comparisons with the LSD means separation test (Sokal and Rohlf, 1995).

RESULTS AND DISCUSSION

We recovered JA from all the eggs, neonates, and diets we analyzed (Figure 1). Mass spectra of meJA recovered from lepidopterans and their diets were nearly identical to that of the pure standard, confirming the identity of JA in our samples (Figure 1A). Moreover, we did not recover meJA in absence of the derivatization agent, verifying that the meJA we measured was derivatized from JA and was not itself present in samples. Remarkably, concentrations of JA in eggs and neonates were in some cases hundreds of times greater than the concentrations in their diets (Table 1) and those typically found per gram of plant tissue (e.g., Schmelz et al., 2003). We suspect that the probable source of JA in eggs and neonates was the larval diet of ovipositing mothers and that the high concentrations resulted from bioaccumulation. If so, the presence of JA could conceivably be a nonadaptive by-product of larval feeding. Still, the presence of JA at such high concentrations in eggs and neonates raises the possibility that it may play some yet to be described functional role in these stages.

Amounts of JA in eggs and neonates varied within and among species (Figure 1B; ANOVA: $F_{18,97} = 26.2$, $P < 0.001$). Levels of JA were highest in eggs of the four noctuid species, and amounts of JA in eggs of these species were greater than amounts found in neonates (LSD, $P < 0.05$). Jasmonate levels in neonates were similar across noctuid species (LSD, $P > 0.05$), suggesting these species process JA similarly.

Ostrinia nubilalis, *D. saccharalis*, and *M. sexta* had low levels of JA that were similar in eggs and neonates (LSD, $P > 0.05$), but *L. dispar* neonates had greater amounts of JA than their eggs (LSD, $P < 0.05$). *Gnorimoschema gallaesolidaginis* neonates also had greater amounts of JA than their eggs, but only at the 0.1 significance level. Neonates did not have available food upon emergence, but consumed a small portion of their egg chorion in the course of emergence. Chorions appear to contain small amounts of JA (unpublished data),

FIG. 1. Jasmonic acid (JA) in lepidopteran species. (A) EI mass spectra of meJA: the pure standard (top); derivatized from JA and recovered from *Spodoptera frugiperda* eggs (bottom). (B) JA recovered from eggs and neonates of nine lepidopteran species. Data shown are untransformed. Species marked with asterisks had significant differences in JA concentrations between eggs and neonates (*$P < 0.1$; **$P < 0.05$; ***$P < 0.001$). See text for details on statistics.

but not enough to account for the elevated levels of JA in neonates of these two species. After emerging, *L. dispar* larvae may also have consumed some of the setae that form the matrix of their egg mass. Setae also contain JA (Figure 1B), and further research will be needed to determine if setae are a source of JA for neonates. If chorions and setae are not significant sources of additional JA in *L. dispar* and *G. gallaesolidaginis* neonates, the possibility remains that larvae of these species synthesize JA. Other insect species are suspected to produce phytohormones to manipulate their host plants (e.g., Mapes and Davies, 2001), and further research should explore this possibility.

All but one of the species we screened originated from laboratory colonies maintained on artificial diets containing JA (Table 1); therefore, the presence of JA in eggs and neonates, or its presence at the high concentrations we measured, could conceivably be artifacts of these man-made diets. The four species of noctuid exhibiting very high amounts of JA in their eggs were all reared on the same diet, which had relatively high concentrations of JA (Table 1). However, JA recovered from field-collected *G. gallaesolidaginis* indicates that artificial diets are not solely responsible for JA in eggs and neonates. Moreover, concentrations of JA in the artificial diets used (Table 1) appear to be comparable to those found in live plants and those used by other researchers (e.g., Li et al., 2002).

Oviposition by some insect species can elicit plant volatile responses that attract natural enemies (Meiners and Hilker, 2000). Similar volatile emissions can be induced by applying exogenous JA, suggesting that JA plays a role in the initiation of plant defensive responses (Meiners and Hilker, 2000). Despite the recent implication of a protein in oviduct secretions as an elicitor of volatiles in response to oviposition (Hilker et al., 2005), our discovery of JA in lepidopteran eggs provides a plausible mechanism that could contribute to volatile production following oviposition.

The consistent presence of JA in lepidopteran eggs and neonates raises the possibility that it may serve some adaptive function for the insects themselves. Significant similarities exist between hormone signals used in plant and animal systems (Schultz and Appel, 2004), so JA might have a signaling function in lepidopterans. Jasmonates convey messages between plants and insects, inducing detoxification genes in feeding caterpillars (Li et al., 2002). Moreover, JA has substantial structural similarities to eicosanoids, and to prostaglandins in particular, which serve a range of functions in invertebrates including mediating oviposition, salivary secretions, and immune responses (Stanley-Samuelson and Pedibhotla, 1996). Further research is underway to determine what role, if any, JA plays in lepidopteran eggs and neonates.

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EXTRACTION OF CONDENSED TANNINS FROM CERVID FEED AND FECES AND QUANTIFICATION USING A RADIAL DIFFUSION ASSAY

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Abstract—A radial diffusion assay was employed to quantify condensed tannins (CT) in feed and feces of mule deer (*Odocoileus hemionus*) and white-tailed deer (*O. virginianus*). This biological assay measures the precipitation of bovine serum albumin (BSA), with the area of the precipitation ring being proportional to the amount of extracted CT applied to the well. CT extracted from the bark of white spruce (*Picea glauca*) was used as the standard. CT were extracted with 70% (v/v) aqueous acetone and precipitated with 50% (v/v) aqueous methanol or 70% (v/v) aqueous acetone. Functional range of CT weights for suitable ring measurement was 0.5–4.0 mg, and equilibrium was achieved within an incubation period of 24 hr. Methanol (50%) was a more effective precipitation solvent than acetone (70%) having 13 ± 4% greater specific activity ($P < 0.05$) and superior capabilities for predicting CT content. Precipitation rings were evaluated on images magnified on a photocopier. Ring diameters measured on a 200% enlarged photocopy provided the most precise estimate of ring area ($R^2 = 0.98$). This convenient method reduced analysis times and enhanced accuracy and precision of tannin quantification. Analytical consequences and future research requirements are considered.

Key Words— Condensed tannins, radial diffusion assay, extraction, quantification, gel plate photocopy, Cervidae, mule deer, white-tailed deer, feed, feces.

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INTRODUCTION

Tannins are polyphenolic compounds found in most plants and are generally thought to function as plant chemical defenses against pathogens and herbivory. Tannins are conventionally classified into two chemically distinct groups: hydrolyzable (gallotannins; HT), which occur mainly in fruit pods and plant galls; and condensed (proanthocyanidins; CT), which are the most common type found in forage legumes, trees, and shrubs (McLeod, 1974). CT can have a profound influence on herbivore nutrition, and hence, this paper will deal primarily with this type, as there is a limited presence of HT in natural feed in temperate regions (Martínez et al., 2004).

Mammal studies have shown that CT-containing forages can cause decreased digestibility and voluntary intake (Panda et al., 1983; Van Hoven, 1984), may reduce liveweight gain (Panda et al., 1983; Barry, 1985; Mehansho et al., 1987), wool growth (Barry, 1985), and mineral absorption (Disler et al., 1975; Roy and Mukherji, 1979), and can damage the intestine (Sandusky et al., 1977) or liver (Jones and Hunt, 1983). Therefore, it was thought that herbivores, particularly browsers, select forage to minimize CT intake (see Waterman and Mole, 1994).

However, recent evidence demonstrates the beneficial effects of CT when ingested in moderate amounts (<5% dry matter) (e.g., Verheyden-Tixier and Duncan, 2000; Min et al., 2003). For example, CT can act as natural detergents to reduce bloat (Waghorn and Jones, 1989; Tanner et al., 1995; Frutos et al., 2002) and decrease dependence on synthetic anthelminthic compounds for controlling internal parasites (Schrägle and Müller, 1990; Robertson et al., 1995; Niezen et al., 1995, 1998, 2002; Aerts et al., 1999; Hoskin et al., 2000; Molan et al., 2000; Kabasa et al., 2000). Furthermore, the biological value of forage may be enhanced with CT through reduced degradation of dietary protein to ammonia by rumen microbes, increasing protein outflow from the rumen, leading to increased absorption of amino acids from the small intestine (Waghorn et al., 1987). For example, improved production efficiency has been demonstrated with animals on diets having moderate concentrations of CT (2–4%), through increased wool growth, liveweight gain, milk yield, ovulation rate, and fecundity, while maintaining voluntary intake (Terrill et al., 1992; Wang et al., 1996a,b; Barry and McNabb, 1999; Min et al., 2003; Kabasa et al., 2004).

A lack of appropriate analytical techniques for extracting and quantifying CT has hindered investigations into tannin–protein associations in ruminants. Current methods, although precise, often involve elaborate sample preparation and tedious analytical procedures [e.g., thiolysis reversed-phase high performance liquid chromatography (HPLC; Guyot et al., 1998), spectrophotometric assay of dye-labeled protein (Asquith and Butler, 1985), precipitation of ribulose-1,5-bisphosphate carboxylase/oxygenase by tannic acid (Martin and

Martin, 1983), precipitation of iodine-125 labeled bovine serum albumin (BSA; Hagerman and Butler, 1980)]. These techniques were primarily used to measure CT in plant material and attempts to extract CT from feces have had little success. Our objective was to modify Hagerman's (1987) radial diffusion assay method for quantifying tannins in plant extracts, and to develop an improved protocol for extracting and quantifying CT in cervid feed and feces. This would facilitate a quick and easy analysis of large numbers of samples.

METHODS AND MATERIALS

Reagents. The assay gel contained BSA (fatty acid-free), agarose (Type I), 50 mM acetic acid, and 60 μM ascorbic acid (Sigma, St. Louis, MO, USA). Solvents used were 70% (v/v) aqueous acetone and 50% (v/v) aqueous methanol. All reagents were HPLC-grade.

Preparation of Standard. The assay was standardized with purified CT prepared from the ground bark of white spruce (*Picea glauca*) using an aqueous base extraction. The bark contained approximately 40% CT and the liquid extract was freeze-dried producing a black tannin powder. CT standards underwent nitrogen analysis (Leco FP-428 Nitrogen Determinator) to determine extent of protein contamination, which could interfere with the precipitation assay. Although not pure, impurities were removed and accounted for during the CT extraction procedure.

Feed and Study Animals. Feed used was standard deer ration (Cargill Nutrena Feeds, Camrose, AB, Canada) containing 31% barley, 25% alfalfa, 16% beet pulp, 14% wheat mince, 8.5% soy bean meal, 4% molasses, salt, and a vitamin/mineral mix. Bark CT extract was added to the ration to produce feeds containing 5% or 10% (w/w) tannin. Captive white-tailed deer (*Odocoileus virginianus*) and mule deer (*O. hemionus*) were divided into two groups: tannin and control. Following long-term maintenance on the standard deer ration, the tannin group was offered 5% CT feed for 5 wk, then 10% CT feed for a further 5 wk. The control group was maintained on CT-free ration prior to, and for the duration of the experiment. Fecal samples were collected from each group immediately before the introduction of the CT diet, at the end of 5 wk, and at the end of 10 wk.

CT Extraction. The same method was used for extracting CT from feed and feces, and preparing bark extract CT standard. Samples were freeze-dried for 48 hr at −60°C and ground through a 20-mesh screen in a Wiley mill. Ground samples (~500–600 mg feed/feces, ~200 mg CT standard) were dissolved in 5 ml extraction solvent (ES) (70% (v/v) acetone), vortexed several times over 20 min, centrifuged (5 min at 3000 rpm), and the supernatant was

transferred to a 15-ml plastic centrifuge tube. In total, three extractions were performed. The supernatant (~15 ml) was evaporated dry using a Multivap Nitrogen Evaporator (Organomation Associates Inc., Berlin, MA, USA). Pellets were resuspended in 3 ml precipitation solvent (PS) [i.e., either 70% (v/v) aqueous acetone or 50% (v/v) aqueous methanol], vortexed several times over 30 min, and centrifuged (5 min at 3000 rpm).

Following the third extraction, after removing the supernatant from the tube containing CT standard, the residue was air-dried and weighed. Also, following the addition of CT standard to gel plate wells, solution was centrifuged (5 min at 3000 rpm), the supernatant removed, and residue air-dried and weighed. Residue weights were cumulated and subtracted from CT standard weighed out to correct for impurities.

Gel Preparation. Gel plates were prepared according to Hagerman (1987). A solution of 50 mM acetic acid and 60 μM ascorbic acid was mixed, forming a solution of pH 2.8. This was adjusted to pH 5.0 by adding 0.1 M NaOH, as this has been reported as the optimum pH for maximum BSA precipitation (Hagerman and Klucher, 1986; López et al., 2004). The solution was heated to boiling while stirring, as 1% (w/v) agarose was added. The solution was cooled to 45°C and 0.1% (w/v) BSA added, while stirring. Aliquots of 9.5 ml were added to 8.5-cm-diam Petri dishes on a level surface (to obtain a gel of uniform thickness) and cooled. Gel plates were stored at 4°C to prevent bacterial growth.

Radial Diffusion Assay. Wells measuring 5.0 mm in diam were punched in the gel plates, spaced 1.5 cm apart. Using a micropipette, prepared solutions were added to wells (in duplicate) in 10-μl aliquots. As the solution was absorbed by the gel, several successive 10-μl aliquots were added, depending on the dilution of the solution. Wells were rinsed with successive 10-μl aliquots of PS [i.e., either 70% (v/v) aqueous acetone or 50% (v/v) aqueous methanol] to ensure all tannin was available to complex with BSA. Petri dishes were covered and sealed with Parafilm, and incubated at 30°C for 24–120 hr.

Ring Measurement. Following incubation, gel plates were photocopied at actual size, 200% enlargement, and 400% enlargement, and compared. Perpendicular ring diameters were measured to account for nonuniform ring development (rare) using microcalipers (actual size) and a standard mm ruler (enlargements). CT was quantified by squaring the mean of the perpendicular diameters (hereafter referred to as "area") and substituting this value into a calibration equation. This is the best-fit equation when ring area (mm^2) is regressed against known quantities of CT (mg) from feed and CT standard.

Calculations. All statistical analyses were performed using SPSS Base 12.0 (SPSS Inc., 2003). Simple linear regression was utilized to examine the predictive capabilities for quantifying CT and comparing ring measurement techniques. Student's paired *t*-test was used to compare ring areas when

determining specific activity of PS. Probabilities of $\alpha < 0.05$ were accepted as significant. Means are reported with standard errors.

RESULTS

The incubation period required for precipitation rings to achieve equilibrium (i.e., maximum size) was 24 hr for the range of CT weights used in this study. This time varies according to CT quantity, with longer incubation time required for higher CT concentration in wells.

Precipitation ring area (D^2, in mm^2) demonstrated a linear relationship with CT applied to wells (mg) (Figure 1). The calibration equations from experiments run separately were similar (Exp. 1: $y = 97.3 + 43.0x$, $R^2 = 0.98$, $P < 0.001$; Exp. 2: $y = 98.4 + 44.0x$, $R^2 = 0.88$, $P < 0.001$). All assays of extracts of feed not containing CT, fecal samples collected prior to experiment commencement, and fecal samples from deer in the control group, did not form precipitation rings (Table 1). Based on a presumed feed digestibility of approximately 70%, fecal CT concentrations from animals on CT diet were lower than expected (Table 1). The detection threshold for accurate measure-

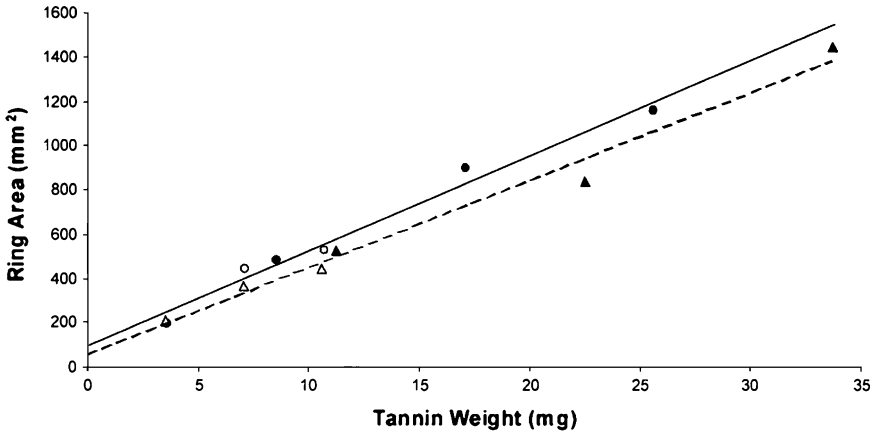

FIG. 1. Comparison of precipitation solvents for quantifying tannins. Ring area (mm^2) (measured from 200% enlargement) is regressed against known tannin weight (mg). Solid line represents 50% across aqueous methanol (●/○) ($y = 97.3 + 43.0x$, $R^2 = 0.98$, $P < 0.001$). Dashed line represents 70% acetone (▲/△) ($y = 56.0 + 39.4x$, $R^2 = 0.98$, $P < 0.001$). Closed symbols represent tannin standard bark extract from *Picea glauca*, whereas open symbols represent tannin extracted from cervid feed. Each point is the mean of duplicate ring areas.

TABLE 1. ESTIMATED TANNIN CONCENTRATIONS IN CERVID FECES

Cervid diet	N^a	Tannin concentration (%)[b]
0% Tannin	23	0.0 ± 0.0
5% Tannin	7	6.6 ± 2.5
10% Tannin	7	11.0 ± 1.6

[a] Indicates number of samples from which mean ± SE was calculated.
[b] The calibration equation area = 98.4 + 44.0 (CT weight) was used to estimate tannin weight. Tannin concentration is expressed as the percentage estimated tannin of tannin applied to well.

ment of precipitation rings is approximately 0.5 mg. It is recommended that CT applied to wells does not exceed 4.0 mg, as ring overlap precludes accurate measurement.

The Microanalytical Service Laboratory in the Department of Chemistry at the University of Alberta performed nitrogen analysis on the purified CT. Crude protein content (N × 6.25) was 1.3%, indicating negligible protein contamination. Removal of nontannin residue from bark extract after CT extraction revealed a purity of 75 ± 5%. This impurity was accounted for when determining standard calibration curves.

Greater specific activity was demonstrated with 50% aqueous methanol as a precipitation solvent (PS), than 70% aqueous acetone ($P < 0.05$), and hence, methanol was a more effective PS (Table 2). When equal quantities of CT were dispensed in wells, methanol formed rings with 13 ± 4% greater area than

TABLE 2. COMPARISON OF SPECIFIC ACTIVITY OF PRECIPITATION SOLVENTS

Sample type	Tannin Weight (mg)[a]	Diameter^2 (mm^2)[b]		
		70% acetone	50% methanol	50% Me/70% Ac
Tannin standard	14.1	529	484	0.91
Tannin standard	28.3	841	900	1.07
Tannin standard	28.3	841	1089	1.29
Feed (10% tannin)	3.5	210	196	0.93
Feed (10% tannin)	7.1	361	441	1.22
Feed (10% tannin)	10.6	441	529	1.20
Feed (10% tannin)	7.1	324	380	1.17
Fecal	3.6	169	182	1.08
Fecal	7.3	225	256	1.14
Fecal	10.9	256	324	1.27
				Mean 1.13 ± 0.04[c]

[a] Tannin weights for fecals are estimated.
[b] Values are means of duplicates.
[c] Greater ring area using 50% methanol is significant ($P < 0.05$).

TABLE 3. COMPARISON OF PHOTOCOPY METHODS FOR MEASURING PRECIPITATION RINGS

Sample	Tannin Weight (mg)	Diameter^2 (mm^2)[a]		
		Actual size	200% enlarged	400% enlarged
Tannin standard	8.6	153	484	3364
Tannin standard	17.1	252	900	5929
Tannin standard	25.6	281	1156	6724
10% Tannin feed	3.6	44	196	784
10% Tannin feed	7.1	96	441	1764
10% Tannin feed	10.7	113	529	2070
Regression equation[b]		$y = 24.4 + 10.9x$ $R^2 = 0.89$ ($P < 0.01$)	$y = 97.3 + 43.0x$ $R^2 = 0.98$ ($P < 0.001$)	$y = 35.5 + 281.1x$ $R^2 = 0.88$ ($P < 0.005$)

[a] Values are means of duplicates.
[b] Represents simple linear regression of ring area (mm^2) against tannin weight (mg).

acetone. Methanol also exhibited superior predictive capabilities to acetone for estimating CT concentration (Table 2). All ring area measurements included the area of the well. At 200% enlargement, the diameter of the well is 10 mm; therefore, the well area (D^2) is calculated as 100 mm^2. This suggests that the best-fit equation for perfect predictability when ring area (mm^2) is regressed against CT weight (mg), would have a y-intercept of 100. The best-fit regression equation, when 50% aqueous methanol was incorporated as the PS, had a y-intercept of 97.3 [area = 97.3 + 43.0(CT weight), $R^2 = 0.98$, $P < 0.001$] (Table 2), which is similar to that expected. Furthermore, precipitation rings with methanol PS had a distinct, clearly visible outer ring, facilitating easy, rapid measurement, whereas rings with acetone PS faded towards the outer edges.

Measuring ring diameter with a standard mm ruler on a photocopy enlarged 200% provided the most precise estimate of ring area ($R^2 = 0.98$) (Table 3). Accuracy of microcaliper measurements did not overcome the difficulty in visualizing the outer boundary of precipitation rings directly measured on the plate nor on an actual size photocopy ($R^2 = 0.89$). Rings on the photocopy enlarged 400% lacked the clarity necessary to accurately distinguish ring perimeter ($R^2 = 0.88$).

DISCUSSION

Radial diffusion assay reflects the ability of tannins to precipitate proteins. It is derived from the principle of radial immunodiffusion (e.g., Vaerman,

1981), in that tannin–protein interactions are similar to antigen–antibody reactions. For tannin quantification, more ecologically significant gel proteins could be substituted for BSA; however, Hagerman (1987) selected BSA for its homogeneity, solubility, and relatively low cost. The simplicity of the technique and the lack of complex reagents enhances reproducibility.

An advantage of the radial diffusion assay method is its specificity for tannins over other phenolic compounds. Other tannin quantification techniques depending on functional groups have been unreliable, as these functional groups are often not unique to tannins [e.g., Folin–Denis assay (Burns, 1963), Prussian blue (Price and Butler, 1977)]. Our samples that did not contain CT did not form precipitation rings. This suggests that the method is tannin-specific and experiences negligible interference from nontannin phenolics.

The 24-hr incubation period required for precipitation rings to reach equilibrium using this assay method is shorter than that recorded by Hagerman (1987) using a similar technique. Hagerman found that a 0.5-mg aliquot of tannic acid took 48 hr to reach equilibrium, whereas 1.0 mg required 96 hr. Within 24 hr, precipitation rings for the entire range of CT weights analyzed in this study (0.5–4.0 mg) achieved maximum diameter. This is convenient in that many analytical runs may be completed in a short time. The 70% aqueous acetone PS exhibited a faster diffusion rate through the BSA gel than 50% aqueous methanol; however, this is likely a result of the greater precipitation activity of methanol.

The tannin detection threshold of our technique (0.5 mg) was considerably higher than the 0.025 mg described by Hagerman (1987). This should not present a problem, as difficulties in measuring rings precipitated by a dilute sample can be obviated by dispensing more aliquots to the well until a suitable measurement may be obtained. Conversely, problems may also be encountered with solutions of high concentrations, as precipitation rings become so large that they interfere with the protein being precipitated from the adjacent well (ring overlap). This may be overcome by reducing the volume of solution dispensed in the well or increasing space between wells. Adjustments in volumes of solution dispensed in wells will provide optimal ring sizes for accurate measurement.

Acetone is considered to be one of the most efficient solvents for extracting tannins (Fletcher et al., 1977). For example, Yu and Dahlgren (2000) reported that 50% aqueous acetone extracted 20–59% more CT than 50% aqueous methanol. However, some precipitation techniques are highly sensitive to acetone interference (e.g., Hagerman and Butler, 1978; Asquith and Butler, 1985), and the presence of even small quantities of acetone may inhibit protein precipitation, requiring further procedures to remove the acetone (e.g., rotary evaporation). Hagerman (1987) reported that this radial diffusion assay method is unaffected by the presence of acetone. In our study, the combination of 70% aqueous acetone for extracting and 50% aqueous methanol for precipitating CT

provided maximum CT extraction and BSA precipitation. Furthermore, the clearly defined outer ring produced when precipitating CT using methanol (i.e., acetone-precipitated rings had faded outer edges) facilitates rapid, accurate measurement.

CT analysis is complicated by the complexity and variability of structures found with this group of compounds (Schofield et al., 2001). Structural variation among CT can introduce error when assays are used for quantification, and as such, selection of the standard is paramount to the success of tannin assays. Nelson et al. (1997) noted that errors can arise from an incorrect choice of external standard, particularly when it differs from the tannin being analyzed. In this study, we had the advantage of the CT standard and analyzed CT being the same, which likely contributed to the superior predictive capabilities demonstrated.

Although the bark extract employed as the standard in this study contained contaminants, removal of impurities during CT extraction was simple. In order to reliably predict CT quantities extracted from fecal samples, for every batch it is necessary to have duplicate standard reference wells on each plate, as well as producing precipitation rings from a suitably wide range of CT standard weights. From this, the simple regression calibration equation is developed by examining precipitation ring area (mm^2) in relation to known quantities of CT standard in each well. Several methods reviewed by Maxson and Rooney (1972) had significant variation in standard curves between days and/or laboratories. This highlights the importance of preparing individual standards for each analytical run. This ensures that samples and standards are prepared and run under identical conditions. Although variation in calibration curves among our batches was negligible, each batch should be restandardized with purified CT.

The unique, yet simple technique of ring measurement developed in this study provides an accurate and rapid method for determining ring area. Assay methods used in the past have depended on colorimetric measurement of absorbance using spectrophotometric laboratory equipment [e.g., dye-labeled protein assay (Asquith and Butler, 1985), vanillin-HCl assay (Price et al., 1978), Prussian blue (Price and Butler, 1977), butanol-HCl hydrolysis (Porter et al., 1986)]. This “gel plate photocopy” technique is convenient in that laboratory instruments are unnecessary. In fact, gel plates can be photocopied immediately after incubation and measurement carried out anywhere and at anytime thereafter. Also, photocopies can be reaccessed later, if further analysis is desired. Perhaps measurer bias may be reduced by scanning gel plates with a gel scanner or photocopies with a conventional image scanner, followed by computer analysis of the digitized images.

Fecal tannin concentrations reflected that of diet and perhaps could be used to monitor diets of wild deer using easily collected samples. However, lower than expected fecal CT concentrations suggest that differences in extraction

efficiency between feed and feces preclude its use as an internal marker for estimating feed digestibility. Feces may contain CT that has formed associations with proteins while passing through the gastrointestinal system. This radial diffusion assay technique does not have the ability to detect these bound tannins, as BSA cannot form associations with them to produce a precipitate. Therefore, further research into radial diffusion assays should attempt to account for the presence of bound tannins in feces. Phenol is a strong solvent that dissociates tannin–protein complexes (Hagerman and Butler, 1980). Perhaps simultaneous comparison of precipitation rings from CT solutions that have been exposed to phenol fractionation, to those that have not, will reveal ratios of bound to unbound proteins.

Effects of antioxidants on specific activity of protein precipitation should also be investigated further. Peng and Jay-Allemand (1991) found a 20% increase in protein precipitation when the antioxidant diethyldithiocarbamic acid was added to the agarose gel. They also demonstrated protein precipitation increases of 30% and 75% when the antioxidants ascorbic acid and sodium metabisulfite ($Na_2S_2O_5$), respectively, were added to the 50% aqueous methanol extraction solvent.

Hagerman (1987) found the radial diffusion assay method for quantifying tannins in plant extracts to be simple, sensitive, and specific, facilitating analysis of large numbers of samples. Complex reagents and elaborate instruments were not necessary and there was no apparent interference from nonphenolic compounds. Our experiments yielded comparable results, whereas modifications to the technique provided a more convenient method with augmented precipitation specific activity leading to shorter analysis times and enhanced accuracy and precision of CT quantification. Methods employed in this study had high reproducibility and exhibited excellent linear relationships between protein precipitation area and CT concentrations in wells. One must be aware of the limitations of this procedure for extracting and quantifying fecal tannins, because of its inability to reliably account for bound tannins. Modifications of this highly reliable technique will prove useful in future investigations into the tannin content of plants and nutritional effects on animals consuming these plants.

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RAPID ABSORPTION OF DIETARY 1,8-CINEOLE RESULTS IN CRITICAL BLOOD CONCENTRATION OF CINEOLE AND IMMEDIATE CESSATION OF EATING IN THE COMMON BRUSHTAIL POSSUM (*Trichosurus vulpecula*)

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Abstract—The blood concentration of 1,8-cineole and its metabolites was measured in six male brushtail possums while they voluntarily fed on diets laced with varying concentrations of cineole for 3 d. On the third day, blood samples were collected during and after each bout of feeding for 3 hr. Blood cineole was measured by using headspace solid-phase microextraction (SPME), while cineole metabolites were measured by liquid–liquid extraction followed by gas chromatography-mass spectroscopy. Feeding patterns were measured by continual recording of residual food weight and time. Cineole absorption was rapid, resulting in a peak blood concentration at the end of each feeding bout. The blood concentration of cineole did not exceed a critical value (51.8 ± 14.1 μmol/l) regardless of the concentration in the diet. Food and, therefore, cineole intake was regulated. The amount of food ingested in the first feeding bout decreased from 236 ± 52 g on the control diet to 36 ± 20 g on the 4% cineole diet. The amount of cineole ingested in the first bout (1.18 ± 1.10 g) was the same regardless of the dietary concentration and was controlled by the size of the meal. Total food eaten during the 7-hr feeding session decreased by 64% from 368 ± 94 g (control diet) to 131 ± 52 g (4% diet). Total cineole intake increased from 2.47 ± 0.60 g (1% diet) to 5.05 ± 2.41 g (4% diet). Cineole metabolites accumulated throughout the sampling period and were generally still rising at the end of blood sampling period. Blood levels of metabolites were at least 10-fold higher than cineole levels.

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The immediate control of feeding seems to be regulated by blood levels of cineole, whereas metabolites are likely to be more important in regulating the chronic ingestion of cineole.

Key Words—Absorption, antifeedant, blood concentrations, Brushtail possum, 1,8-cineole, cineole metabolites, plant secondary metabolites, terpenes.

INTRODUCTION

Diet selection is a complex process for herbivores that must strike a balance between meeting nutritional requirements and avoiding harm from plant defenses. Herbivores need to select a diet of foliage with sufficient nutritional content for optimal health and survival. Meanwhile, plants are armed with physical or chemical defense systems that deter browsing (Southwell, 1973; Cork, 1984; Raven et al., 1986; Palo and Robbins, 1991). Each herbivore has its own specific strategies for coping with plant defenses. Strategies are reflected and often identified as measurable alterations in feeding behaviors, particularly after the ingestion of toxic chemical plant defenses (Wiggins et al., 2003; Boyle and McLean, 2004; Villalba and Provenza, 2005).

In this study, we examined the voluntary ingestion of 1,8-cineole (1,3,3-trimethyl-2-oxabicyclo[2,2,2]octane), a monoterpene synthesized by many *Eucalyptus* species. 1,8-Cineole is a known antifeedant (Lawler et al., 1999; Boyle and McLean, 2004). We used the brushtail possum (*Trichosurus vulpecula*), a marsupial commonly found across the majority of vegetated Australia, as our model herbivore. The brushtail possum is considered a generalist herbivore and it includes *Eucalyptus* foliage in its diet.

There have been many studies observing and measuring changes in feeding behavior with the introduction, manipulation, or withdrawing of dietary toxins in herbivore diets (Guglielmo et al., 1996; Dearing and Cork, 1999; Dearing et al., 2000; Mangione et al., 2000; Atwood et al., 2001; Marsh et al., 2003; Wiggins et al., 2003; Boyle and McLean, 2004). Generally, the underlying physiological or toxicological mechanisms causing the change in feeding behavior have been speculative. Considering that cineole is a small lipophilic molecule and will readily cross the blood–brain barrier, it is likely to exert antifeedant effects as a consequence of low-grade toxicity associated with central nervous system (CNS) depression. We hypothesized that (1) the blood concentration of plant secondary metabolites (PSMs) is an important determinant of PSM ingestion, and (2) feeding behavior is modified as a result of critical concentrations of cineole and/or its metabolites. To address these hypotheses, we measured the concentrations of 1,8-cineole and its metabolites

in blood, as possums voluntarily consume a diet containing varying concentrations of 1,8-cineole.

METHODS AND MATERIALS

Animals. Six male brushtail possums (*T. vulpecula*) were trapped from bushland around the city of Hobart, Tasmania, Australia. Animals were housed in individual enclosed pens with wooden nest boxes (28 × 28 × 36 cm) attached to the walls. They were fed daily a freshly prepared maintenance diet of grated silverbeet (*Beta vulgaris*), carrot, apple, lucerne chaff, and sugar (McArthur et al., 2000). Cineole was added to this diet in appropriate amounts and mixed in well.

Prior surgical implantation of Vascular Access Ports (model TI200; titanium reservoir, reservoir volume 0.25 ml, silicone septum, preattached silicone catheter (5 Fr) cut to 15 cm; Access Technologies, Skokie, IL, USA) provided reliable long-term access to the jugular vein for blood sampling (Cleva et al., 1995). Animals were sedated with Zoletil (12 mg/kg, i.m.; Virbac Australia Pty Ltd, Peakhurst, NSW, AU) and anesthetized with isoflurane (Rhodia Australia Pty Ltd, Notting Hill, Victoria, AU). The surgical procedure was similar to that used in the koala (Cleva et al., 1995). Animals were allowed at least 1 wk to recover before experiments commenced. Ports were maintained by flushing them twice weekly with saline, and were kept filled with heparin saline (125 units/ml).

Experimental Design. Six possums were used in the experiments. There were five treatment periods of 3 d. Each started on the same day of the week for 5 consecutive wk. The first was for acclimation to the procedure, daytime feeding, and the feeding setup while receiving the 1% cineole diet (wet weight). The remaining four treatment diets were: (1) control diet (regular maintenance diet), (2) 1% cineole diet, (3) 2% cineole diet, and (4) 4% cineole diet. These were presented in a randomized crossover design.

Possums were fasted the night before the start of each treatment, and the treatment was presented each day for the next 3 d. Each morning possums were taken from their enclosures to the feeding apparatus in their usual nest boxes. They stayed in their nest boxes throughout the experiments. The treatment diet was presented for 7 hr. The length of the feeding session was arbitrarily chosen, balancing maximum exposure to food with convenience. The length of the session was appropriate, as it allowed time for possums to consume sufficient food, from multiple bouts if required, to maintain body weight. Possums were returned in their nest boxes to their usual pens each night. A single possum (BP8) became uncharacteristically active and agitated when offered the 4% cineole diet, and was withdrawn from this treatment.

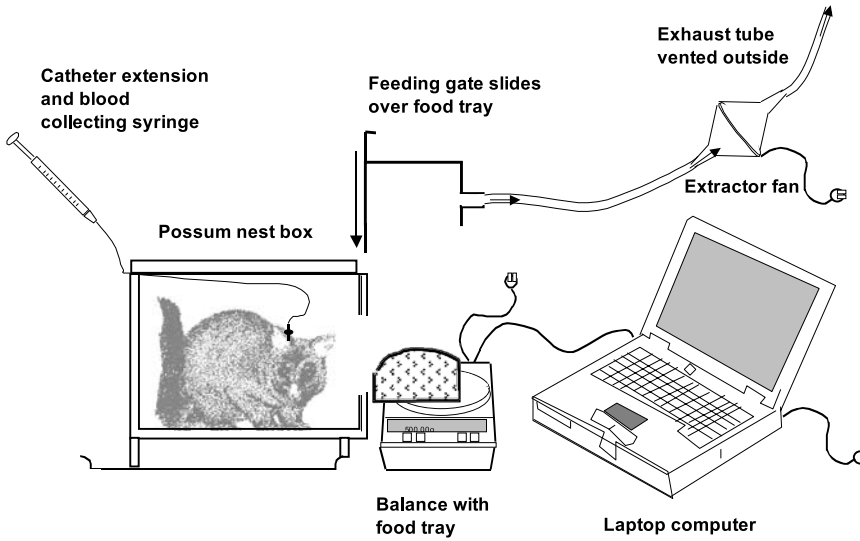


FIG. 1. Feeding and blood sampling apparatus. Possums remained in their own nest boxes. The port was accessed with a Huber needle and catheter that extended out of the box. The diet was weighed into a steel tray and secured onto the balance. The feeding gate slid over the entrance allowing possums *ad libitum* access to the diet. The tube at the rear of the feeding slide went to an extractor vented fan. The balance recorded time and weight into a laptop computer.

Feeding Setup. The feeding apparatus was designed so that detailed measurements of food intake in freely feeding possums could be taken throughout the day, while allowing regular blood samples to be collected at the same time. The setup, shown in Figure 1, was fitted to the nest box. Food was measured into a stainless steel dish that was placed on a balance positioned at the front of the nest box. The digital output from the balance was fed into a laptop computer, and a program (written in Qbasic) recorded the time and the weight of the food dish every 45 sec. A gate was slotted onto the front of the nest box and fitted snugly around the food tray. This ensured that the possum remained within the nest box while providing *ad libitum* access to food. Food intake was calculated from the weight of food lost from the feeding dish. From these data, the number, length, and size of feeding bouts were calculated.

A transparent top of the feeding gate was usually covered but, if necessary, allowed visual inspection of the possum as it fed. At the back of the feeding chamber, a vacuum hose (diam 32 mm) was attached to an extractor fan (flow rate ranged from 0.9 to 1.1 l/sec) and vented to a fume cupboard. This was necessary to prevent the accumulation of cineole vapor in the nest box and feeding area.

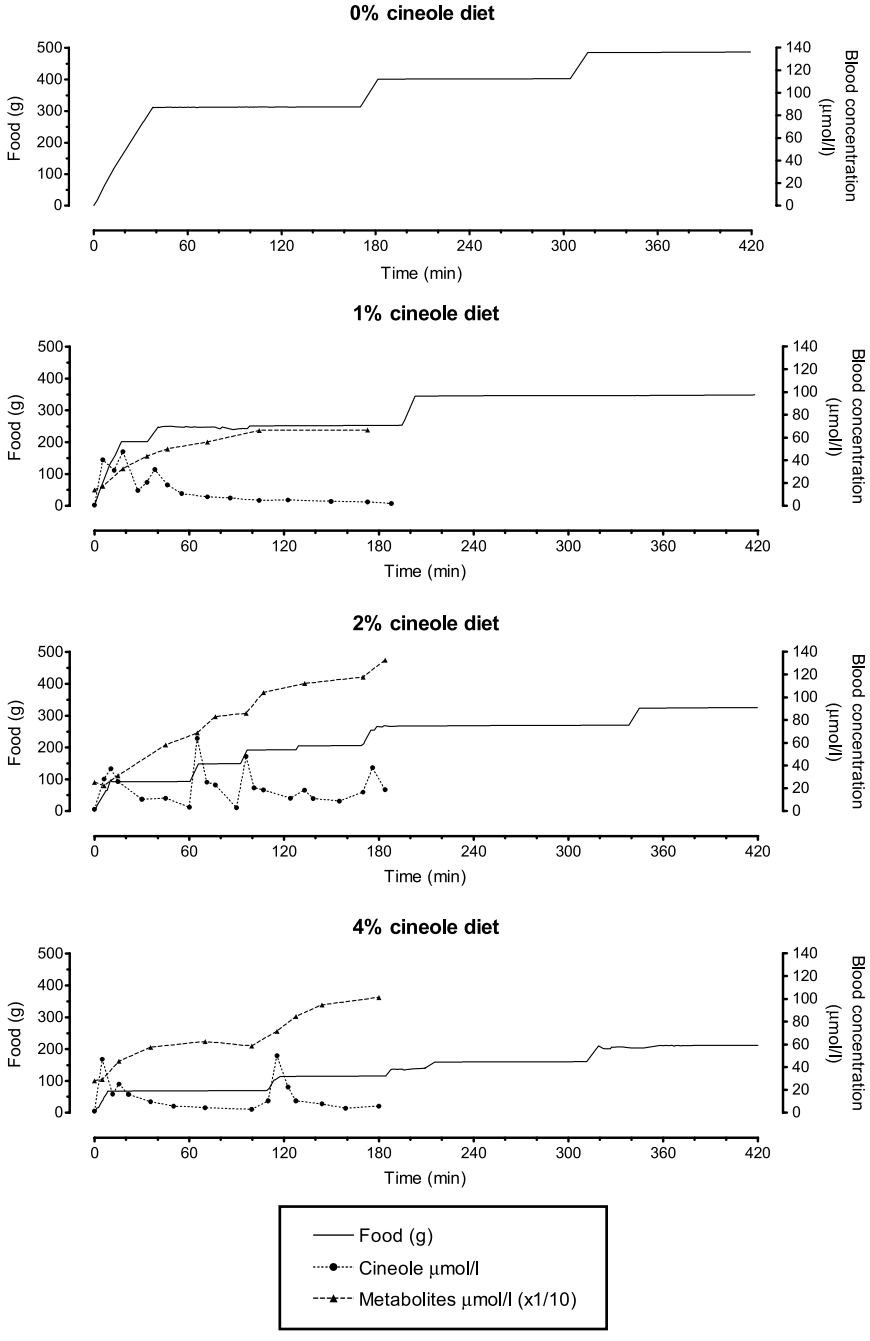
Blood Sampling. On the third day of each cineole treatment, blood was collected during the first 3 hr of feeding. The vascular access port was accessed and tested before the possum was allowed to start feeding. The port site was first cleaned with 70% sterile alcohol [Pharmacia (Perth) Pty Ltd., Bentley, Western Australia, AU], 0.05% chlorhexidine acetate, and 0.5% cetrimide aqueous antiseptic solution (Baxter Healthcare Pty. Ltd., Old Toongabbie, NSW, AU), and finally 10% povidone iodine (Betadine Antiseptic Solution, Faulding Pharmaceuticals, Salisbury, South Australia, AU). A saline-filled right angle noncoring Huber Infusion Set (22-ga., right angle, 1/2" tip-to-bend, 45 cm catheter tubing; Access Technologies, Skokie, IL, USA) was inserted into the port reservoir. The tail end of the catheter was capped and left outside the closed lid of the nest box, allowing blood to be collected as often as required without disturbing the possum. The dead volume of the port (0.5 ml) was withdrawn and discarded, a control blood sample (0.4 ml) was collected, and the port and catheter volume was refilled with heparinized saline [50 U/5 ml; Pharmacia and Upjohn (Perth) Pty. Ltd., Bentley, Western Australia, AU].

Blood samples were collected at the start of each feeding bout and then every 5 min until 10 min after the end of the bout. Samples were taken every 15–30 min thereafter, until the start of the next feeding bout or the end of the 3-hr collecting period. The volume of blood collected depended on the analyses to be performed. Blood cineole was measured in every sample collected, while metabolites were only measured in every other sample. Therefore, blood volumes collected alternated between 0.1 and 0.3 ml. The port and catheter were filled with heparinized saline between each blood sample to prevent blood clotting in the catheter.

Blood samples were transferred directly to a heparinized screw cap glass vial (2 ml volume). Vials were prepared by diluting heparin injection 25,000 U/5 ml [Pharmacia and Upjohn (Perth) Pty. Ltd.] to 25 ml and transferring 100 μl to each vial before drying in an oven at 60°C. After collection of blood, the vials were sealed with a PTFE/silicon septum (Bonnet Equipment Pty Ltd, Tarren Point, NSW, AU) and temporarily stored in an insulated box filled with salted ice. At the end of each experiment, all samples were frozen at −18°C until thawed for analysis.

Blood Analysis

Cineole. Blood cineole concentrations were measured by solid phase microextraction (SPME) using the method previously described by Boyle et al. (2002). Blood was thawed to room temperature, and 50 μl were measured into a 17 × 60 mm glass screw cap vial (Packard Bioscience, AU). The internal standard (250 ng linalool in 25 μl water) and 25 μl water were also added to the vial, which was capped using PTFE-lined silicon septa and then vortexed. The


0% cineole diet
1% cineole diet
2% cineole diet
4% cineole diet
Food (g)
Blood concentration (µmol/l)
Time (min)
0
60
120
180
240
300
360
420
100
200
300
400
500
20
40
60
80
120
140
Food (g)
Cineole µmol/l
Metabolites µmol/l (x1/10)

fiber sheath was inserted through the septum, and the fiber was exposed to the headspace above the blood. The vial was heated at 35°C, and the fiber was exposed for 12 min. After absorption, the fiber unit was immediately transferred to the GC injector for desorption and GC analysis.

Cineole Metabolites. The method is based on our method used to analyze urinary metabolites of cineole (Boyle et al., 2000). Briefly, cineole metabolites, isolated from possum urine, were used as internal standards to prepare calibration curves for each metabolite. Metabolites were extracted from both unhydrolyzed and hydrolyzed blood samples by using liquid–liquid extraction and subsequently derivatized with *N*,*O*-bis(trimethyl)trifluoroacetamide (BSTFA; Alltech Associates, Deerfield, IL, USA) to form trimethylsilyl (TMS) derivatives. The derivatized extracts were analyzed by gas chromatography-mass spectroscopy (GC-MS), and the amount of metabolite was calculated by using prepared calibration curves for each metabolite.

Data Analysis. The balance output data were manipulated by subtracting each weight recording from the starting weight to give the cumulative amount of food eaten. Evaporation of water from the diet was measured during periods where the food was undisturbed for prolonged periods (>1 hr) on 20 occasions. The evaporation rate was 0.63 ± 0.25 g/hr. The amount of food presented to possums was 500 gm. Therefore, less than 1% of the starting weight of food was lost due to evaporation over the 7-hr feeding session. Evaporation of cineole from the wet bulky diet was also insignificant. The same diet, laced with 4% cineole, was analyzed by hexane extraction and GC for cineole concentration before and after it had been offered overnight to possums kept in metabolism cages. There was no significant difference in concentration between the fresh and the residual food (Boyle et al., unpublished data). Therefore, no correction for evaporation of water or cineole was needed. Each time reading was also converted to time from the start of the experiment.

We arbitrarily defined a feeding bout as an intake of more than 3 g, while rests of up to 2 min were accepted as part of the same feeding bout. From these data, we calculated the total food and cineole intake, the percentage of the food eaten in bouts, the size of the largest feeding bout, the total time spent eating, and the mean duration of feeding bouts. The rate of food and cineole consumption in each bout was also calculated by dividing the amount of food eaten by the time taken to eat it. We also determined the amount of food, and therefore cineole, consumed in the first feeding bout, the duration of the first

FIG. 2. Food consumption and blood concentrations of cineole and metabolites for each of the four dietary concentrations of cineole for a single possum (BP 11). Possums were offered the diet for 7 hr, and blood samples were collected during the first 3 hr of that period.

feeding bout, and the percentage of the daily food intake ingested in the first bout.

Although feeding data were recorded and calculated for each day of experiments, feeding behavior is reported only for the third day in order to compare directly to the measured blood levels.

Statistical comparisons used repeated-measures (RM) ANOVA, except where specifically stated otherwise. Separate RM ANOVA were performed for the following parameters: maximum cineole blood concentrations, amount of food ingested in the first bout, the amount of cineole ingested in the first bout, the time spent feeding on the first bout, the percentage of total food eaten in the first bout, the percent of total food eaten in bouts exceeding 3 gm, total food intake, total cineole intake, rate of food consumption, and rate of cineole consumption. The computer software program GraphPad Prism (version 4 for Windows, GraphPad Software, San Diego, CA, USA) was used for statistical analysis and graphing. Where data are summarized, values are expressed as mean ± SD.

RESULTS

Figure 2 overlays food ingested (and thus cineole ingestion) with both blood cineole and metabolite concentrations for each treatment for a single possum (BP 11). The control diet was eaten in 3 bouts, whereas with increasing cineole concentrations the feeding bouts became smaller and total food consumption progressively fell. Similar patterns were seen in all other possums.

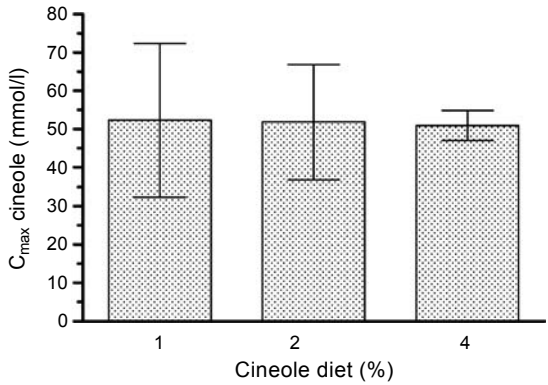


FIG. 3. The mean maximum blood cineole concentration after any feeding bout for each of the three dietary cineole concentrations. Data are shown as mean ± SD ($N = 6$, except for the 4% diet where $N = 5$).

For every feeding bout on a cineole diet, there was an associated rise in blood cineole levels. Cineole absorption was rapid with peak concentrations occurring before, or just at, the end of the feeding bout. Cineole blood levels then declined until the beginning of the next feeding bout.

The mean maximum cineole blood concentrations (C_{max}) for each cineole treatment were similar, regardless of the dietary concentration (51.8 ± 14.1 μmol/l, $N = 17$; repeated-measures ANOVA $F_{2,10} = 0.15$, $P = 0.87$; Figure 3). Variability in the mean C_{max} decreased with increasing percentage of cineole in the diet.

Each feeding parameter is summarized in Table 1 for all 3 d of data collection. Repeated-measures ANOVA for the 3 d was performed for each parameter, and significant results are indicated. The main differences across the 3 d was that food intake increased progressively on the cineole diets and the number of feeding bouts increased at the higher cineole concentrations.

TABLE 1. SUMMARY OF FEEDING PARAMETERS (MEAN ± SD) COLLECTED OVER THREE DAYS OF FEEDING

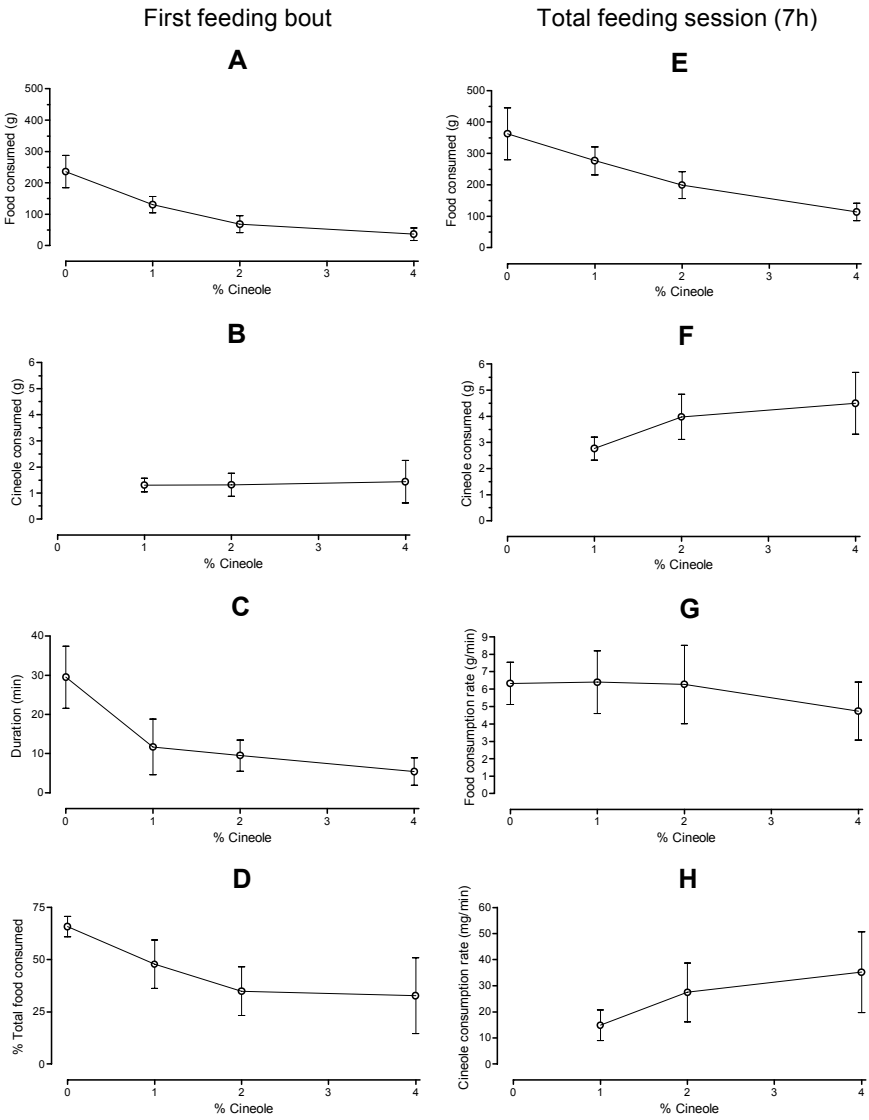
	Values recorded over three days (mean ± SD)			
	0%	1%	2%	4%
Total measurements				
Food consumed (g)	362 ± 88	277 ± 65*	199 ± 66**	112 ± 40**
Cineole consumed (g)	0	2.77 ± 0.60*	3.98 ± 1.30**	4.4 ± 1.68**
Percent eaten in bouts (>3 g)	97.6 ± 1.7	97.3 ± 2.1	94.8 ± 3.5**	87.5 ± 7.1*
Maximum bout (g)	266 ± 80	133 ± 37	72 ± 49	40 ± 23
Number of bouts	4.8 ± 1.9	7.3 ± 2.9	6.3 ± 2.1**	4.8 ± 1.9**
Time spent eating (min)	46.8 ± 5.1	41.4 ± 15.4	32.8 ± 8.1	25.8 ± 12.0
Mean duration of bouts (min)	10.7 ± 4.9	6.4 ± 2.6	5.2 ± 2.7	4.3 ± 2.2
Rate of food consumption (g/min)	6.0 ± 2.0	6.4 ± 1.9	6.3 ± 3.5	4.7 ± 1.6
Rate of cineole consumption (mg/min)	0	64.1 ± 19.2	127.3 ± 65.8	215.7 ± 149.1
First feeding bout				
Food consumed (g)	236 ± 58	130 ± 45	68 ± 51.5	36 ± 24
Cineole consumed (g)	0	1.30 ± 0.45	1.40 ± 0.10	1.44 ± 1.00
Duration of first bout min)	26.9 ± 8.9	15.6 ± 7.6	9.9 ± 5.1	6.8 ± 6.6
% Food eaten in first bout	62 ± 17	48 ± 17	35 ± 20	33 ± 21

Repeated-measures ANOVA were performed on the means of each day for each parameter to identify significant changes in that parameter over the 3 d.

*Significantly different means.

**Linear trend in significantly different means over 3 d.

Feeding parameters recorded on the third day for each cineole concentration are the averages for all possums and are shown in Figure 4. This figure summarizes data for both the first bout and the total feeding session of 7 hr. The order of treatment was not associated with any trends in the amount of cineole consumed in the first bout.



In 18 of the 23 experiments, the first feeding bout was the largest meal of the day. The amount of food ingested in the first bout decreased from 236 ± 52 g (mean ± SD) on the control diet to 36 ± 20 g on the 4% diet (repeated-measures ANOVA $F_{3,15} = 40.7$, $P < 0.001$; Figure 4A). The amount of cineole ingested in the first bout was the same regardless of the dietary concentration (1.18 ± 1.10 g; repeated-measures ANOVA $F_{2,10} = 0.12$, $P = 0.89$; Figure 4B).

Time spent feeding on the first bout decreased with increasing cineole concentration (repeated-measures ANOVA $F_{3,15} = 0.94$, $P < 0.001$; Figure 4C). The percentage of total food eaten in the first bout also decreased progressively from 66% of the control diet to 33% of the 4% diet (repeated-measures ANOVA $F_{3,15} = 7.08$, $P = 0.004$; Figure 4D).

Most of the food consumed in the 7-hr session was accounted for in bouts of greater than 3 g, although the percentage decreased with increasing amounts of cineole (repeated-measures ANOVA $F_{3,15} = 0.87$, $P = 0.02$). While feeding on the control diet, 98% of the food loss throughout the feeding session was accounted for in feeding bouts (>3 g), whereas this value decreased to 88% on the 4% cineole diet. Total food intake decreased with increasing concentrations of cineole (repeated-measures ANOVA $F_{3,15} = 22.43$, $P < 0.001$; Figure 4E). Overall food intake on the 4% cineole diet (131 ± 52 g) was reduced by 64% compared to the control diet (368 ± 94 g). Cineole intake, however, increased with increasing concentrations of cineole in the diet (repeated-measures ANOVA $F_{2,10} = 7.27$, $P = 0.01$; Figure 4F). The amount of cineole eaten in the 1% diet (2.47 ± 0.60 g) was approximately half of that eaten in the 4% diet (5.05 ± 2.41 g). The rate of food consumption, measured during feeding bouts only, did not slow significantly with increasing cineole concentration (repeated-measures ANOVA $F_{3,15} = 1.71$, $P = 0.2$; Figure 4G), and this in turn resulted in an increasing rate of cineole intake (repeated-measures ANOVA $F_{3,15} = 17.61$,

FIG. 4. Summaries of feeding patterns on day 3 of each treatment. A, B, C, and D relate to the first feeding bout only, whereas E, F, G, and H relate to the total or overall feeding patterns. (A) The amount of food eaten in the first feeding bout of the day decreased as the percentage of cineole in the diet increased. (B) The amount of cineole ingested in the first bout was the same across all concentrations of dietary cineole. (C) The amount of time spent feeding during the first bout declined with increasing cineole. (D) The percentage of the total daily intake of food eaten in the first bout dropped with increasing cineole content. (E) The total amount of food eaten during the feeding session decreased with increasing cineole concentrations. (F) Total cineole intake increased with increasing cineole concentrations. (G) Possums ate the food at a similar average rate regardless of the concentration of cineole, although the rate was lower on the 4% diet. (H) Since the rate of food intake was consistent, the rate of cineole ingestion must increase with increasing concentration in the diet. Data are shown as mean ± SD ($N = 6$, except for the 4% diet where $N = 5$).

TABLE 2. CINEOLE METABOLITE CONCENTRATIONS (MEAN ± SD) MEASURED APPROXIMATELY 3 HOURS AFTER COMMENCING CINEOLE DIETS

Cineole diet (%)	Metabolite concentration (μmol/l)	Cumulative cineole dose (g)
1	570.8 ± 260.0	1.72 ± 0.71
2	968.3 ± 345.2	3.44 ± 1.47
4	980.5 ± 492.1	4.28 ± 1.78

$N = 4$.

$P < 0.001$; Figure 4H), although there were large fluctuations noted in the rate of cineole intake.

Eighteen oxidized metabolites of cineole have been found excreted in possum urine (Boyle et al., 2000). Thirteen of these were easily detected and quantified in the blood, and each was analyzed and quantified separately. The metabolites quantified were four hydroxycineoles (2-, 3-, 7-, and 9-hydroxycineole), two carboxycineoles (7- and 9-cineolic acid), and seven hydroxy carboxycineoles (positions of oxidations were not elucidated). The carboxycineole and hydroxy carboxycineole metabolites were generally measured at similar levels, and together they accounted for the majority of metabolites. The hydroxycineole metabolites accounted for only a small proportion of total metabolites in the blood. There was no marked change in the proportions of individual metabolites over the sampling periods, so the molar concentrations were summed to simplify interpretation of results. Cineole metabolites accumulated throughout the blood sampling period and were still rising after 3 hr (see Figure 2). The maximum concentrations of cineole metabolites are recorded in Table 2. These values were measured in the final blood sample (taken approximately 3 hr after the start of the experiment).

DISCUSSION

These are the first data on blood concentrations of an antifeedant and its metabolites during feeding, and are coupled with continuous food consumption data. Furthermore, we measured these parameters after manipulating the concentration of cineole in the diet, thereby changing feeding behavior. We are able to make some observations about the antifeedant mechanisms of terpenes such as 1,8-cineole in herbivores.

The example shown in Figure 2, the feeding pattern of one possum, overlaid with the respective blood cineole and metabolite concentrations, shows the association between eating and circulating blood levels of cineole and its metabolites. The conclusions and their implications on feeding are discussed below.

There were sharp peaks in blood cineole concentrations associated with every feeding bout. Cineole was found in the first blood sample collected after feeding commenced. Cineole concentration during each feeding bout peaked within a few minutes of the start of the bout and was associated with the time that the animal stopped feeding (Figure 2). From the possums' perspective, every time it eats a diet containing cineole it has to deal with a rapid surge of circulating blood cineole. Cineole is a small lipophilic molecule that readily crosses the blood–brain barrier, directly exerting its toxicity on brain function resulting in CNS depression (Patel and Wiggins, 1980; Webb and Pitt, 1993; Whitman and Ghazizadeh, 1993; Barnes, 1996). Surges in blood cineole are likely to rapidly transpose into adverse effects on the CNS that would not only compromise the functioning ability of the animals but also provide a rapid feedback mechanism to stop feeding on that diet.

The rapidity of cineole uptake was surprising, as the dose was dispersed in food. We had expected the food bolus to be retained in the stomach for digestion. It is possible that the immediate cineole peaks arise from absorption directly through the stomach. Rapid C_{max} have been seen after oral dosing of terpenes in other studies (Boyle et al., 2002; Sorensen and Dearing, 2003).

In Figure 3, we see that the maximum cineole concentration resulting from any feeding bout was the same regardless of the dietary concentration. In 11 of the 17 cineole diet experiments, C_{max} resulted from the first feeding bout of the day. The first day was when possums were at their hungriest, as they were starved the previous night. In these cases, subsequent feeding bouts resulted in substantially lower cineole blood levels associated with smaller feeding bouts and cineole intake.

Possums also adjusted their food intake in the first feeding bout such that the amount of cineole ingested was the same regardless of the dietary concentration (Figure 4B). Combined, these observations provide evidence that possums are able to titrate blood cineole concentrations, and that they stop cineole intake at a critical blood concentration (about 50 μmol/l).

The circulating blood cineole concentration dropped rapidly after feeding had stopped, yet feeding did not resume upon the levels returning to a low level. It is likely that cineole concentration in the brain lags behind circulating blood concentration, and it is the concentration in this organ that is important for resuming feeding. The degree of satiation after the initial feeding bout and the memory of the aversive experience may delay the possum from resuming feeding until driven by hunger.

It is interesting that there was less variability in C_{max} values measured on the 4% cineole diet compared to diets with a lower percentage of cineole. Several factors may account for this. First, the 1% diet consists of four times the bulk for the same cineole dose compared to the 4% diet. The food may act as a buffer and slow cineole absorption and delay the relay time between

food ingestion and peak blood concentrations leading to the higher blood concentrations. We attempted to reconcile the timing of C_{max} to the end of the respective feeding bout to show this effect, but we were unsuccessful, as individual possum feeding behavior was variable, making it difficult to identify patterns in such detail. Also, while feeding on diets with lower cineole concentrations, possums may have been satiated before the critical amount had been ingested, resulting in smaller blood cineole peaks. However, at 4%, possums are hungry and appear to eat as much cineole as they can tolerate before stopping. We suggest that the smaller bulk of food on 4% diets resulted in more controlled feedback of food intake. This was supported by food and cineole intake measured in the first feeding bout; food intake decreased with increasing cineole concentration, but the amount of cineole ingested remained constant.

A more general cause of variability in measuring the peak concentrations of cineole in the blood was the time interval between blood samples. The sample interval of 5 min was used as the minimum time interval we could reliably manage between samples. However, the true peak of C_{max} may not have been captured in each case, as the concentration rose and fell so rapidly.

In contrast to the reactivity in cineole blood levels during feeding, cineole metabolite levels rose steadily throughout the sampling period. Overall levels of metabolites increased with increasing dietary concentrations. The maximum metabolite concentrations measured in each experiment were at least 10-fold higher than cineole C_{max} values. Most cineole metabolites are acidic, and the accumulation and excretion of such large acid loads provides a significant challenge for the cineole-eating possum (Foley et al., 1995). We suggest that metabolite levels are also important in regulating cineole intake. Although blood cineole levels produce immediate feedback to the possum regulating feeding, metabolites levels are likely to control the total daily or chronic intake of cineole by limitations in excretion of the acid load.

Ideally, these experiments should be performed during the possums' active nighttime phase. However, possums are wild, agile animals with feisty characters. Although they are difficult to handle during their active phase, they are relatively compliant during the day, and for this reason the experiments were performed during the daytime. It might be expected that active possums stop feeding at a lower blood cineole titer, as the consequences of CNS depression in active and arboreal animals are more serious than in sleepy, nest-box-bound possums.

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FEEDING RESPONSES OF FREE-FLYING HONEYBEES TO SECONDARY COMPOUNDS MIMICKING FLORAL NECTARS

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Abstract—The role of secondary compounds (SC) in deterring herbivores and pathogens from vegetative parts of plants is well established, whereas their role in plant reproductive organs such as floral nectar is unclear. The present study aimed to reveal the response of free-flying honeybees to naturally occurring concentrations of four SC in floral nectar. We selected nicotine, anabasine, caffeine, and amygdalin, all of which are found in nectar of various plants. In repeated paired-choice experiments, we offered 20% sucrose solution as control along with test solutions of 20% sucrose with various concentrations of the above SC. Except for anabasine, naturally occurring concentrations of SC did not have a deterring effect. Furthermore, low concentrations of nicotine and caffeine elicited a significant feeding preference. SC can, therefore, be regarded as postingestive stimulants to pollinators, indicating that the psychoactive alkaloids in nectar may be a part of their mutualistic reward. Further studies are needed to test our hypothesis that psychoactive alkaloids in nectar impose dependence or addiction effects on pollinators.

Key Words—Nectar, secondary compounds, naturally occurring concentrations, honeybees, attraction, deterrence.

INTRODUCTION

Many studies have focused on elucidating the role of secondary compounds (SC) in deterring herbivores and pathogens from plants (e.g., Rosenthal and Berenbaum, 1991). Others have determined the costs and benefits of producing

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these compounds in the context of plant–herbivore interactions (Van Dam et al., 1996; Agrawal et al., 1999). The current concept is that the wide varieties of SC produced by higher plants play a multifunctional role in the complex biotic and abiotic interactions of plants (Izhaki, 2002; Harrison and Baldwin, 2004; Holopainen, 2004). The myriad challenges that plants face seem to promote natural selection for SC that possess multiple functions (Wink, 1999; Adler et al., 2001; Gronquist et al., 2001; Izhaki 2002). Although the role of SC in deterring herbivores and pathogens is well established (Karban and Baldwin, 1997), their role as mediators of plant–pollinator mutualistic relationships has been widely overlooked (Adler, 2000).

SC are not uncommon in floral nectar. Depending on the specific compound, SC have been found in 9–55% of surveyed species (Baker and Baker, 1975; Baker, 1977, 1978). The nectar of some plants may be deterrent or even toxic to floral visitors (Adler, 2000), and widespread "toxic nectar" is puzzling considering its attractive role in pollination (Faegri and van der Pijl, 1979). The deterrence and toxicity of SC in nectar may be activated through unpalatability or through an effect on the central nervous system of the flower visitors (Baker and Baker, 1975). Consequently, SC in nectar may mediate plant–pollinator interactions affecting the fitness of both plants and pollinators (Baker, 1977).

Several adaptive hypotheses have been proposed to explain the ecological and evolutionary roles of SC in nectar (Rhoades and Bergdahl, 1981; Adler, 2000). The most common claims that SC deter nectar robbers and generalists, or inefficient pollinators. Baker and Baker (1975) suggested that the level of tolerance to SC in nectar by pollinators is related to their efficiency in transferring conspecific pollen. This "pollinator fidelity" hypothesis holds that the nontolerant pollinators are also less efficient in transferring conspecific pollen in comparison to pollinators that are more tolerant to SC (Adler, 2000). However, toxic nectar may have no adaptive function but instead be a consequence of production and mobilization of SC in other plant tissues (Adler, 2000).

Most SC studied so far (e.g., alkaloids, glycosides, phenolic substances) actually deter bees (*Apis mellifera*) within a wide range of high concentrations (Detzel and Wink, 1993). The effects of SC on bees are dose- and season-dependent (e.g., Singaravelan et al., 2006). Low concentrations of phenolic substances such as caffeic and genistic acids elicited preference, whereas high concentrations deterred honeybees (Hagler and Buchmann, 1993). Likewise, bees preferred low concentrations of amygdalin during early summer but not later (London-Shafir et al., 2003). Some alkaloid-containing nectars attracted bees in the field even when alternative nectar sources were available (Ish-Am and Eisikowitch, 1998). This circumstantial evidence indicates that bees cope with naturally occurring concentrations of SC in nectar. Despite evolutionary and ecological implications, the interaction between bees and SC in nectar has not

been widely studied. Specifically, this study was designed to test the responses of honeybees to natural concentrations of SC in nectar, with *a priori* prediction that the latter will not impose strong deterrent effects.

In repeated paired-choice experiments with artificial nectar, we studied feeding preferences of free-flying honeybees (*A. mellifera*) under natural conditions. We offered the bees artificial nectar of 20% sucrose solution as a control, simultaneously with test solutions of 20% sucrose containing various concentrations of four SC. We tested nicotine and anabasine, naturally occurring in the nectar of *Nicotiana* spp. (Detzel and Wink, 1993; Tadmor-Melamed et al., 2004), caffeine that is most common in the nectar of *Citrus* spp. (Kretschmar and Baumann, 1999), and amygdalin that characterizes almond (*Amygdalus* sp.) nectar (London-Shafir et al., 2003). We examined the effects of naturally occurring concentrations of these SC on foraging behavior of honeybees.

METHODS AND MATERIALS

Experimental Arena and Training Procedures. We conducted the experiments on a flat rooftop of a building in the Oranim campus of University of Haifa, Israel, between January and April 2004. Hourly temperatures were recorded with a maximum–mininum thermometer. We conducted the experiments only when the ambient temperature was above 18°C. Honeybees were trained to feed on sucrose solutions (20% sucrose) from 250-ml translucent plastic beakers (6.5-cm diam). The mouth of the solution-filled beakers was covered with a Petri dish (8.6-cm diam and 1.3 cm deep) and turned upside down. The bees fed from the nectar trough formed around the beaker's mouth. Nectar spontaneously filled the trough whenever its level dropped below the mouth. This feeder allowed 70–80 bees to feed simultaneously. Each feeder was placed on a colored plastic plate (14-cm diam) that was placed on a white plastic tray (36 $\times$ 26 cm) on the floor. We started by training bees to feed from a 20% sucrose solution in one station. Then, we split them into five separate groups, each feeding from a feeder that was placed on different colored plate. Later, we gradually separated five feeders about 20 m apart. In a preliminary experiment, we marked 50 bees at each feeding station and monitored their visits for about a week to ensure that they established independent feeding groups. Indeed, about 85% of the bees fed only in the feeding station where they were marked, whereas only some (<15%) were observed also in another feeding station.

Secondary Compounds. We tested the bees' response to four SC: nicotine (Aldrich Ltd), anabasine, caffeine, and amygdalin (Sigma Ltd). We chose these SC because honeybees frequently visit flowers and feed on the nectar of *Nicotiana* spp., *Citrus* spp., and *Amygdalus* sp. that contain them, and their natural concentration in floral nectar is known (Table 1).

TABLE 1. NATURALLY OCCURRING CONCENTRATIONS OF SC AND THE CONCENTRATIONS TESTED IN EXPERIMENTS I AND II OF THE PRESENT STUDY

Secondary compounds	Plant species	Naturally occurring concentration in nectar (ppm)	References	Concentration tested in the present study (ppm)	
				Experiment I	Experiment II
Nicotine	*Nicotiana tabacum*	0.166[a]	Detzel and Wink (1993)	2.5, 5, 10, 20	0.5, 1, 2, 5
	Nicotiana glauca	0.56 ± 0.12 (0 to 2.5)	Tadmor-Melamed et al. (2004)		
Anabasine	*Nicotiana tabacum*	0.166[a]	Detzel and Wink (1993)	NT	2.5, 5, 10, 25
	Nicotiana glauca	5.4 ± 0.90 (0 to 50)	Tadmor-Melamed et al. (2004)		
Caffeine	*Citrus paradisi*	94.26 ± 2.90	Kretschmar and Baumann (1999)	50, 100, 150, 200	12.5, 25, 50, 100
	Citrus maxima	17.61 ± 0.97			
	Citrus limon	11.61 ± 0.39			
Amygdalin	*Amygdalus communis*	4 to 10	London-Shafir et al. (2003)	5, 10, 25, 50	2.5, 5, 7.5, 10

Natural concentration: mean ± SE; range, when available, is given in parentheses.
NT: not tested.
[a]Total alkaloid concentration.

Food Preference Trials. At each feeding station, we offered the bees simultaneously one feeder with a control solution (20% sucrose) and one feeder with a test solution (20% sucrose with a known concentration of one SC). In each experimental session, only one SC was tested, and in each experimental trial, the same concentration of the same SC was tested against the control in all five feeding stations. Thus, each test was replicated five times. We preferred the paired-choice design, as it is a compromise between multiple-choice tests, which simulate the natural situation but suffer from lack of independence among observations, and single-choice tests, which often underestimate preferences because of the lack of real choice (Manly, 1993). In the first experiment, we examined the response of bees to a wide range of concentrations (experiment I, Table 1) to obtain concentration–response information. This enabled us to determine the threshold minimal deterring concentration for each SC. In the second experiment, we repeated the same experimental design testing the range of naturally occurring concentrations of each SC (experiment II, Table 1).

To determine the consumption rate, we weighed (Precisa Instruments Ltd, Switzerland, electronic balances) each feeder before and after 1 hr of bee feeding. Simultaneously with the control solution, a particular concentration of SC test solution was offered in all stations simultaneously for 1 hr. The tested concentrations were changed after each hour. Whenever we changed the tested concentrations, we also changed the relative position of the control and test feeders randomly on the plastic tray to shun any possible association of any solution type with a certain position by bees. Each experimental session that tested a range of concentrations of a SC lasted 3–5 consecutive days. Between any two experimental sessions, we had an average time lag of 5 d, during which we offered only control solutions to the bees. On each experimental day, we tested all concentrations of a particular SC for its selected range. We changed the order of presentation of the various concentrations during each experimental day.

To detect correlations between number of bees and consumption rate of test solutions, we counted the number of bee visits in each station with a tally counter and stopwatch. Bees were counted for 1 min at the control feeder and 1 min at the tested solution feeder.

Data Analyses. We considered each experimental station as an independent replicate, as >85% of marked bees remained feeding only in one station. Thus, we obtained five replicates for each concentration of each SC. We calculated the percentage differences in food intake and in the number of bees per minute between control and experimental solutions for each preference trial and each station. We averaged the differences for the three experimental days. We analyzed these differences by two-tailed one-sample t test. One-way ANOVA was used to detect effects of SC concentrations on food consumption followed by Tukey's multiple comparison test ($P < 0.05$). We related the number of bees that visited the feeders and the consumption rate of test solutions with Pearson's

correlation. All proportions were arcsin-square-root-transformed prior to statistical analyses for normal distribution. Results are presented as mean ± SE.

RESULTS

Experiment I—Wide Range of Concentration Series. In this experimental series, the bees were not deterred from naturally occurring concentrations of nicotine, caffeine, and amygdalin (Figure 1). On the contrary, bees consumed these concentrations more than that of the control solutions. Bees were deterred by concentrations of nicotine and caffeine that were higher than natural concentrations in nectar (>2.5 and >100 ppm, respectively). The deterrence effect tended to increase with concentration by an order of magnitude. Notably, bees were not deterred by any of the tested amygdalin concentrations. The relative differences in consumption of treated solutions per hour from that of control varied significantly across concentrations for all SC except for amygdalin (Figure 1).

Experiment II—Natural Range of Concentration Series. Of the four SC tested within their natural range of concentration, bees significantly preferred the lower concentrations of nicotine and caffeine over the control. They were significantly deterred by three of four concentrations of anabasine. Although bees consumed more amygdalin at all tested concentrations than controls, the differences were not significant (Figure 1). This preferential intake of experimental solutions was significant for 0.5 and 1 ppm of nicotine and for 25 ppm of caffeine. Moreover, in this experiment, bees were not deterred by any of the tested concentrations of caffeine and amygdalin. The consumption of tested solutions relative to that of controls varied significantly across concentrations for all SC except amygdalin (Figure 1).

Consumption Rate vs. Number of Bees. We found a positive and significant correlation between number of bees feeding and consumption rate across concentrations and SC (nicotine: $R = 0.89$, $N = 40$, $P < 0.001$; caffeine: $R = 0.85$, $N = 40$, $P < 0.001$; amygdalin: $R = 0.40$, $N = 40$, $P < 0.01$; anabasine: $R = 0.82$, $N = 20$, $P < 0.001$). The number of bees feeding on the tested solutions relative to control solutions showed a similar pattern to that of the relative consumption of the solutions and is, therefore, not presented here.

DISCUSSION

Preference vs. Deterrence. Honeybees use multiple cues to identify food. They associate and/or memorize many chemical stimuli with sucrose to recognize or discriminate among differing mixtures of reward (Jakobsen et al.,

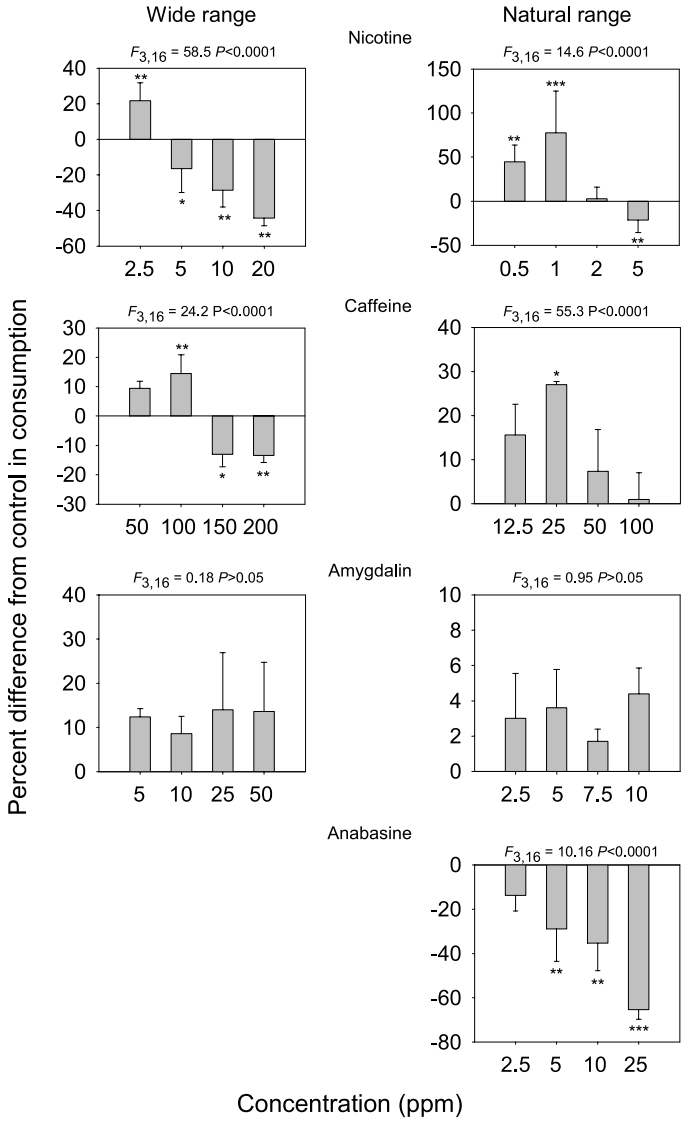

FIG. 1. Responses of *Apis mellifera* to various concentrations of four secondary compounds in artificial nectar of 20% sucrose. The relative differences (%) in nectar intake of the test solutions from controls were subjected to one sample, two-tailed t test, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Bars represent mean ± SE; positive bars indicate preference, and negative bars indicate deterrence. The effects of concentrations are presented as F and P values of one-way Anova.

1995; Laska et al., 1999; Paldi et al., 2003). For foraging bees, natural concentrations of SC are associated with artificial reward imitating natural floral nectar. Our results indicate that except for the strong deterrent effect of anabasine, which acts as a selective nicotinic acetylcholine receptor (nAChR) agonist for insects (Sultana et al., 2002), honeybees were not deterred by nicotine, caffeine, or amygdalin in their natural range of nectar concentrations. Furthermore, honeybees significantly preferred solutions with low concentrations of nicotine and caffeine over control (20% sucrose) solution. A similar but nonsignificant pattern was detected also for all concentrations of amygdalin (Figure 1). When offered a wide range of concentrations, bees consumed nicotine and caffeine solutions only within their natural concentration range and were deterred by higher concentrations. For amygdalin, bees were not deterred even by higher concentrations. It appears that some SC in nectar may furnish foraging cues to mutualistic pollinators as has been hypothesized for fruits and their frugivores (Cipollini and Levey, 1997).

Bees appeared to modulate their response to the differing concentration spectra of SC, as they chose the lower concentrations of nicotine and caffeine in both ranges. Such modulatory and/or differential responses across different concentration spectra are known for nectar compounds in general (Masson et al., 1993; Menzel, 1993) and SC in particular (London-Shafir et al., 2003). Nonetheless, the differential responses cannot be an outcome of choice behavior or side bias by bees to the feeders during choice experiments, as we noted a consistency in deterrence response to concentrations deviating from the natural range of nicotine and caffeine.

Response to Nicotine. The biphasic, dose-dependent response in nicotine intake might be the result of a dual motivational effect of nicotine (Laviolette and van der Kooy, 2004), rewarding (preference) at low concentrations on the one hand and aversive (deterrence) at higher concentrations on the other. Nicotine acts on endogenous nAChR prevalent in the central and peripheral nervous systems in almost all animal species (Laviolette and van der Kooy, 2004). The highly inducible nicotine, which acts as a feeding deterrent to herbivorous insects, can be found in vegetative plant parts in relatively high (300–5000 ppm) concentrations (Ohnmeiss and Baldwin, 2000). Our results demonstrated that honeybees were deterred even by much lower concentrations (≥5 ppm) of nicotine in sucrose solution.

Low concentrations of nicotine act as positive reinforcers both through intravenous and oral self-administration (Halladay et al., 1999; Laviolette and van der Kooy, 2004). Nicotine drinking has induced active nicotine preference in rats (Halladay et al., 1999). In insects, the actions are likely to be CNS-specific, where they appear to play a major excitatory role (Wolf and Heberlein, 2003). Repeated exposure to nicotine enforces subsequent neuronal changes in the mesolimbic dopamine system of the brain, which, in turn, evokes com-

pulsive nicotine-seeking behaviors in mammals (Laviolette and van der Kooy, 2004). Although the mechanism in insects has yet to be elucidated, there are some indications that invertebrates such as the nematode *Caenorhabditis elegans* (Schafer, 2004) and insects such as *Drosophila* (Bainton et al., 2000) adopt addictive behaviors when exposed to low concentrations. Given this, it is paramount to test rigorously whether nicotine in nectar imposes dependence or addiction effects on pollinators. The mammalian literature on addiction characterizes it as a progressive increase in preferential intake of psychoactive substances despite its toxic effects and/or even after deprivation of drugs over a stipulated period (Heyne and Wolffgramm, 1998). However, addiction (if any) to substances such as nicotine in nectar by pollinators needs to be studied in detail. It should be noted that natural concentrations of nicotine (Table 1) do not affect the fitness of caged honeybees (Singaravelan et al., 2006).

Response to Anabasine. The bees in our experiments were deterred even by naturally occurring concentrations of anabasine. Thus, we cannot rule out the possibility that certain SC at their natural concentrations deter honeybees. Indeed, anabasine is a selective nAChR agonist for insects with insecticidal activity at relatively high concentrations (Sultana et al., 2002) and an effective antifeedant (Gonzalez-Coloma et al., 2004). Nonetheless, anabasine and nicotine are both constituents of *Nicotiana* nectar; anabasine is the predominant compound in *N. glauca*, whereas nicotine is the major one in *N. tabacum* (Bush and Crowe, 1992). Notably, honeybees visit only the flowers of the latter. It would be of interest to study the response of bees to combinations of nicotine and anabasine simulating the natural situation.

Response to Caffeine. Caffeine acts as a mild reinforcer and psychostimulant to mammals such as rats (Vitiello and Woods, 1977). In contrast, it may function as an antifeedant to insects (Bernays et al., 2000), although it is not efficiently effective against insect pests of coffee (Guerreiro and Mazzafera, 2000). Caffeine in relatively high concentrations is deterrent (ED_{50} at 300 ppm) and even toxic to honeybees (LD_{50} at 2000 ppm; Detzel and Wink, 1993). In our study, bees preferred caffeinated 20% sucrose solutions, within its natural concentration range in nectar, over control 20% sucrose (Table 1). In natural situations, honeybees collect caffeine containing *Citrus* nectar (Ish-Am and Eisikowitch, 1998) and even prefer it to alternative nectar resources. Moreover, in Israel, during winter, when nectar resources are limited, honeybees often forage in trash bins on sweetened Coca-Cola (personal observations) that contains 103 ppm of caffeine (http://www.coca-cola.com; accessed 24 March 2005). These factors may help bees in dealing with caffeine in floral nectars.

Response to Amygdalin. Bees showed a nonsignificant higher intake of amygdalin-laced 20% sucrose solutions than 20% sucrose controls in both natural and wider concentration ranges. A previous study showed a variable seasonal response of honeybees to amygdalin. The intake of amygdalin-laced sucrose

varied with the availability of other seasonal nectar sources (London-Shafir et al., 2003). The cyanogenic glycoside amygdalin also did not have strong deterrent effect on folivorous orthopterans (Bernays, 1983), but reduced food intake in two noctuid caterpillars (Glendinning and Slansky, 1994). The preference and performance of a frugivorous cedar waxwing bird (*Bombycylla cedrorum*) were not affected by even high concentrations of amygdalin (Struempf et al., 1999).

SC in Nectar vs. Pollen. Detzel and Wink (1993) found that bees were deterred by many SC, but mostly at higher concentrations that we tested. Our concentration range was based on naturally occurring concentrations in floral nectar, whereas Detzel and Wink (1993) examined higher ones that occur mainly in pollen. However, foraging bees probably do not encounter in nectar high ED_{50} concentrations of SC (mentioned in their study). From an evolutionary perspective, to increase fitness, plants might have evolved higher concentrations of SC in pollen to deter pollen eaters and lower concentration in nectar to increase attractiveness to pollinators.

Possible Mechanisms. We found that honeybees can discriminate well among various concentrations of SC (Figure 1), as reported earlier (Hagler and Buchmann, 1993; London-Shafir et al., 2003). Such discrimination might be based on the universally bitter taste of alkaloids (Kingsbury, 1964). How do honeybees overcome this unpalatability? The presence of carbohydrates (sugars and sugar alcohols) can "mask" the unpleasant taste of some SC to herbivorous insects (Glendinning, 2000), as carbohydrates inhibit the response of deterrent taste cells (Shields and Mitchell, 1995). It appears that sucrose (20%) might have masked the unpalatable nature of low concentrations of nicotine and caffeine. Further studies should reveal the full spectrum of this tradeoff by evaluating the bee's responses to various concentrations of SC in various concentrations of sugar.

Ecological and Evolutionary Implications. As predicted, naturally occurring concentrations of nectar SC do not have a strong deterrent effect on bees (with the exception of anabasine); rather, some low concentrations of nicotine and caffeine even significantly stimulate them. Although honeybees are generalist pollinators, a few *Nicotiana* sp., *Citrus* spp., and *Amygdalus* spp. depend on bees for pollination (Detzel and Wink, 1993; Kretschmar and Baumann, 1999; London-Shafir et al., 2003). Thus, our results provide some support for the "pollinator fidelity" hypothesis, as honeybees are not deterred by SC and were even stimulated by the natural concentrations of nicotine and caffeine mimicking nectar. Notably, nicotine and caffeine are not restricted to nectars of *Nicotiana* spp. and *Citrus* spp. These alkaloids are distributed in nectars of other plant species (Naef et al., 2004). Thus, further studies should focus on the hypothesis that plants produce these compounds in nectar to "addict" faithful pollinators. Many insects are addicted to SC (Boppré, 1999; Renwick and

Lopez, 1999; Renwick, 2001), and plants may use SC to mediate various insect–plant relationships by a method of differential allocation of SC concentrations to different plant parts (Harborne, 1993; Boppré, 1999). Thus, SC in nectar may govern the selection of the best mutualistic partners. The prediction of a pollinator fidelity hypothesis remains to be studied.

In summary, pollinators are stimulated by a variety of constituents in nectar at substance-specific spectra of concentrations. They are stimulated mainly by substances such as sugars and amino acids to fulfill their energetic and nutritional demands (Baker and Baker, 1975) and are controlled by taste thresholds (Gardener and Gillman, 2002). They are also stimulated by essential oils (Detzel and Wink, 1993) and other volatiles/scents mediated by olfactory sense (Heinrich, 1979). These may be considered as "preingestive stimulants." In a similar manner, some SC, particularly the psychoactive alkaloids in nectar, may act as "postingestive stimulants" mediated possibly by their concentration-specific rewarding (pleasuring) effects on flower visitors. Conceivably, a considerable number of alkaloids in nectar (e.g., nicotine, caffeine, cannabinoids) have both addictive and aversive properties and have not yet been studied in an ecological context. It is a question of considerable interest whether preferential intake of low concentrations of nicotine and caffeine could impose dependence or addiction effects on bees.

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NUTRIENT COMPOSITION OF LARVAL NECTAR SECRETIONS FROM THREE SPECIES OF MYRMECOPHILOUS BUTTERFLIES

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Abstract—A comparative chemical analysis of the larval nectar secretions and hemolymph from three unspecifically and facultatively ant-attended lycaenid species (*Polyommatus coridon*, *P. icarus*, and *Zizeeria knysna*) was performed by using high-performance liquid chromatography techniques. Sucrose was the main sugar component in all three species. In half of the samples of *P. coridon*, it was accompanied by glucose, whereas other sugars occurred only rarely. In *P. icarus* and *Z. knysna*, melezitose was the second-most important component, followed by fructose and glucose. Total sugar contents were 43.6 ± 14.8 g/l (mean ± SD) for *P. coridon*, 74.2 g/l for *P. icarus*, and 68.3 ± 22.6 g/l for *Z. knysna*. Up to 14 different identified amino acids were found in *P. coridon* nectar, with a total content of 9.7 ± 3.4 g/l. Leucine was always the major component (contributing 50% of overall amino acid content). Other important amino acids were tyrosine, proline, arginine, and phenylalanine. *P. icarus* nectar contained up to six amino acids with a total content of 1.2 g/l, dominated by tyrosine and phenylalanine. *Z. knysna* nectar contained alanine and proline, with only 0.3 ± 0.17 g/l total content. In the hemolymph of all species, up to 16 different amino acids occurred relatively regularly, with histidine dominating, followed by serine and proline. The amino acid pattern in hemolymph was considerably different from that of the nectar secretions. Larval diet weakly influenced *P. coridon* nectar sugars, and with a semisynthetic diet, a more homogeneous amino acid

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pattern was detected. Comparison with reports from other lycaenid species shows that secretions rich in amino acids are related to intimate, often obligate ant associations, whereas facultative, unspecific myrmecophiles rely on carbohydrates.

Key Words—Lycaenidae, ants, myrmecophily, facultative mutualism, nectar secretion, sugars, amino acids.

INTRODUCTION

Lepidopteran caterpillars are slow-moving insects, with a soft body enclosed in a weakly sclerotized cuticle. Because they accumulate resources for the adult phase of life, they are full of nutrients and an attractive target for predators. Particularly, ants, as the leading terrestrial invertebrate predators, prey on caterpillars unless these are protected by mechanical (e.g., hairiness) or chemical (e.g., deterring or poisonous substances) means. Caterpillars in the butterfly family Lycaenidae provide a remarkable exception because they are either ignored or may even enter into stable associations with these aggressive social insects. More than half of the lycaenid species associate with ants during development (Fiedler, 1991; Pierce et al., 2002). The habit of lycaenid larvae (and many other arthropods) to associate with ants is termed myrmecophily. The intimacy of such associations is defined by the degree of dependence on the ant partner. Species that require specific ants for their survival, as protectors against enemies or as a food source, are obligate myrmecophiles. Species that can thrive with or without ants are facultatively myrmecophilous.

Caterpillar–ant interactions are mediated by several glandular organs (Malicky, 1969; Cottrell, 1984; Kitching and Luke, 1985). Most important for closely ant-associated, nonparasitic lycaenid caterpillars is the dorsal nectar organ (DNO, nomenclature after Cottrell, 1984), a gland situated on the seventh abdominal segment. Upon tactile stimulation of the surroundings of this gland by attendant ants (Malicky, 1969), larvae secrete droplets of an attractive clear fluid (herein called "nectar''). Nectar secretions are essential to induce food recruitment behavior in ants that have found a caterpillar (Fiedler and Maschwitz, 1989), and the importance of a DNO for maintaining stable ant associations is emphasized by comparative data (Kitching and Luke, 1985; Fiedler, 1991).

The delivery of nectar secretions is under the control of the caterpillar. Secretory behavior is finely tuned to the particular circumstances (Leimar and Axén, 1993), and only minute amounts are spent. This is different from ant associations of aphids and other Homoptera, to which Lycaenidae are often compared. Honeydew of aphids is a modified waste product that is excreted in

large amounts. It is easily available for chemical analysis, and its composition is well known for many homopteran species (e.g., Yao and Akimoto, 2002; Blüthgen et al., 2004; Woodring et al., 2004). In contrast, the collection of lycaenid larval nectar secretion is an arduous process (Daniels, 2004), and only a few species have been examined (Nomura et al., 1992; Cushman et al., 1994; Pierce and Nash, 1999). These studies are based on small sample sizes, and usually few quantitative data are reported. Furthermore, apart from two studies (Maschwitz et al., 1975; Wagner, 1994), all others deal with obligate myrmecophilous lycaenids. These larvae secrete comparatively large amounts of nectar, but represent the extreme end of a large range of different degrees of myrmecophily. Only 20% of all lycaenid species worldwide appear to be obligate myrmecophiles. In most geographical regions and phylogenetic lineages, facultative myrmecophiles are far more numerous (Fiedler, 1991, 1998; Pierce et al., 2002). For a better understanding of the evolutionary and functional ecology of butterfly–ant associations, precise data on the quality and quantity of nectar in facultative myrmecophiles are needed. This would also enable testing the hypothesis proposed by Pierce (1987) that more generalized myrmecophiles will secrete carbohydrates, whereas intimate and host-specific myrmecophiles will have a more nitrogen-biased nectar.

Here, the first comparative analysis of the secretions of three facultatively myrmecophilous lycaenid species was undertaken. As the major study organism, one of the most strongly ant-associated species in Europe, *Polyommatus coridon*, was chosen. It occurs in southern and central Europe, and larvae feed on *Hippocrepis* and *Coronilla* species (Fabaceae). A second species, *P. icarus*, has a less intimate ant association, a wider host plant range (various herbaceous Fabaceae), and is distributed throughout the Palearctic region. Finally, *Zizeeria knysna* from Africa and southern Europe was investigated. Its larvae are more polyphagous and have only occasionally been reported to be attended by ants (Fiedler, 1991). Within the subfamily Polyommatini, *P. coridon* and *P. icarus* are closely related representatives of the *Polyommatus* section, whereas *Z. knysna* is a more distant relative in the *Zizeeria* section (Wiemers, 2003). We aimed at answering the following questions:

- What are the nutrient contents of the nectar secretions, and how do these compare to other ant-visited nectar sources?
- To what extent is the nectar composition influenced by the diet that caterpillars consume during their development (cf. Burghardt and Fiedler, 1996)?
- Does the allocation of carbohydrates vs. amino acids differ among lycaenid species in relation to the intimacy of ant associations?
- Does nectar composition reflect phylogenetic relatedness, or ecological similarities, among species?

METHODS AND MATERIAL

Insect Rearing. Caterpillars for nectar samples were raised in captivity under standardized conditions from eggs laid by captive females or from individuals collected as second and third instars in the wild (*P. coridon*). *P. coridon* and *P. icarus* originated from northern Bavaria (vicinity of Bayreuth), and *Z. knysna* originated from Gran Canaria and Fuerteventura. Females were allowed to oviposit on host plants in cages lined with moist cellulose tissue (26:15°C, 75% r.h., 16-hr light/8-hr dark period). Butterfly cultures were maintained in climatic chambers over various generations.

Every 3 d, plant shoots with eggs were transferred to closed transparent plastic boxes (volume 250 or 1000 cm^3, dependent on group size) that were lined with moistened cellulose tissue for optimal humidity. In these boxes, caterpillars were raised at 25°C (16-hr light/8-hr dark) until pupation. *P. coridon* was reared on its two natural host plants [*Hippocrepis comosa*, *Securigera* (*Coronilla*) *varia*] and on a semisynthetic diet (Daniels, 2004; herein called "artificial food''). *P. icarus* and *Z. knysna* were reared on *Medicago sativa* and artificial food.

Sampling and Processing. Single caterpillars were associated in a certain time scheme together with laboratory-reared *Lasius flavus* ants (Daniels, 2004). Secreted droplets were collected with microcapillaries (0.5 µl, Hirschmann). The length of the fluid column obtained was immediately measured with a calibrated micrometer eyepiece (Wild M5A stereomicroscope, 25× magnification), allowing later calculation of the total secretion amount in the (convoluted) samples. Capillaries were immediately dispensed into 50 µl 70% ethanol–millipore–water and rinsed with 70% ethanol–millipore–water. Preliminary measurements showed that evaporation from the viscous fluid column started only after 1 min, so this did not constitute a problem. Although the association procedure allowed a comparatively efficient sampling, secretions were still dispensed only in minute amounts (Daniels, 2004). Thus, most samples had to be pooled from several individuals to achieve a volume amenable to reliable high-performance liquid chromatography (HPLC) analysis (*P. coridon* 4.2 ± 2.1, *P. icarus* 4–10, *Z. knysna* 22.2 ± 13.1 individuals, respectively). Only in *P. coridon* could four samples be obtained from single individual caterpillars. Individuals used to collect a cumulative nectar sample were always taken from the same food treatment. Late fourth instars and still moving prepupae were sampled, as these secrete more nectar than younger instars (Fiedler and Maschwitz, 1989; Burghardt and Fiedler, 1996). With the optimized harvesting method, one complete *P. coridon* sample (volume 0.4–2.0 µl, consisting of ca. 100–600 droplets) required repeated sampling over 1 wk (provided the appropriate instars were available). In *P. icarus* or *Z. knysna*, harvesting similar nectar volumes (0.4–1.3 µl, 300–1500 droplets) took up to 2 mo per sample.

Hemolymph was obtained by punctuating the dorsal vessels of late fourth instars with a sterile insect pin and subsequently collecting the fluids with microcapillaries. All samples were stored at −20°C until analysis.

Chemical Analysis. This was performed by using HPLC. Ethanolic samples were vacuum centrifuged, diluted in pure ion-free water, filtered (Spartan 3/20, 0.45-μm pores, Schleicher & Schuell), and split for separate sugar and amino acid analyses. For sugar investigation, Sentry Guard (3.9 × 150 mm), and Waters carbohydrate (4.6 × 250 mm) columns were used with isocratic 72% acetonitrile solvent at 35°C (Waters 510 pump, flow 1.4 ml min^{-1}). Sugar detection was performed with a refractive index detector (Waters 410).

Amino acid samples were derivatized with borate-buffered 6-amino-quinolyle-*N*-hydroxysuccinimidyle-carbamate. Sentry Guard (3.9 × 150 mm), and Waters AccQtag columns were used with a trinary solvent system (TEA/phosphate buffer with pH 5.5, acetonitrile, and water) at 37°C (Waters 600E pump, flow 1.0 ml min^{-1}). Amino acid detection was performed with a fluorescent detector (Waters 470). Nine carbohydrates (Fluka, Germany) and 17 amino acids (Waters) were used as standards. Tryptophan could not be identified by the derivatization method chosen. Fatty acids and proteins were not determined. Waters Millenium 3.0 software controlled the HPLC and created the respective chromatograms.

Statistical Analysis. Statistical analysis was performed in Statistica 6.0 and Primer v5 (Clarke and Warwick, 2001). If not mentioned otherwise, values are given as mean ± SD. Bonferroni correction, if required, follows Hochberg (1988) and is explicitly noted where applicable. Testing for differences in the coefficient of variation between groups was performed as described by Sokal and Braumann (1980). The principal component analysis (PCA) was calculated with the amino acid spectrum data as proportions of total amino acid content (100%). Two axes were extracted, and rotation procedure adopted was varimax standard. Various other versions of principal component analyses were performed with raw data, with subsamples and extracting more axes, delivering all the same general picture.

RESULTS

Sugar Composition and Concentration. Altogether, nine different sugars were identified in nectar secretions of the three study species (Table 1). Five additional peaks were detected but only one occurred more than twice (retention time 4.92 min, 11 times in 32 *P. coridon* samples).

The carbohydrate content of larval nectars of *P. coridon* caterpillars reared on natural food plants ($N = 23$) consisted mainly of sucrose. In less than half of the samples, sucrose was accompanied by glucose. Arabinose, fructose, maltose,

TABLE 1. SUGARS AND AMINO ACIDS IN LYCAENID LARVAL NECTAR SECRETIONS AND HEMOLYMPH, AS REVEALED BY HPLC ANALYSIS

		Sugars											Amino acids															
Source	*N*	Arabinose	Fructose	Glucose	Sucrose	Maltose	Trehalose	Lactose	Melibiose	Melezitose	Total (g/l)	*N*	Aspartate	Serine	Glutamate	Glycine	Histidine	Arginine	Threonine	Alanine	Proline	Tyrosine	Valine	Methionine	Lysine	Isoleucine	Leucine	Phenylalanine	Total (g/l)
P. coridon fed with																												
H. comosa	11	○	▽	○	■	▽	○	▽	–	–	47.4 ± 19.3	11	–	▽	–	▽	▼	▽	▽	▽	▼	●	▽	▽	▽	▽	■	▼	11.2 ± 3.1
S. varia	12	–	–	○	□	–	–	–	–	–	40.0 ± 8.3	21	–	▽	○	○	▽	○	▽	▽	▽	●	▽	▽	▽	▽	●	▼	8.9 ± 3.6
artificial food	10	–	▽	○	■	–	–	–	–	–	60.4 ± 21.8	10	–	▽	–	▽	▼	▽	▽	▽	●	●	▽	▽	▽	▼	■	▼	9.6 ± 3.6
P. icarus fed with																												
M. sativa	2	–	●	▽	●	–	–	–	▽	●	74.2 ± 1.9	2	–	○	–	–	–	●	–	▼	○	●	–	–	–	–	–	●	1.2 ± 1.0
Z. knysna fed with																												
M. sativa	3	–	●	○	●	–	–	–	–	●	82.6 ± 8.8	3	–	–	–	–	–	–	○	■	□	–	–	–	–	–	–	–	0.2 ± 0.2
artificial food	5	–	○	▽	■	–	–	–	–	○	59.7 ± 24.7	3	–	–	–	–	–	○	○	□	□	–	–	–	–	–	–	–	0.4 ± 0.1
Hemolymph																												
P. coridon	3	–	▽	–	●	–	■	–	○	–	26.3 ± 6.5	4	▽	●	▽	▽	●	▼	▼	▼	●	▽	▼	▽	▼	▼	▼	▽	8.6 ± 5.4
P. icarus	2	–	–	–	●	–	■	–	–	–	33.9 ± 0.3	3	–	●	▽	▼	●	▼	▼	▼	▼	▽	▼	▼	▼	▼	▼	▼	8.8 ± 2.9
Z. knysna	3	–	–	□	●	–	□	–	–	–	13.6 ± 1.8	4	–	○	▽	▼	●	▼	▼	▼	●	▽	▼	▽	●	▼	▼	▼	14.6 ± 12.0

Symbols indicate sugar and amino concentration: ▼, <10%; ●, 10–50%; ■, >50% (mean percent of total weight of all identified sugars or amino acids, respectively). Open symbols (▽, ○, □) are given for substances that were not present in all samples of a source. Total concentrations as mean ± SD. *N* = number of samples.

trehalose, and lactose occurred rarely and in minor quantities (Table 1). Melezitose was not found in *P. coridon* nectar. Total sugar concentration was 43.55 ± 14.76 g/l. *P. coridon* nectar was neither clearly acidic nor alkaline. The pH value ranged from 5.0 to 8.0 (measurement with pH paper, pH 1–10, Merck).

The larval nectar secretions of *P. icarus* (N = 2, *M. sativa* fed) and *Z. knysna* (N = 8, 3 reared on *M. sativa*, 5 fed with artificial food) caterpillars were similar with regard to sugar content and composition. Main components were sucrose and melezitose, accompanied by fructose and glucose in successively lower concentrations. Additional uncommon sugars were melibiose (in one *P. icarus* sample) and probably erlose (in one *P. icarus* sample and one *Z. knysna* sample, not quantified). Total carbohydrate concentration was 74.2 g/l (median) for *P. icarus* and 68.3 ± 22.6 g/l for *Z. knysna*, respectively.

Trehalose was the dominant sugar in hemolymph, accompanied by a lower quantity of sucrose. Three other sugars were detected (glucose, fructose, and melibiose), each in only one hemolymph sample. Total sugar concentrations in hemolymph were 26.75 g/l (median, N = 3) for *P. coridon*, 33.92 g/l (N = 2) for *P. icarus*, and 13.18 g/l (N = 3) for *Z. knysna* hemolymph.

Amino Acid Composition and Concentration. Up to 14 different identified amino acids were found in the larval nectar secretions of *P. coridon*. Almost half of the overall content was contributed by a single amino acid, leucine (Figure 1). The next most important amino acids were tyrosine, proline, arginine, and phenylalanine. Histidine, methionine, alanine, threonine, isoleucine, and valine occurred in lower quantities, but still regularly (in >50% of all samples). Lysine, serine, and glycine were found occasionally; glutamate was rare and in highly variable quantities. The total amino acid content was 9.68 ± 3.39 g/l (N = 42, all food treatments combined).

Larval nectar of *P. icarus* contained much less amino acids. Tyrosine was the major compound, followed by phenylalanine, arginine, and, in minor quantities, proline, serine, and alanine (Table 1). Total amino acid content was only 1.2 g/l (median N = 2), i.e., roughly one tenth of the total amino acid content of *P. coridon* nectar. In *Z. knysna* nectar, alanine or proline either occurred alone or together, sometimes accompanied by threonine or arginine in low quantities (Table 1). The total amino acid content was lower (0.30 ± 0.17 g/l, N = 6), corresponding to 25% of *P. icarus* and 3% of *P. coridon* total amino acid content.

In hemolymph, up to 16 free amino acids were found, almost all of them regularly (in >75% of all samples). Only tyrosine, glutamate, and asparagine occurred irregularly (Figure 2). The dominant amino acid was histidine, followed by serine and proline. All other amino acids contributed less than 10%. Total amino acid content was high, with an average across all species and samples of 10.81 ± 7.92 g/l. Hemolymph composition was similar in the three

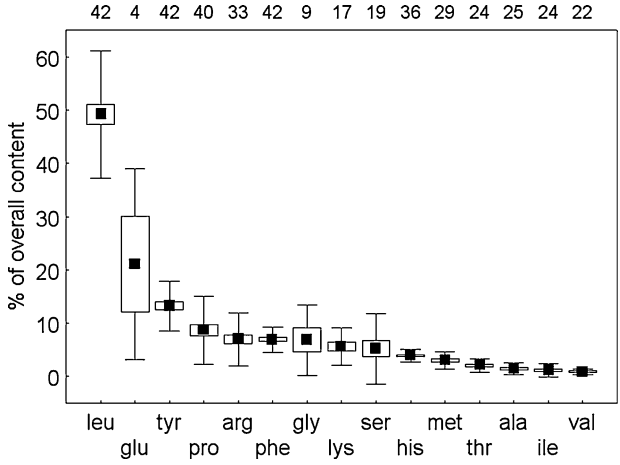

FIG. 1. Mean proportion of overall amino acid content (box: ±SE, whiskers: ±SD) contributed by different amino acids in *P. coridon* larval nectar secretions, ranked after their mean abundance. Figures above the graph denote the number of occurrences of each amino acid in the 42 secretion samples.

species. The pattern of amino acids in hemolymph differed considerably from the patterns found in the secretions. The mean content of the individual amino acids in nectar secretions and hemolymph correlated neither in *P. coridon* (Spearman $R_s = -0.06$, $P = 0.82$, $N = 16$) nor in *Z. knysna* ($R_s = 0.1$, $P = 0.73$, $N = 15$). The main components of hemolymph differed from the main components found in the secretions, as indicated by the arrows in Figure 2 (see also Figure 1 and Table 1).

Differences between Species. Nectar secretions of *Z. knysna* had a higher overall sugar content than *P. coridon* plant-fed caterpillars (Kruskal–Wallis ANOVA, $H_{2,33} = 9.17$, $P = 0.01$, *P. coridon* reared on plants vs. *P. icarus* vs. *Z. knysna*). Conversely, *P. coridon* nectar secretions had a higher content of amino acids ($H_{2,50} = 19.88$, $P < 0.001$), as well as a higher number of different amino acids ($H_{2,52} = 22.76$, $P < 0.001$). The sample size for *P. icarus* nectar secretions was too low to allow for meaningful statistical validation of results, but the pattern follows the same trend as *Z. knysna*.

Hemolymph of *Z. knysna* had a significantly lower overall sugar content than in *P. icarus* ($H_{2,8} = 6.25$, $P = 0.044$). There was neither a difference between the three species for total amino acid content ($H_{2,11} = 0.96$, $P = 0.62$) nor for the number of amino acids in hemolymph ($H_{2,11} = 2.09$, $P = 0.35$).

The Influence of Larval Food. Diet consumed by *P. coridon* caterpillars during their development had minor influence on sugar contents in the se-

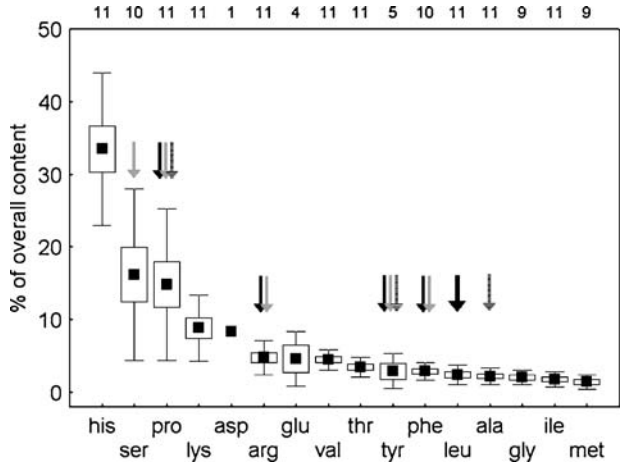

FIG. 2. Mean proportion of overall amino acid content (box: ±SE, whiskers: ±SD) contributed by different amino acids in hemolymph, ranked by their mean abundance. Black arrows indicate the main amino acids found in *P. coridon* nectar, gray arrows the main ones in *P. icarus*, and dashed arrows the main ones in *Z. knysna* nectar. Figures above the graph denote the number of occurrences of each amino acid in 11 hemolymph samples (*P. coridon*: 3 *H. comosa*-fed, 1 artificial food; *P. icarus*: 3 *M. sativa*-fed; *Z. knysna*: 3 *M. sativa*-fed, 1 artificial food).

cretions. Caterpillars fed with artificial diet secreted more sucrose than caterpillars fed with *H. comosa* (one-way ANOVA, $F_{2,29} = 17.78$, $P < 0.001$; Scheffé, $P < 0.001$) and those fed with *S. varia* (Scheffé, $P = 0.005$). The latter secreted as a trend slightly less than those fed with artificial diet (Scheffé, $P = 0.066$). However, concerning total sugar content, the pattern changed. Nectar from caterpillars reared with either *H. comosa* or artificial diet had higher total content than *S. varia* nectar, albeit nonsignificantly (one-way ANOVA, $F_{2,29} = 3.13$, $P = 0.058$). *H. comosa*-fed individuals secreted more often additional sugars (fructose, arabinose, maltose, trehalose; Table 1). For *Z. knysna*, no effects of the larval diet on the sugar contents of the nectar could be detected (Mann–Whitney *U* test, $U_{1,8} = 3$, $P = 0.18$), but because of the small sample size, the power of the test was low. Patterns of carbohydrate contents in *Z. knysna* nectar were similar irrespective of larval food (Table 1).

In *P. coridon*, larval food had no effect on total amino acid content (one-way ANOVA, $F_{2,39} = 1.65$, $P = 0.2$), but did on the number of amino acids in the nectar (one-way ANOVA, $F_{2,39} = 10.23$, $P < 0.001$). Caterpillars reared on *H. comosa* secreted more different amino acids than caterpillars reared on *S. varia* (Scheffé, $P < 0.001$; artificial diet vs. *S. varia*, $P = 0.055$). The amino

acid composition in secretions from *P. coridon* caterpillars fed with artificial diet was significantly more homogenous than in secretions from *H. comosa*-fed individuals (Figure 3; test for differences in the coefficient of variation in the PCA pattern—PC1: $t_{1,21} = 423.38$, $P < 0.001$; PC2: $t_{1,21} = 147.99$, $P < 0.01$). Feeding with *S. varia* caused higher variation in the amino acid profiles than feeding on artificial diet and *H. comosa* (*S. varia* vs. artificial diet—PC1: $t_{1,31} = 424.4$, $P < 0.002$; PC2: $t_{1,31} = 419.8$, $P < 0.002$; *S. varia* vs. *H. comosa*—PC1: $t_{1,32} = 597.65$, $P < 0.002$; PC2: $t_{1,32} = 400.65$, $P < 0.01$).

Comparison of the Amino Acid Profiles. Principal components analysis of the amino acid profiles of nectar and hemolymph revealed a clear pattern (Figure 3). The first two PCA dimensions explained 39.3% of the variance. These axes did not correlate with the acidic or alkaline character or the essentialness of the amino acids. Nectar samples harvested from *P. coridon* formed one large group with no segregation because of larval nutrition [one-way analysis of similarity (ANOSIM): $R_{\text{global}} = -0.012$, $P = 0.54$]. *P. icarus* secretions are relatively similar to those of *P. coridon*, whereas *Z. knysna* forms a separate group (Figure 3), with no further subdivision because of larval nu-

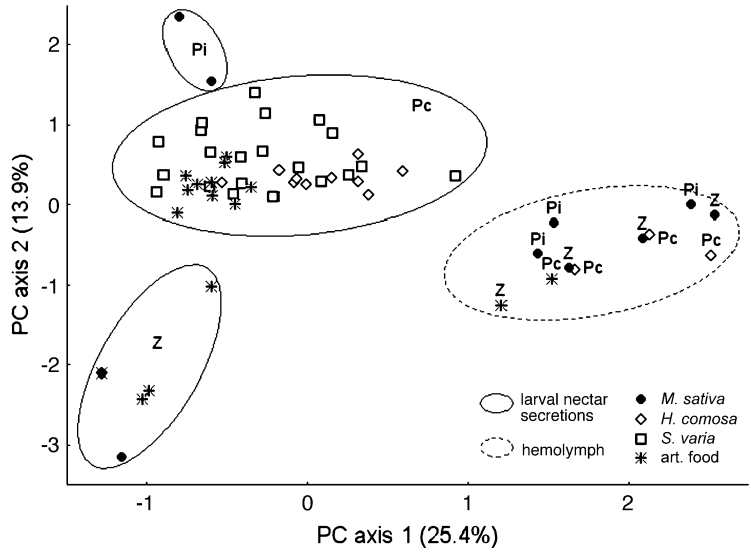

FIG. 3. Ordination of nectar and hemolymph samples from three facultatively myrmecophilous lycaenid species (Pc: *P. coridon*, Pi: *P. icarus*, Z: *Z. knysna*), based on a principle components analysis of amino acid proportions after varimax standard rotation. Caterpillars were reared on four different foods, as denoted in the legend. The contribution of the respective major axes to the explanation of observed variance is shown in parentheses.

trition (one-way ANOSIM: $R_{global} = -0.148$, $P = 0.70$). The secretion amino acid profiles of all species, however, are statistically different [two-way ANOSIM (food treatments nested in species): $R_{global} = 0.987$, $P < 0.001$].

Amino acid composition of hemolymph differs from nectar secretions (one-way ANOSIM: $R_{global} = 0.554$, $P < 0.001$) and is similar for all three species (one-way ANOSIM: $R_{global} = -0.173$, $P = 0.93$).

DISCUSSION

Myrmecophilous lycaenid butterfly larvae foster ant associations through nectar secretion. Here we report on how the precise composition may vary along with a number of factors, and how this correlates with the strength of myrmecophilous associations. Of main interest is the finding that nectar from one species, the strongly facultative myrmecophilous *P. coridon*, has highly elevated amino acid concentrations. As the closely related *P. icarus*, as well as *Z. knysna*, have low amino acid levels, this seems to point toward an overriding influence of the degree of myrmecophily on nectar composition. This is further corroborated by our finding that neither different diets nor hemolymph composition could explain the nectar composition, pointing toward a genetically determined function of the gland in composing nectar secretion. Phylogenetic relatedness seems to be less important than the degree of myrmecophily, as the differences in *P. coridon* and *P. icarus* nectar illustrate. The amino acid content of *P. coridon* secretion is greater than in almost any other alternative source, be it honeydew (Bernays and Klein, 2002; Yao and Akimoto, 2002; Woodring et al., 2004), floral or extrafloral nectars (e.g., Baker and Baker, 1986; Blüthgen et al., 2004), or other lycaenids (Table 2). This suggests an important role of amino acids in the formation of a strong association and supports Pierce's (1987) hypothesis and corroborates findings on other strongly myrmecophilous lycaenid species (DeVries and Baker, 1989; Nomura et al., 1992; Cushman et al., 1994; Table 2).

Nectar of the weaker myrmecophilous *P. icarus* and *Z. knysna* nectar may contain slightly more sugar than *P. coridon* nectar, yet these are still depauperate compared with aphid honeydew (Woodring et al., 2004) and plant nectar (e.g., Baker and Baker, 1986; Blüthgen et al., 2004). *P. icarus* nectar was at least similar to these sources in amino acid content, though. Both *P. icarus* and *Z. knysna* nectars also contained the sugar melezitose. This trisaccharide is commonly found in aphid honeydew (e.g., Hendrix et al., 1992; Blüthgen et al., 2004), probably serving osmoregulatory functions in the gut (Wilkinson et al., 1997). These are presumably not required in the lycaenid nectar gland with its own chitinized reservoir (Malicky, 1969). Melezitose is preferred by a number

TABLE 2. COMPARISON OF SUGAR AND AMINO ACID CONTENTS IN NECTAR SECRETIONS OF EIGHT LYCAENID AND ONE RIODINID SPECIES FROM SEVERAL STUDIES

	Lycaenidae								Riodinidae
	Niphanda fusca [a]	*Jalmenus evagoras* [b]	*Paralucia aurifera* [b]	*Hemiargus isola* [c]	*P. hispana* [c]	*P. coridon* [c]	*P. icarus* [c]	*Z. knysna* [c]	*Thisbe irenea* [c]
Sample size	n.g	n.g	n.g	8 [d]	2	23/42	2	8/6	5
Total sugar	0.16 M + or ca	ca. 10 %	34 %	–	13–19 %	4.4 % [e]	7.4 % [e]	6.8 % [e]	<0.5 %
Content	1.3 M								
Major components	Glucose	Sucrose, fructose [f]	Glucose [g]	–	Sucrose, fructose	Sucrose	Sucrose, melezitose	Sucrose, melezitose	n.g.
Total amino acid content (mmol/l)	57.1 [h]	20–40 [h]	97	4.3 [h]	Trace	108	10	6	10.600 [i]
Major components	gly	ser [j]	[k]	–	met [g]	leu	tyr, phe	ala, pro	gly, gln [l]

Study	Nomura et al. (1992); Chogyoji in Wada et al. (2001)	Pierce (1983); Pierce and Nash (1999)	Cushman et al. (1994)	Wagner (1994)	Maschwitz et al. (1975)	This study	This study	This study	DeVries (1988)

Different analytical methods. Major components are defined as regularly contributing >25% of total content;
– not analyzed; n.g. not given.
[a] Parasitic.
[b] Obligate myrmecophilous.
[c] Facultative myrmecophilous.
[d] Involves unknown amount of pseudoreplication.
[e] % w/v, only plant-fed larvae.
[f] No rank order given.
[g] Single substance.
[h] Originally given as "mM".
[i] Originally given as µM/µl.
[j] Major component, exact contribution not stated.
[k] States six amino acids without rank order.
[l] Rank order 1.8 and 2.6 on scale of 1–16.

of ant species (Völkl et al., 1999; Tinti and Nofre, 2001), although not universally (Cornelius et al., 1996; Blüthgen and Fiedler, 2004; Daniels, 2004). The presence of melezitose uniquely in these two weaker myrmecophilous lycaenids may function to advertise a resource, which is valuable for ants in the normal trophobiotic context. This would circumvent predation and would assure more ant attendance for these lycaenids. In contrast, the more costly amino acids in *P. coridon* nectar secure a unique niche among nectar producers and afford a stronger ant association.

Although the data do support Pierce's (1987) hypothesis, variations and exceptions exist. Total amino acid content in the nectar of the strongly, but facultatively myrmecophilous *P. coridon* is the highest value reported so far for lycaenid species, only surpassed by the riodinid *Thisbe irenea* (DeVries and Baker, 1989; Table 2). Sugar content in the *P. coridon* nectar is comparable with the values reported for the ant-parasitic *Niphanda fusca* (Nomura et al., 1992; but see Chogyoji in Wada et al., 2001). The sugar content in the nectar of the two facultatives *P. icarus* and *Z. knysna* is similar to the nectar of the obligate *Jalmenus evagoras* (Pierce, 1983; Pierce and Nash, 1999; Table 2). Nevertheless, from our analyses and the few values reported in the literature, a pattern emerges. Nectar of myrmecophilous lycaenid species contains about 5–10% sugar, with the possible exception of the Australian obligate ant-mutualist *Paralucia aurifera* (Cushman et al., 1994; Table 2). In all lycaenid and riodinid species analyzed thus far, the main sugar components are uniformly either sucrose or glucose (Table 2). The amino acid content of nectar, in contrast, seems to be determined by the intimacy of associations with attendant ants, more strongly myrmecophilous species providing a richer and more diverse mixture. There are differences among the species with regard to the main nectar amino acids: serine in *J. evagoras*, glycine in *N. fusca*, glycine, glutamine in the riodinid *T. irenea*, and leucine in *P. coridon*. In all facultative myrmecophiles considered here, serine and glycine were not significant, in contradiction to the notion of Yao and Akimoto (2002) who proposed a universally important role for these two amino acids in myrmecophilous insect–ant interactions. One potential explanation for the differences between lycaenid species regarding identity of the leading amino acid as well as contents could be that nectars are specifically tuned to match the gustatory preferences of the major ant visitors. However, only *J. evagoras* (with a few *Iridomyrmex* species) and *N. fusca* (with *Camponotus japonicus*) are host-specific myrmecophiles. In contrast, ant visitors of the numerous facultatively myrmecophilous species are unpredictable. Preferences for amino acids vary tremendously across ant species (Lanza and Krauss, 1984; Kay, 2002) and also have a conditional component (Blüthgen and Fiedler, 2004). Ultimately, preferences of particular ant species for nectar secretions, and, thus, the success of lycaenid larvae in initiating a lasting association, have to be tested experimentally for each combination of

partner species. For facultatively myrmecophilous lycaenid species with their unpredictable ant associates, it is impossible to present an ideal secretion equally attractive to all possible visiting ant species. In general, more strongly associated species can nevertheless use (rich) mixtures of amino acids as currency in the interaction, whereas the weaker associated species cash in on sugar. Further nectar analyses over the whole range of lycaenid myrmecophily would certainly be desirable to corroborate or challenge this pattern.

In addition, further study is required on how diet influences nectar secretion. We could find only minor effects in our study. Several other plant diets tested for *P. icarus* and *Z. knysna* did not yield sufficient nectar for analysis. Whether this was an artifact in our experiments or was caused by suboptimal growth or an inherent property of certain host plants remains to be tested, but would certainly be a significant factor in ant association (compare Burghardt and Fiedler, 1996).

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THE SOUTH AFRICAN AND NAMIBIAN POPULATIONS OF THE RESURRECTION PLANT *Myrothamnus flabellifolius* ARE GENETICALLY DISTINCT AND DISPLAY VARIATION IN THEIR GALLOYLQUINIC ACID COMPOSITION

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Abstract—The polyphenol contents and compositions in desiccated leaves of *Myrothamnus flabellifolius* plants collected in various locations in Namibia and South Africa were analyzed using UV spectroscopy and high-performance liquid chromatography–mass spectrometry. A study of the genetic relatedness of these populations was also performed by determination of the DNA sequence of the intergenic spacer region between the *psbA* and the *trnH* genes in the chloroplast genome. Namibian *M. flabellifolius* plants contained significantly more polyphenols than South African plants. Namibian plants essentially contained a single polyphenol, 3,4,5-tri-*O*-galloylquinic acid, whereas South African plants contained a variety of galloylquinic acids including 3,4,5-tri-*O*-galloylquinic acid together with higher molecular weight galloylquinic acids. Sequence analysis revealed a 1.4% divergence between Namibian and South African plants corresponding to the separation of these populations of approximately 4×10^6 years. The significance of the polyphenol content and composition to the desiccation tolerance of the two populations is discussed.

Key Words—Polyphenol, mass spectrometry, Myrothamnaceae.

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INTRODUCTION

Resurrection plants are a unique group of plants, the majority of which occur in southern Africa, capable of surviving dehydration to an air-dried state (Gaff, 1971, 1977). The resurrection plant, *Myrothamnus flabellifolius*, is a woody shrub with a widespread distribution throughout southern Africa (Kruger, 1998), where it grows in shallow, well-drained crevices in sandstone and in granite outcrops (Child, 1960; Mauve, 1966). Populations of *M. flabellifolius* occur along the west coast of Namibia, in central southern Zimbabwe, and in north-eastern South Africa (see Glen et al., 1999 for a general distribution map). This region experiences high summer temperatures and irradiance, with extremes of water availability (Mauve, 1966). Growing conditions on the rocky outcrops are such that the plants must sometimes survive for a year or more in a dry quiescent state, rehydrating only after summer rainfall (Farrant and Kruger, 2001). In attempting to elucidate the "resurrection" mechanism, we recently noted an extremely high polyphenol content in the leaves of this plant, with the main polyphenol present in plants collected in Namibia determined to be 3,4,5-tri-*O*-galloylquinic acid. In addition, we demonstrated that 3,4,5-tri-*O*-galloylquinic acid was able to protect membranes against desiccation and oxidation damage (Moore et al., 2005). The Namibian and the Zimbabwean/South African populations of *M. flabellifolius* are not only separated by the Kalahari Desert (Puff, 1978; Glen et al., 1999), but these two populations also occur in different biomes, with the Namibian population occurring in a dry Karoo biome and the Zimbabwean/South African population occurring in a moist savannah biome (Sinclair et al., 2002). In this report, we have investigated whether the Zimbabwean/South African population of *M. flabellifolius* also has high concentrations of 3,4,5-tri-*O*-galloylquinic acid present in the leaves. Because this population was found to have a different polyphenolic profile from plants collected in Namibia, we also investigated the genetic variation between the two populations based on nucleotide sequence comparisons of regions of the chloroplast genome.

METHODS AND MATERIALS

Plant Material. Desiccated plant material was obtained from populations in Namibia (N1–N5), namely, the Komas Hochland, near the Cunene River, and around Outjo, as well as from populations in South Africa (S1–S4), namely, Vaalwater in the Limpopo Province, near Lydenberg, and the Blouberg Mountains, Mpumalanga Province (see Figure 1 for a map of the region). The dry leaf material was stored at 20°C in the laboratory until use.

Polyphenol Extraction, HPLC Analysis, and Mass Spectrometry. Phenolic extraction was performed by homogenizing leaf material for 5 min in MeOH/

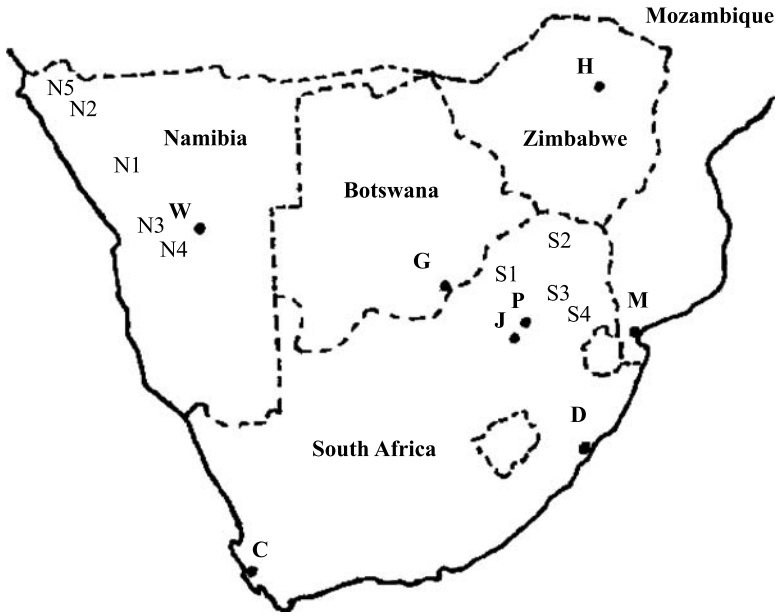

FIG. 1. Map of Southern Africa showing international borders. Major towns are shown as single letters—C: Cape Town; D: Durban; G: Gaberone; H: Harare; J: Johannesburg; M: Maputo; P: Pretoria; W: Windhoek. Collection sites in Namibia (N1–N5) were Outjo (N1), the Komas Hochland (N3 and N4), and near the Cunene River (N2 and N5). Collection sites in South Africa (S1–S4) were Vaalwater (S1), the Blouberg Mountains (S2), and near Lydenberg (S3 and S4).

H_2O (7:3, v/v) using a Polytron Homogenizer (Kinematica PT 2000). The homogenate was then subjected to sonication (Bandelin Sonorex bath sonicator) for 5 min, after which it was centrifuged (6000 × *g* for 5 min). The pellet was twice re-extracted using 3 volumes solvent per mass of sample. The supernatants were pooled, and the spectrum between 200 and 400 nm was determined. The polyphenol content was determined from the UV absorption at 280 nm using the previously measured molar extinction coefficient of 3,4,5-tri-*O*-galloylquinic acid of 21,000 (Altmann and Falk, 1995). Data reported are the mean ± standard deviation of three plants from each population analyzed in duplicate. Means were compared using a Student's *t* test with the level of significance set at $\alpha = 0.05$ (Microsoft Excel).

A 20-µl aliquot of the supernatant was subjected to analytical high-performance liquid chromatography (HPLC), performed on a Shimadzu LC-10A binary gradient system equipped with Photo-Diode Array detection (Shimadzu, Kyoto, Japan; Moore et al., 2005). Fractions were eluted from a

Jones chromatography RP-C18 (250 × 4 mm) column using a linear gradient between TFA/H_2O (1:1000, v/v) and TFA/acetonitrile (1:1000, v/v) over 40 min at a flow rate of 0.7 ml/min. MALDI-TOF mass spectra were obtained on a Perseptive Biosystems DE-PRO MALDI mass spectrometer equipped with a TOF analyzer (Perseptive Biosystems, Framingham, MA, USA). A 1-μl sample aliquot was dissolved in acetonitrile : water (1:1), mixed with 1 μl 2,5-dihydroxybenzoic acid matrix, and applied to a gold sample plate. The mixture was air-dried prior to use. The spectrometer was operated in positive and negative ion mode.

DNA Extraction, PCR Amplification, Purification, and Sequencing. Leaf material was frozen, lyophilized, and ground in a mortar using liquid nitrogen prior to DNA extraction. Total DNA was extracted using a modified CTAB DNA extraction protocol (Gawel and Jarret, 1991). The standard CTAB buffer was supplemented with 20 mg poly-(1-vinyl-pyrrolidine-2) (PVP; Merck, Darmstadt, Germany) and 1 ml of 2-mercaptoethanol : H_2O (1:1000, vol : vol; Gawel and Jarret, 1991). DNA purity was assessed using the A_{260}/A_{280} ratio and by agarose gel electrophoresis. The polymerase chain reaction (PCR) reaction mixture consisted of SuperTherm buffer, 0.025 U SuperTherm *Taq* polymerase, 5 mM $MgCl_2$, 0.1 mM dNTP, 0.33 μM each of the forward and reverse primers, and 3 μl template DNA in a total volume of 30 μl. The amplification program consisted of 95°C for 2 min, 30 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 2 min with a final extension of 72°C for 7 min. The forward and reverse primers were 5′GTTATGCATGAACGTAATGCTC3′ and 5′CGCGCATGGTGGATTCACAAATC3′, respectively. PCR reactions were supplemented with various amounts of soluble PVP (Merck) to reverse polyphenol inhibition (Koonjul et al., 1999). Amplified products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Product quantity and purity were estimated using agarose gel electrophoresis. Purified products were sequenced on a MegaBACE 1030 sequencer (Amersham BioSciences, Amersham, UK). DNA sequences were edited using Chromas 2.01 software (Technelysium PtyLtd., Helensvale, Queensland, AU). Alignment of sequences was performed using DNAMAN (Lynnon Corporation, Vaudreuil-Dorion, Quebec, CA) and MegAlign 3.08 software (DNASTAR, Madison, WI, USA).

RESULTS

We initially quantified the total leaf polyphenol content from plants collected in Namibia and in South Africa. Leaf material from each population was exhaustively extracted in MeOH/H_2O (7:3, vol : vol), and the polyphenol content was determined from the UV absorption at 280 nm. A significant difference in the total mean polyphenol content was observed between plants

TABLE 1. PERCENTAGE POLYPHENOL CONTENT IN DESICCATED LEAVES OF PLANTS COLLECTED FROM VARIOUS LOCATIONS IN NAMIBIA (N1–N5) AND SOUTH AFRICA (S1–S4)

Namibian locations	% Polyphenol content[a]	South African locations	% Polyphenol content
N1 Outjo	41.7 ± 6.7	S1 Limpopo	26.7 ± 0.7
N2 Cunene	30.1 ± 5.2	S2 Blouberg	21.7 ± 5.3
N3 Komas Hochland	28.3 ± 1.3	S3 Lydenberg	25.3 ± 4.6
N4 Komas Hochland	32.7 ± 6.7	S4 Lydenberg	22.9 ± 1.0
N5 Cunene	30.9 ± 0.1		
Average	32.7 ± 5.2	Average	24.4 ± 2.2

[a]Polyphenol content is expressed in 3,4,5-tri-*O*-galloylquinic acid equivalents in % g polyphenol per g dry weight. A minimum of three plants from each population was analyzed in duplicate. The data are expressed as the mean ± standard deviation; $P < 0.05$.

from Namibia and from South Africa. Plants collected in Namibia contained 32.7 ± 5.2% in contrast to plants collected in South Africa, which contained 24.4 ± 2.2% (Table 1, $P < 0.05$).

HPLC analysis of the extracted polyphenols was performed to determine whether South African *M. flabellifolius* plants contained the same polyphenol species as Namibian plants. The chromatograms obtained (Figure 2) revealed distinct compositional differences, the major difference being an abundance of a single polyphenol in Namibian plants (Moore et al., 2005). In contrast, South African plants contained a wide variety of different polyphenols. The main polyphenol present in leaves of Namibian plants (peak 1, Figure 2A) was ascribed to 3,4,5-tri-*O*-galloylquinic acid (Moore et al., 2005) and confirmed by mass spectrometry of the extract; this yielded a mass of 647 Da. The remaining phenolic peaks (peaks 2 and 3) in the chromatogram represented gallic and ellagic acid esters of 3,4,5-tri-*O*-galloylquinic acid. These assignments were made after mass spectrometry of the material eluting in these peaks yielded masses that varied from one another by an integral mass of either 152 Da, the mass addition of one extra galloyl group to the core molecule, or 298 Da, the mass addition of one extra ellagic acid moiety to the parent structure. A mass of 799 Da was found for the material eluting in peak 2 and a range of masses, namely, 799, 936, 953, 1570, and 1722 Da for that in peak 3. These latter masses were assigned to represent tetra-, penta-, nona-, and decagalloylquinate esters (Moore et al., 2005). In contrast, HPLC analysis of polyphenols extracted from leaves of South African *M. flabellifolius* showed that no single polyphenol was present in abundance. Instead, a variety of polyphenols were present, some of which were identical to those present in Namibian plants, but the majority of which eluted with longer retention times (Figure 2B). In general, there was a relationship between the molecular weight of the polyphenol and the HPLC retention time (Table 2). Fractions 1, 2, and 3 from South African plants had

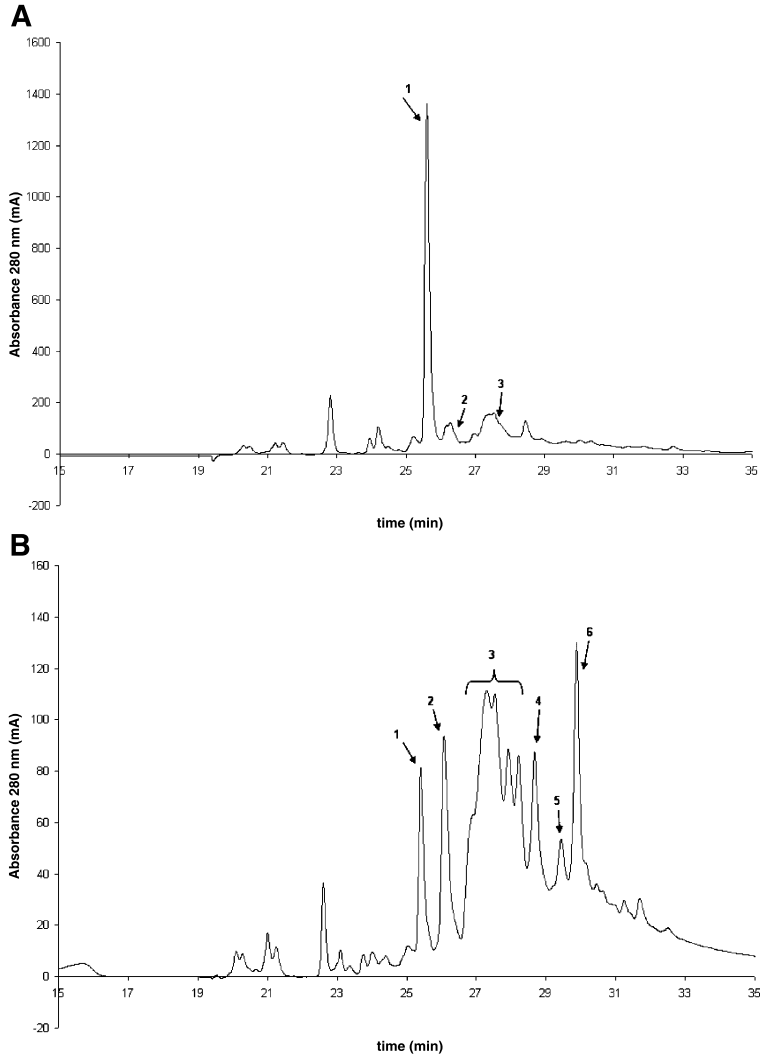

FIG. 2. High-performance liquid chromatography (HPLC) chromatograms of polyphenols extracted from leaves of *M. flabellifolius* collected in (A) Namibia and (B) South Africa and separated on a C_{18} column using a linear gradient between TFA/H_2O and TFA/acetonitrile as described in text. Fractions were pooled and lyophilized prior to analysis. The absorbance is shown in milliabsorbance units (mA). Note that the ordinate scale for (B) is 10^{-1} times of that shown in (A).

TABLE 2. ION SPECIES PRESENT IN VARIOUS HPLC FRACTIONS OF POLYPHENOLS EXTRACTED FROM LEAVES OF NAMIBIAN AND SOUTH AFRICAN PLANTS

Fraction number	R_t (min)	Galloyl groups	Quinic acid groups	M_r	Molecular ion[a]
1[b]	25.8	3	1	647[c]	$[M–H]^-$
		4	1	675[c]	$[M–C_6H_5O_3–H]^-$
2[b]	26	3	1	647[c]	$[M–H]^-$
		4	1	675[c]	$[M–C_6H_5O_3–H]^-$
		4	1	783[c]	$[M–OH]^-$
		5	1	936[c]	$[M–OH]^-$
		9	1	1570[c]	$[M–H]^-$
		10	1	1722[c]	$[M–H]^-$
3[b]	27	4	1	783[c]	$[M–OH]^-$
		4	1	799[c]	$[M–H]^-$
		5	1	936[c]	$[M–OH]^-$
		5	1	953[c]	$[M–H]^-$
		9	1	1570[c]	$[M–H]^-$
		10	1	1722[c]	$[M–H]^-$
4	29	4	1	767	$[M–2OH]^-$
		4	1	783[c]	$[M–OH]^-$
		5	1	936[c]	$[M–OH]^-$
		6	1	1171	2054 fragment
		7	1	1322	2203 fragment
		9	1	1570[c]	$[M–H]^-$
		10	1	1722[c]	$[M–H]^-$
		11	1	1874	$[M–H]^-$
5	29.7	4	0	636	$[M+CO–OH]^-$
		4	1	675[c]	$[M–C_6H_5O_3–H]^-$
				751	Unassigned
		6	0	912	$[M–OH]^-$
		5	1	936[c]	$[M–OH]^-$
		6	1	1088	$[M–OH]^-$
		7	1	1322	2203 fragment
		9	1	1570[c]	$[M–H]^-$
		10	1	1722[c]	$[M–H]^-$
		12	1	2054	$[M+K–2H]^-$
		13	1	2203	$[M+K–2H]^-$
6	30.1	4	0	636	$[M+CO–OH]^-$
		4	1	675[c]	$[M–C_6H_5O_3–H]^-$
				751	Unassigned
		5	0	882	2203 fragment
		5	1	936[c]	$[M–OH]^-$
		6	1	1088	$[M–OH]^-$
		7	1	1322	2203 fragment
		9	1	1570[c]	$[M–H]^-$
		10	1	1722[c]	$[M–H]^-$
		13	1	2203	$[M+K–2H]^-$

[a] $C_6H_5O_3$ refers to the pyrogallol fragment.
[b] Peak fractions common to both South African and Namibian plants.
[c] Molecular ions common to both South African and Namibian plants.

similar polyphenols as the same fractions from Namibian plants. Polyphenols that eluted in fractions 4–6 were unique to South African plants (Figure 3). As expected, the polyphenols in the later eluting fractions had higher masses, brought about by the addition of either gallic acid or ellagic acid moieties to the core molecule, 3,4,5-tri-*O*-galloylquinic acid (Table 2). A range of masses between *m*/*z* 636 and 2203 was evident; other masses present, e.g., 1322 Da, could be ascribed to the fragmentation of one of these parent molecules. Peaks displaying masses of 1874, 2054, and 2203 Da represent novel octa-, nona-, and decagalloylquinate ester molecules (assuming 3,4,5-tri-*O*-galloylquinic acid as the core molecule), respectively, each with the addition of a single ellagic acid moiety. These species were only found to be present in South African plants (Figure 2; Table 2). The final major peak eluting after approximately 30 min displayed masses of 882, 939, 1088, and 1322 Da. The mass of 1088 Da represents a hexagalloylquinic acid that was not found in plants collected in Namibia. We observed no change in galloylquinate ester composition between desiccated and hydrated plants (data not shown).

Because the two *M. flabellifolius* populations have distinct differences in their polyphenol content and composition, we investigated their genetic relatedness. The intergenic spacer region between *psbA* (photosynthetic subunit A)

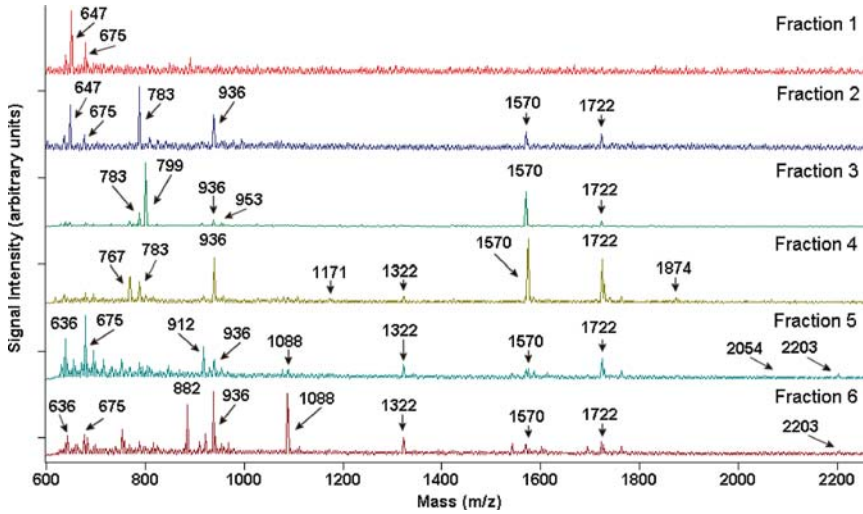

FIG. 3. Mass spectral analysis of HPLC fractions containing polyphenols extracted from leaves of *M. flabellifolius* collected in South Africa. Note that the ordinate scale is in arbitrary units and varies from fraction to fraction depending on the amount of material present.

and *trnH* (histidine amino acid biosynthesis) on the chloroplast genome was chosen. This region has been shown to evolve at a rate suitable for assaying population level differences (Aldrich et al., 1988; Sang et al., 1997). An approximately 400-bp fragment was consistently amplified from DNA extracts of both populations. Sequence analysis revealed that this fragment represented 288 bp of the intergenic spacer region with the remaining sequence derived from the 3′ and 5′ ends of *psbA* and *trnH*, respectively (Figure 4). The DNA

```
S          CTAGCTGCTGTCGAAG.............CTCCATCTACAA      28
N          ----------------CCCCATCTACAA------------      40

S          ATGGATAAGACTTTGGTCTTAGTGTATACGAGTTTTTGAA      68
N          ----------------------------------------      80
                           3'↓
S          CTTAAAGGAGCAATAACCAATTTCTTGTTCTATCAAGAGG     108
N          ----------------------------------------     120

S          GTTGGTATTGCTCCTTTATTTAGTATTCTTTTATTTCTAT     148
N          ----------------------------------------     160

S          TTTCTTTTCATTTCTTTGCTTCAGCATAAGAAAAAAGAAA     188
N          ----------------------C-----------------     200

S          AAGTATTCTTATGGGTATTGAGTATCATACTTTTTTTCTG     228
N          ----------------------------------------     240

S          TACTAATCTCTAATTTTTCAAATAGAAAAATATTTTTCTG     268
N          ---------G----------------------C--------     280

S          ACTAATATTTGTATCTAAGAAGGAAGATAAGAAAGACTAA     308
N          ------------------------------------A---     320

S          ATGAAATAATTATAAATGGAATCCTTTCTAATTTGTAACT     348
N          ----------------------------------------     360
                                      ↓5'
S          TTCTAATTTGTAAATAGTATAGGGGCGGATGTAGCCAAGT     388
N          ----------------------------------------     400

S          GGATCAAGGCAGT                               401
N          -------------                               413
```

FIG. 4. Chloroplast DNA sequence from leaves of *M. flabellifolius* representing the 3′ end of *psbA*, the intergenic spacer, and the 5′ end of *trnH* from plants collected in South Africa (S) and Namibia (N). Arrows denote the 3′ end of *psbA* and the 5′ end of *trnH*, respectively.

sequence of this region was identical for all plants collected from South Africa, but distinct from plants collected from Namibia, all of which displayed an identical sequence. Alignment of the 288-bp intergenic sequences obtained from all samples revealed four consistent differences between plants from South Africa and from Namibia. These differences occurred at positions 88, 154, 176, and 221 in the intergenic spacer, and represented a sequence divergence of 1.4% between the two populations. We also observed an insertion of 12 bp in *psbA* from all plants collected in Namibia (Figure 4).

The genus *Myrothamnus* is distantly related to the genus *Melianthus*, which has been proposed to have diverged from its sister group *Greyia* at a mutation rate of 0.0035 substitution per site per 10^6 years (Henning, 2003). Assuming an equal mutation rate, a divergence rate of 0.014 substitution per site yields a time of divergence for the Namibian and South African *M. flabellifolius* populations of approximately 4×10^6 years.

DISCUSSION

This work reports that the Namibian and South African populations of *M. flabellifolius* have distinct differences in their polyphenol content and composition. Whereas plants collected in Namibia essentially contained a single polyphenol, 3,4,5-tri-*O*-galloylquinic acid, South African plants contained a mixture of polyphenols that were derivatives of this molecule. Although both the Namibian and South African populations occur in similar geological environments, the rainfall patterns of the regions in which these populations occur are very different. South African plants grow in a region that experiences dry winters and regular annual summer rainfall. In contrast, Namibian plants grow in a more arid region of dry winters and irregular summer rainfall patterns. Farrant and Kruger (2001) reported that South African *M. flabellifolius* plants are only capable of surviving 9 months in a desiccated state. Whereas South African plants must only survive the dry winter months, Namibian plants must be capable of surviving not only a dry winter but also the ensuing dry summer and the following dry winter. In this instance, "dry" means that no rainfall whatsoever is experienced. Anecdotal evidence suggests that Namibian *M. flabellifolius* plants are capable of surviving for extended periods (3 or more years) in a quiescent state. We propose that the presence of the single polyphenol 3,4,5-tri-*O*-galloylquinic acid contributes toward this degree of desiccation tolerance. In support of this hypothesis, Moore et al. (2005) demonstrated that 3,4,5-tri-*O*-galloylquinic acid protects membranes against desiccation-induced damage as well as against free radical-induced oxidation.

M. flabellifolius in Africa south of the Sahara was originally divided on morphological grounds into three subspecies, namely, *M. flabellifolius sensu*

stricto, *elongata*, and *robusta*, the latter only occurring north of the Zambesi River in Zambia and Mozambique (Weimarck, 1936). A later revision resulted in the abandonment of *M. flabellifolius elongata* as a subspecies because of it being indistinguishable from *M. flabellifolius sensu stricto* (Puff, 1978). Because polyphenols do not display rapid turnover during plant growth and metabolism (Boudet et al., 1985; Haslam and Lilley, 1985), these molecules serve as useful chemotaxonomic markers for studies of plant systematics and evolution. Our data, therefore, suggest a new division of *M. flabellifolius* based on the phenolic compounds. This division would correlate with the biogeography of the species. We propose that there are two distinct populations on either side of the Kalahari Desert, respectively. This hypothesis is supported by the genetic data presented here that show that plants collected in Namibia were genetically identical and distinct from plants collected in South Africa, which were also genetically identical. The 1.4% genetic variation within the *psbA*/*trnH* intergenic region corresponds to a time of divergence of approximately 4 million years. This figure correlates with the climate change that occurred approximately 5 million years ago when the Karoo region along the western part of southern Africa changed from a wet to an arid region (Klak et al., 2004). Our hypothesis is that this climate change resulted in a requirement for Namibian population of *M. flabellifolius* to remain in a quiescent state for extended periods. The separation of the two populations by over 1000 km of desert presumably prevented cross-pollination and wind dispersal of the seeds (Child, 1960), resulting in two separate populations. Current research is focused on the relationship between the polyphenol content and the ability of Namibian plants to survive in a quiescent state.

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PHENOLOGICAL VARIATION IN CHEMICAL DEFENSE OF THE PIPEVINE SWALLOWTAIL, *Battus philenor*

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Abstract—Larvae of the pipevine swallowtail, *Battus philenor*, feed on plants in the genus *Aristolochia*, which contains aristolochic acids, toxic alkaloids unique to the Aristolochiaceae. Pipevine swallowtails sequester these compounds and, as a consequence, are chemically defended against many natural enemies. In California, the primary aristolochic acid present in the butterfly is aristolochic acid I. Newly eclosed adult females possess greater amounts of these sequestered toxins compared to males. However, over the course of the flight season, the aristolochic acid content of females in the population declines, whereas male aristolochic acid content remains relatively constant. Transference of sequestered aristolochic acids to eggs by females might explain the decline of these sequestered chemical defenses observed over time. We found no evidence that males transfer aristolochic acids to females via the spermatophore. The possibility that females at the end of the flight season may be automimics of males is discussed. Temporal variation in the aristolochic acid defenses exhibited by this pipevine swallowtail population is both age- and sex-dependent.

Key Words—Aristolochic acid, chemical defense, sequestration, phenological variation, pipevine swallowtail, *Battus philenor*, automimicry.

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INTRODUCTION

Many phytophagous insects, such as some Lepidoptera, sequester plant toxins that they use for their own chemical defense (Nishida, 2002). Plant-derived chemical defenses of insects are known to vary geographically and temporally. Geographic and temporal variation in sequestered chemical defense can reflect intra- and interspecific variability in the amount and types of toxins provided by host plants (Nelson et al., 1981; Urzua and Priestap, 1985; Ritland, 1994), phenological shifts in host use patterns (Malcolm and Brower, 1989; Malcolm et al., 1989; Moranz and Brower, 1998), or changes in plant toxicity over time (Nelson et al., 1981; Fordyce, 2000). Additionally, the quantity and quality of sequestered chemical defenses can vary over the life span of an individual. For example, (Alonso-Mejía and Brower 1994) showed that the concentration of cardiac glycosides in the monarch butterfly, *Danaus plexippus*, decreases as the animal ages. Variation in chemical defense may affect the defensive efficacy of sequestered plant compounds and can potentially affect inter- and intraspecific mimicry relationships (Malcolm and Brower, 1989; Alonso-Mejía and Brower, 1994; Ritland, 1994; Moranz and Brower, 1998; Fordyce, 2001).

Variation in toxicity might also reflect differences in sequestration ability between the sexes; adult female butterflies often contain higher concentrations of sequestered toxins (Dixon et al., 1978; Rothschild et al., 1979; Alonso-Mejía and Brower, 1994; Sime et al., 2000). Transference of specific sequestered compounds to mates, such as pyrrolizidine alkaloids found in the spermatophore of some arctiids (Gonzalez et al., 1999) and danaids (Dussourd et al., 1989), might also result in the sex-specific decline of sequestered compounds following successive matings. Similarly, female transmission of sequestrates to eggs (Nishida and Fukami, 1989; Sime et al., 2000) may result in a substantial loss of these compounds following multiple oviposition events. Here, we examine this possibility by assessing sex-dependent loss of sequestered toxins over time in the spring brood of the pipevine swallowtail, *Battus philenor* L. (Papilionidae, Troidini), in California.

Like all members of its genus, *B. philenor* is a specialist on plants in the genus *Aristolochia* (Aristolochiaceae) (Racheli and Pariset, 1992), commonly called pipevines. The *Aristolochia* contain nitrophenanthrene carboxylic acids called aristolochic acids, which are unique to the Aristolochiaceae (Chen and Zhu, 1987). *B. philenor* sequester these toxins as larvae; adults have no means of obtaining these compounds. The presence of aristolochic acids render larvae and adults unpalatable to many invertebrate and vertebrate predators (Brower, 1958; Codella and Lederhouse, 1989; Uesugi, 1996; Fordyce, 2001). As a consequence, *B. philenor* is an effective model for many mimetic butterfly species, including *Papilio troilus*, *P. polyxenes*, *P. glaucus* (Papilionidae), *Limenitis astyanax*, and *Speyeria diana* (Nymphalidae) (Brower, 1958; Brower and

Brower, 1962; Platt et al., 1971; Jeffords et al., 1979; Codella and Lederhouse, 1989). Aristolochic acids are present on the eggs of *B. philenor* (Sime et al., 2000), presumably conferring some defensive benefit to their clutch (Brower, 1984). In California, there is only one naturally occurring host plant available for *B. philenor*, *Aristolochia californica*, an endemic liana characteristic of riparian habitats in the northern Central Valley and surrounding foothills of the Coast Range and Sierra Nevada mountains. There are at least two broods of *B. philenor* in California. The largest brood occurs in the spring following pupal diapause, with maximum densities of adult butterflies and larvae occurring between late March and early May. Later broods occur at much lower densities and larvae are rarely observed after May.

This study focused on the large, spring brood of *B. philenor*. We examined whether aristolochic acids present in the butterflies change over time as the butterflies age, mate, and lay eggs. We predicted that changes in male and female aristolochic acids over time should differ as a result of parental investment of sequestrates. However, we did not have an *a priori* expectation as to what sex might show a decline in aristolochic acids, because the origin of these toxins coating the eggs, whether donated in the spermatophore or directly from the female, was heretofore unknown. Additionally, we examined differences in aristolochic acids in newly eclosed males and females to assess sex-related differences in the amount of these sequestered compounds.

METHODS AND MATERIALS

Butterfly Samples. Captive reared butterflies were used to assess differences in aristolochic acid sequestration between the sexes. In the spring of 2002, eggs were collected in the field from naturally laid clutches. One egg from each of 67 naturally laid clutches was placed in a common cage and fed fresh, field-collected *A. californica* as needed. Field-collected plants were provided to larvae within 24 hr of collection, and replaced with fresh material within 72 hr. During the third instar, larvae were haphazardly dispersed among separate containers consisting of groups of 5–7 individuals and were continually fed fresh *A. californica* until pupation. Larvae and pupae were maintained under ambient photoperiod between 21 and 27°C. Emergence following diapause occurred between March and June of 2003. Butterflies were collected within 24 hr after eclosion, after they had expelled meconium and their wings had hardened. Butterflies were stored in glassine envelopes at −20°C until chemical extraction and analysis. Differences between males and females in total aristolochic acid content and concentration were determined using a *t*-test.

To examine phenological changes in aristolochic acid content, wild adult *B. philenor* were collected in March through May, 2000–2003 ($N = 76$). This period corresponds to the first brood following winter pupal diapause. To ensure that collection was restricted to the first brood, two independent lines of evidence were used to estimate *B. philenor* phenology. First, naturally laid clutches were monitored in the field to assess the development of larvae. Second, captive larvae, reared from eggs collected in the beginning of the flight season, were monitored in the laboratory until pupation. All butterflies included in this study were collected before ultimate instars in the field were observed pupating, and before the laboratory-reared caterpillars pupated.

Butterflies were stored in glassine envelopes at −20°C until chemical extraction and analysis. All were collected adjacent to, or at Stebbins Cold Canyon Ecological Reserve (Solano Co.), part of the University of California Natural Reserve System. Phenological changes in aristolochic acid content were determined using an ANCOVA, with day of capture, sex, year, and all interactions included in the model. To account for among-year variation in emergence date, which can be influenced by climatic variation, we used a phenological date based on the first day *B. philenor* was observed in the field. All aristolochic acid data were log-transformed prior to statistical analysis to meet the assumption of normality.

Additionally, we explored the possibility that variation in adult aristolochic acid content might be a consequence of reproductive life history, specifically female transference of aristolochic acid to the eggs and male transference of aristolochic acid via the spermatophore. We obtained seven recently mated female *B. philenor* from a population in Knox County, TN, USA; including one captive reared female that was mated in captivity. The exact time since mating of the wild-caught females was unknown; however, based on physical condition, they appeared to be newly eclosed, and their corpus bursa was swollen with a spermatophore. Older females and unmated females have a conspicuously smaller corpus bursa. The corpus bursa, each containing a spermatophore, and all ova were removed from each female by dissection. Aristolochic acids were extracted from each corpus bursa ($N = 7$), unfertilized ova ($N = 45$), and laid eggs ($N = 18$) as described above.

Extraction Procedure. Butterflies were dried under reduced pressure prior to extraction, and weighed to the nearest milligram. Each entire butterfly was placed in a 15-ml centrifuge tube, homogenized in 5 ml of hexane, and sonicated for 20 min at 50°C to remove fat. Samples were centrifuged, and the fat-containing hexane was removed. This procedure was repeated once to ensure fat removal from each sample. Analysis of a subset of the hexane extracts indicated that no aristolochic acids were present. Defatted butterfly tissue was extracted two times with 5 ml of 100% ethanol and sonicated for 30 min at 50°C. The ethanol extract was dried under reduced pressure in a test tube

leaving a yellow residue. The residue from entire butterfly samples was resuspended in 0.5 ml of 100% methanol and passed through a 0.45-μm filter into an autosampler vial for high-performance liquid chromatography (HPLC) analysis. The residue from the corpus bursa, ova, and laid eggs was resuspended in 0.03 ml of 100% methanol.

HPLC Procedure. Sample analyses were performed using a Waters Alliance HPLC system with a 2996 diode array detector and Empower Pro Software. Each injection was 10 μl eluted isocratically with a mixture of methanol, water, and 1% acetic acid (52:47:1) at a rate of 1 ml/min on a Symmetry® C-18 reverse phase column (3.5 μm, 4.6 × 75 mm) (Waters Corp.). Aristolochic acid content was determined using retention time and absorption spectra compared to known standards.

A mixture of aristolochic acid (AA)-I and AA-II was obtained from Sigma Chemical. These are the primary aristolochic acid constituents of California *B. philenor* and *A. californica* (Fordyce, 2000). The aristolochic acids provided in this mixture were separated and isolated through successive HPLC runs using a sample fractioner. These fractions were dried and weighed (in μg) and separate standard curves based on a dilution series were generated for AA-I and AA-II. Aristolochic acids were quantified by absorbance at two separate wavelengths, 320 nm for AA-I and 248 nm for AA-II.

RESULTS

The aristolochic acid content of newly eclosed, captive-reared butterflies differed between the sexes. Females ($N = 32$) sequestered more aristolochic acids than males ($N = 26$), measured both as concentration ($t = 5.130$, $df = 56$, $P < 0.001$, Figure 1a) and total aristolochic acids ($t = 3.736$, $df = 56$, $P < 0.001$, Figure 1b). All captive reared butterflies emerged between March 21 and May 23 (except one female that emerged on September 25). We explored the possibility that time of emergence might explain variation in aristolochic acid content by using an ANCOVA that included emergence date, sex, and the interaction in the model. We found no effect of date of emergence ($F_{1,53} = 0.046$, $P = 0.832$), or its interaction with sex ($F_{1,53} = 0.771$, $P = 0.384$), on total aristolochic acid content. Excluding the single late emerging female from the analysis did not alter the conclusion. Analysis of aristolochic acid concentration gave the same qualitative results (not shown).

The dominant aristolochic acid found in our samples was AA-I, consistent with previous examinations of *B. philenor* aristolochic acid chemistry (Fordyce, 2000; Sime et al., 2000). AA-1 constituted over 90% of the total aristolochic acids present. On average, field-collected individuals had 22.01 ± 3.13 μg

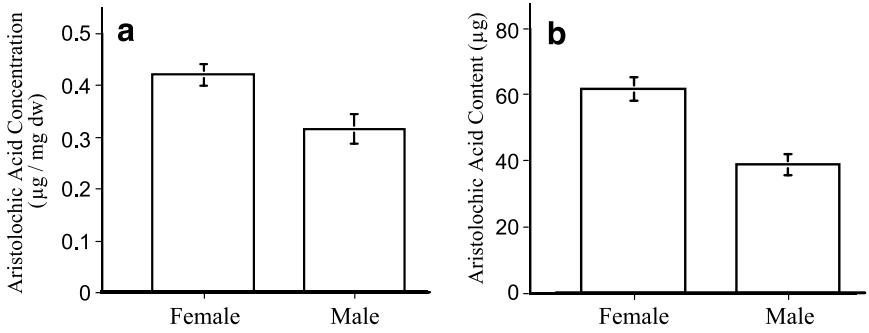

FIG. 1. The difference between male and female *B. philenor* aristolochic acid concentration (a) and total aristolochic acid content (b). Sexes are significantly different at $\alpha < 0.05$ for both measures of aristolochic acid content. Error bars are ±1 SE.

(mean ± SE) of AA-I, and only 1.55 ± 0.24 μg (mean ± SE) of AA-II. Based upon the absorbance spectra of another peak, AA-Ia might also be present in some of the butterflies.

Aristolochic acid content of *B. philenor* declined in population over the course of the flight season. This decline was evident when measured as total amount (Table 1) and amount per mg dry weight (Table 2). Both of these analyses showed that loss of aristolochic acids over time differed between sexes, indicated by the significant interaction term. Subsequent regressions for each sex indicated that females showed a greater decline over time compared to males. Only butterflies captured in 2001 and 2002 could be included in the full ANCOVA model because low numbers of females captured in 2000 and 2003 resulted in insufficient degrees of freedom. Phenological date was used to adjust for among-year variation in emergence date, although using Julian date gives the same qualitative answer based on these analyses. Figure 2 shows the change

TABLE 1. ANCOVA ON TOTAL ARISTOLOCHIC ACID CONTENT (μg) OF MALE AND FEMALE *Battus philenor* OVER TIME, BLOCKED BY YEAR

Source	*df*	MS	*F*	*P*
Phenological date (*P*)	1	3.282	44.182	<0.001
Sex (*S*)	1	0.002	0.023	0.880
$P \times S$	1	0.781	10.510	0.002
Year (*Y*)	1	1.702	22.905	<0.001
$Y \times S$	1	0.289	0.289	0.593
$Y \times P$	1	0.248	3.340	0.073
$Y \times P \times S$	1	0.210	2.825	0.099
Error	55	0.074		

TABLE 2. ANCOVA ON ARISTOLOCHIC ACID CONCENTRATION (μg/mg dw) OF MALE AND FEMALE *B. philenor* OVER TIME, BLOCKED BY YEAR

Source	*df*	MS	*F*	*P*
Phenological date (*P*)	1	0.076	37.72	<0.001
Sex (*S*)	1	0.008	4.119	0.047
$P \times S$	1	0.028	13.84	<0.001
Year (*Y*)	1	0.051	25.517	<0.001
$Y \times S$	1	0.006	2.988	0.090
$Y \times P$	1	0.010	5.034	0.029
$Y \times P \times S$	1	0.010	5.198	0.027
Error	55	0.018		

in total aristolochic acids over time for each sex and includes all individuals collected for this study, including the years excluded from the ANCOVA model.

The results of ANCOVA on total aristolochic acids are more straightforward in interpretation than the analysis on aristolochic acid concentration, which has multiple significant interaction terms. This is likely a consequence of

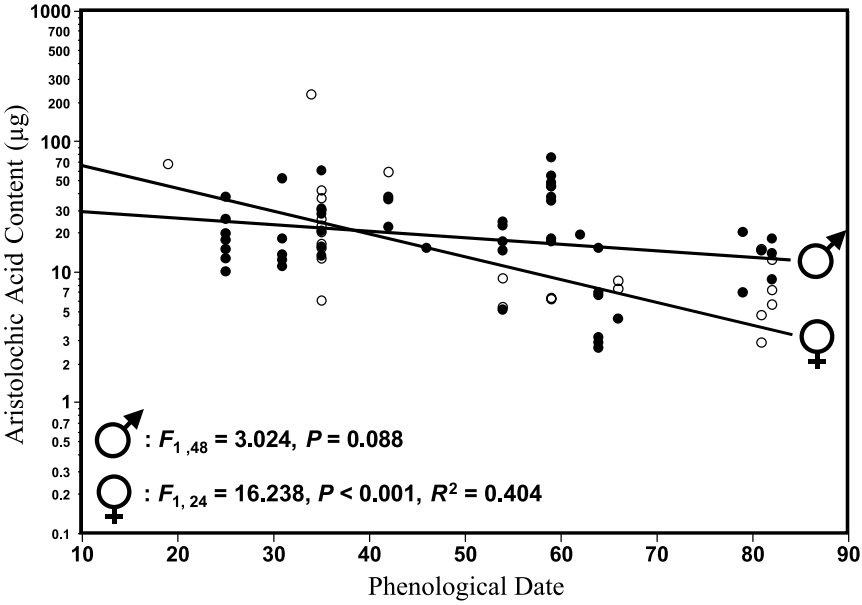

FIG. 2. Change in total aristolochic acid content observed in *B. philenor* sexes over phenological time. Open circles indicate females and solid circles indicate males. Log scale for aristolochic acid content.

the nature of concentrations, which can be affected by changes in the numerator (total amount of aristolochic acids) or the denominator (dry weight) for each individual. A model including dry weight as the response variable indicated that females lose significantly more weight (dry weight) over the course of the flight season compared to males (phenological date × sex: $F_{1,55} = 6.085$, $P = 0.017$).

We found no evidence that males transfer a substantial amount of aristolochic acids to females during mating. The average amount of aristolochic acids found in the corpus bursa, which contained a spermatophore, was 0.300 μg (range: 0.082–0.727 μg). The average aristolochic content of laid eggs was 0.452 μg (range: 0.002–0.951 μg) and unfertilized ova dissected from females was 0.224 μg (range: 0.008–0.415 μg). Thus, the total amount of aristolochic acids associated with the spermatophore was comparable to that found in a single egg.

DISCUSSION

The greater amounts of sequestered aristolochic acids detected in females compared to males at eclosion is consistent with previous analyses of *B. philenor* from other populations (Sime et al., 2000) and other butterfly species sequestering toxic plant chemicals (Dixon et al., 1978; Rothschild et al., 1979; Alonso-Mejía and Brower, 1994). There are two, not necessarily mutually exclusive, explanations for this phenomenon. First, female *B. philenor* adults and pupae weigh more than males (Fordyce, unpublished data), presumably because of their required reproductive investment in egg production. Females might accumulate greater amounts of aristolochic acids if prolonged larval feeding is required to obtain greater mass. Alternatively, females may possess a greater ability for sequestering aristolochic acids.

Females transfer aristolochic acids to their eggs. We found no evidence that males donate a substantial quantity of aristolochic acids to females via the spermatophore. In fact, our analyses indicate that the total amount of aristolochic acids associated with the spermatophore is comparable to that of a single egg. The greater quantities found in newly eclosed females may be a consequence of selection favoring efficient sequestration of these compounds by the female, thus providing her eggs with adequate chemical defenses. Females exhibited a significant decline in aristolochic acids over the course of the flight season, further supporting the hypothesis that the source of egg aristolochic acids is the female. If males were donating significant amounts of these compounds, we would expect them to show a similar decline in aristolochic acids over time. The general effectiveness of egg aristolochic acids as defenses against natural enemies is not known, however, and ants have been frequently observed as predators on *B. philenor* eggs in California (Fordyce, personal

observations). The eggs of *B. philenor* are orange, generally regarded as aposematic, and in California are laid in clusters (Fordyce, 2003). The effectiveness of egg chemical defenses might be enhanced when eggs are laid in clusters; egg clustering is frequently observed in species with toxic, warningly colored eggs (Stamp, 1980; Brower, 1984).

It is important to consider that the phenological change in aristolochic acid content indicated here was observed at the population level. Although *B. philenor* larvae were monitored in the laboratory and in the field until pupation as a means to avoid collecting wild adults from a second brood, the exact age of field-collected butterflies was unknown. It is likely that none of the females collected in May had emerged in early March. We have no estimates for adult longevity in the field, although captive individuals can survive up to 4 wk. However, it is likely that individuals collected late in the flight season are, overall, older than individuals collected early in the season. The time, specifically the order, of eclosion in captive reared butterflies did not explain the variation in aristolochic acid content, nor did it explain the phenological changes in toxicity observed between males and females. Further investigation is required to determine if the observed decline in female toxicity is unequivocally due solely to egg transference, or if female metabolism might also be involved. In general, however, females emerge with higher levels of aristolochic acids than males, but by the end of the flight season males are the more toxic sex observed in the population.

Although *B. philenor* is generally regarded as unpalatable to predators (Brower, 1958), there exists variation among individuals and populations in their acceptability and toxicity to predators (Codella and Lederhouse, 1989). Variation in butterfly toxicity likely reflects variation in aristolochic acid content of host plants, which can vary substantially within and among individual plants and, thus, directly affect butterfly chemical defense (Fordyce, 2001), in addition to the age-associated decline in toxicity observed in this study. Throughout most of its range, *B. philenor* is an effective model in Batesian mimicry complexes (Platt et al., 1971; Jeffords et al., 1979). However, there are no mimics in California. The absence more likely reflects the recent arrival of *B. philenor* to California (Fordyce and Nice, 2003), rather than its palatability (Codella and Lederhouse, 1989; Fordyce, 2000). The substantial decline in female toxicity over time might have important implications in regard to intersexual mimetic relationships, where females possibly become automimics. The development of automimicry as adults lose sequestered defenses over time, or as a consequence of phenological host shifts, has been suggested for *Danaus gilippus* (Ritland, 1994; Moranz and Brower, 1998) and *D. plexippus* (Alonso-Mejía and Brower, 1994). Unlike the examples in the danaines, where both sexes lose chemical defenses as they age (Alonso-Mejía and Brower, 1994), only female *B. philenor* show a decline in chemical defense over time. For

California *B. philenor*, males might function as the effective, honest signal for deterring predators as the females become less toxic over the course of the flight season.

Females showed a decline in toxicity measured as both total amount of aristolochic acids present, and as a measure of concentration. The relative importance of each of these measures as it relates to chemical defense will undoubtedly depend on how a predator samples the butterflies. Beak marks on the wings of butterflies are traditionally construed as evidence of bird rejection following tasting the presence of noxious compounds in the wings (Brower, 1984; but see Kassarov, 1999). Regardless of whether beak marks are a consequence of rejection or escape, if such a small volume of butterfly tissue is to be an effective signal to predators, the concentration of chemical defenses will be important. If, however, predators consume the entire butterfly, the total amount of chemical defenses may be more relevant than the concentration alone, unless there is a dilution effect associated with the biomass of the prey. Regardless of which measure of the amount of chemical defenses is most biologically relevant, both measures of aristolochic acid chemistry declined over time in female *B. philenor* included in this study.

The phenological variation observed for *B. philenor* in California indicates that determining the average amount of sequestered toxins present in the population is sensitive to the time at which individuals are collected. Similarly, determining the difference in aristolochic acid content between males and females is sensitive to the age at which individuals are examined. Female *B. philenor* begin the flight season containing more aristolochic acids. However, by the end of the season, they contain substantially less of these plant derived toxins and, thus, may be less chemically defended against natural enemies compared to males.

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CALCIUM TARTRATE CRYSTALS IN THE MIDGUT OF THE GRAPE LEAFHOPPER

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Abstract—Calcium tartrate crystals were observed in the midgut of grape leafhoppers. This unique compound was found for the first time in insects. The size of the crystals varied strongly between and within individuals with a mean length of 153 ± 87 μm and a mean width of 71 ± 46 μm. In addition, the number of crystals per individual showed a broad variation and ranged from 1 to 150 crystals/individual. The occurrence of calcium tartrate crystals as well as the number of crystals per individual followed the same seasonal pattern as seasonal vine leaf concentrations of tartaric acid found in a previous study, indicating that calcium tartrate is formed to neutralize the tartaric acid in the gut system. It further implies that the grape leafhopper, rather than being a pure phloem sucker, employs a mixed feeding strategy to satisfy its demands for calcium uptake.

Key Words—Calcium tartrate crystals, midgut, grape leafhopper, *Empoasca vitis*.

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INTRODUCTION

Various insects contain crystalline structures such as calcium oxalate or calcium urate that are predominantly found in the Malpighian tubules (Clark, 1958; Teigler et al., 1972; Wigglesworth, 1965). In particular, the urine of plant-feeding caterpillars is loaded with granules and crystals of calcium carbonate and calcium oxalate (Wigglesworth, 1965) that are derived from the diet (Takahashi et al., 1969; Teigler et al., 1972) and are thought to either bind and eliminate excess calcium (Clark, 1958; Teigler et al., 1972) or, in the case of calcium oxalate, to neutralize and eliminate the oxalic acid (Wigglesworth, 1965). However, some caterpillars, e.g., the sphinx moth *Pergesa elpenor*, feed exclusively on plants containing calcium oxalate raphides that are passed unchanged through the digestive tract (Clark, 1958).

Storage of calcium oxalate has been found in the fat body of *Phytomyza* sp. and *Bombyx mori* (Clark, 1958) and calcium urate in specialized urate cells in the fat body of hymenopteran larvae and cockroaches (Dettner and Peters, 1999). Calcium salts have also been reported in the eggs of various insects adding to the rigidity of the shell. Ito (1924) reported calcium oxalate crystals embedded in the eggshell of the cockroach that were produced in the left accessory gland of the females. Calcium citrate crystals that derive from the ventral body wall gland were found adhering to the ootheca of the praying mantis (Parker and Rudall, 1955).

A different type of crystalline structures called brochosomes has been reported in the Malpighian tubules of some leafhoppers consisting mainly of protein and lipids (Rakitov, 2002). The minute dodecahedral crystals are secreted and spread over the body with the hind legs, thus serving as a water repellent (Vidano and Arzone, 1984; Rakitov, 2002).

The grape leafhopper *Empoasca vitis* is an ubiquitous polyphagous phloem sucker (Vidano, 1963, 1967) that reproduces on vine. It overwinters as an adult on evergreens such as yew and spruce (Cerutti et al., 1991, personal observation). In early spring, the grape leafhopper feeds on a variety of host plants (Cerutti et al., 1991). Shortly after bud burst of the vines, it invades the vineyards and deposits its eggs in the veins of the vine leaves (Böll and Herrmann, 2004a). At the beginning of flowering, the first leafhopper larvae emerge, passing through five instar stages before they turn into adults. In contrast to most other German wine regions, in Franconia, only one generation of grape leafhoppers is produced each year (Böll and Herrmann, 2004b). The majority of the leafhoppers of the new generation leave the vineyards to feed on a broad variety of woody host plants before they retreat to overwintering sites in autumn (Cerutti et al., 1991).

In this paper, we report that grape leafhoppers (*E. vitis*) were found to carry translucent crystals in the midgut (Figure 1).

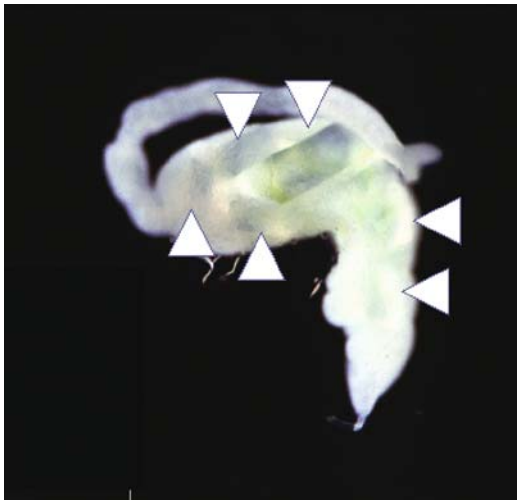

FIG. 1. Isolated midgut of a grape leafhopper packed with calcium tartrate crystals (see arrows).

METHODS AND MATERIALS

Sampling. In 2002 and 2003, grape leafhoppers were collected at least once per month, except for April and May 2002, when no collections were made. From July to October 2002 and May to December 2003, collections were taken on a weekly basis. For this purpose, overwintering sites as yew and spruce (during winter), various shrub and deciduous plant species (during spring and autumn), and vines (during the vegetation period) were beaten, and their specimens were caught in a funnel-shaped scooping net. All sampled specimens were killed either with CO_2 and dissected immediately thereafter or by deep-freezing, in which case dissections took place one to several days later. Abdominal preparations were carried out in 70% alcohol under a stereomicroscope (Leica M420) at a magnification rate of 60×. The sex of each specimen was determined by genital preparations. If present, crystals were extracted from the midgut, counted, and subsequently stored in 70% alcohol. Altogether, in 2002, 981 and in 2003, 756 specimens, respectively, were dissected. Captures with less than five specimens of either sex or developmental stage were not included in the analysis.

The length and width of 100 crystals from six grape leafhoppers were measured under a stereomicroscope (Leica M420) at a magnification rate of 60× to the nearest micrometer.

Crystal Structure Analysis by HPLC-MS. Analyses were performed on a triple-stage quadrupole TSQ 7000 liquid chromatography–tandem mass spectrometry (MS/MS) system (Finnigan MAT, Bremen, Germany). Data acquisition and data evaluation were carried out on a Personal DEC station 5000/33 (Digital Equipment, Unterföhring) and ICIS 8.1 software (Finnigan MAT). The data of the product ion spectra were collected within a mass range between 20 and 180 units. For high-performance liquid chromatography (HPLC), an Applied Biosystems dual syringe pump model 140B (BAI, Bensheim) was used. Crystals from dissected specimens and from purchased (+)-calcium L-tartrate dihydrate (Sigma, Taufkirchen) were dissolved in water, and 10 µl were directly injected [loop–electrospray ionization (ESI)–MS/MS] using 50% 5 mM NH_4Ac in H_2O (LiCrosolv, Merck, Darmstadt, Germany) and 50% MeCN (LiCrosolv, Merck) as mobile phase. ESI in negative mode was used. The temperature of the heated capillary was set to 230°C, and the capillary voltage was set to 3.4 kV. Nitrogen served both as sheath (60 psi) and auxiliary gas (10 l/min).

Crystal Structure Analysis by X-Ray Diffraction. Several crystals isolated from grape leafhoppers were investigated by X-ray diffraction. Each crystal was mounted in inert oil (perfluoroalkylether, ABCR) on a glass fiber and transferred to the cold nitrogen stream of the diffractometer (Stoe-IPDS, graphite-monochromated MoKα-radiation, λ = 0.71073 Å, T = 243 K). The structure was solved (program SHELXS; Sheldrick, 1990) and refined (program SHELXL97; Sheldrick, 1990, 1997) except for the position of hydrogen atoms.

RESULTS

Crystal Structure Analysis by HPLC-MS. The HPLC-MS data of diluted crystals from the leafhoppers were compared with the data from purchased Ca-tartrate. The product ion mass spectra in negative ion mode showed identical fragments for both m/z 149 $[M]^-$; m/z 105 $[M - CO_2]^-$, 87 $[M - CO_2–H_2O]^-$, 73, 59, 43. The anion was identified as tartrate.

Crystal Structure Analysis by X-Ray Diffraction. $C_4H_4O_6Ca{\cdot}4H_2O$, colorless translucent block, size 0.05 × 0.05 × 0.07 mm, orthorhombic, space group $P2_12_12_1$ (no. 19), a = 9.2141(18), b = 9.6434(19), c = 10.589(2) Å, V = 940.9(3) $Å^3$, Z = 4, $F_{(000)}$ = 528, μ = 0.708, $\rho_{(calc)}$ = 1.808 g/cm^3, 9846 refls. (R_{int} = 0.16), $-12 < h < 11$, $-13 < k < 13$, $-14 < l < 14$, completeness: 98.5%, 2509 independent refln, $1834 > 2\sigma_{(I)}$, 145 params., 3 restraints, GooF 0.958, Flack param. 0.12(8), largest difference peak and hole: 0.59/−0.75 e/$Å^3$, $R_{1(obs)}$ = 0.0627, $R_{1(all)}$ = 0.088.

The crystals were identified as Ca-tartrate $C_4H_4O_6Ca{\cdot}4H_2O$ (Figure 2), the structure of which is already known (Boese and Heinemann, 1993). The Flack parameter 0.12(8) obtained in the refinement of the model indicates that the

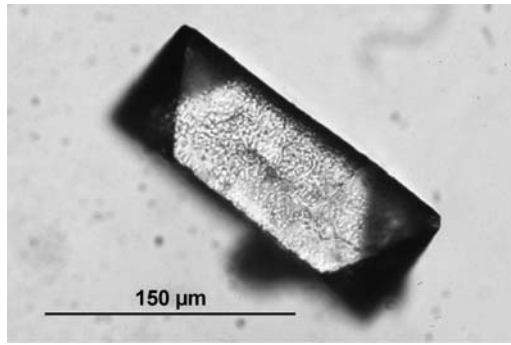

FIG. 2. Calcium tartrate crystal isolated from the midgut of a grape leafhopper.

tartrate crystal investigated is of the normal configuration (2R,3R), provided it is an enantiomerically pure crystal (Flack and Bernadinelli, 2000).

Seasonal Occurrence of Calcium Tartrate Crystals in Grape Leafhoppers. In 2002, calcium tartrate crystals were found in the midgut of grape leafhoppers from July through October, whereas in 2003, crystals already appeared in May, but ceased abruptly in September (Figure 3). In this exceptionally hot and dry

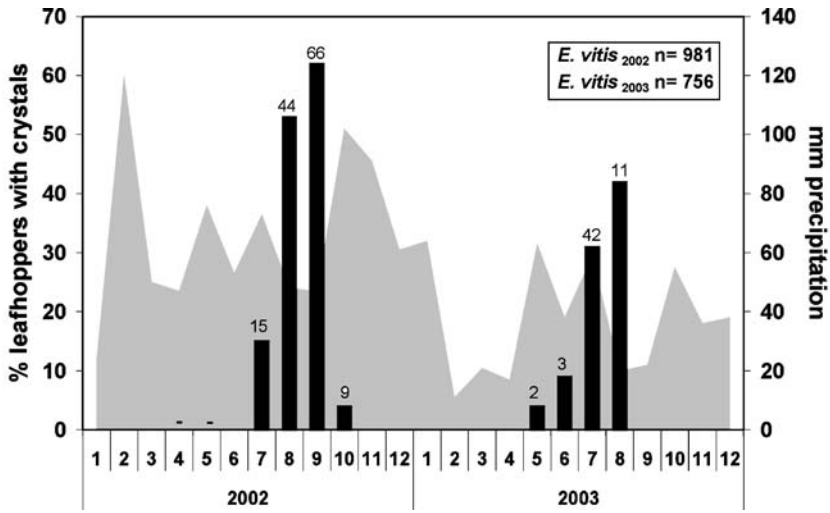

FIG. 3. Percentage of adult grape leafhoppers with calcium tartrate crystals during the seasons 2002 and 2003. The shaded area shows the rainfall pattern over the two seasons. –: No collections were carried out.

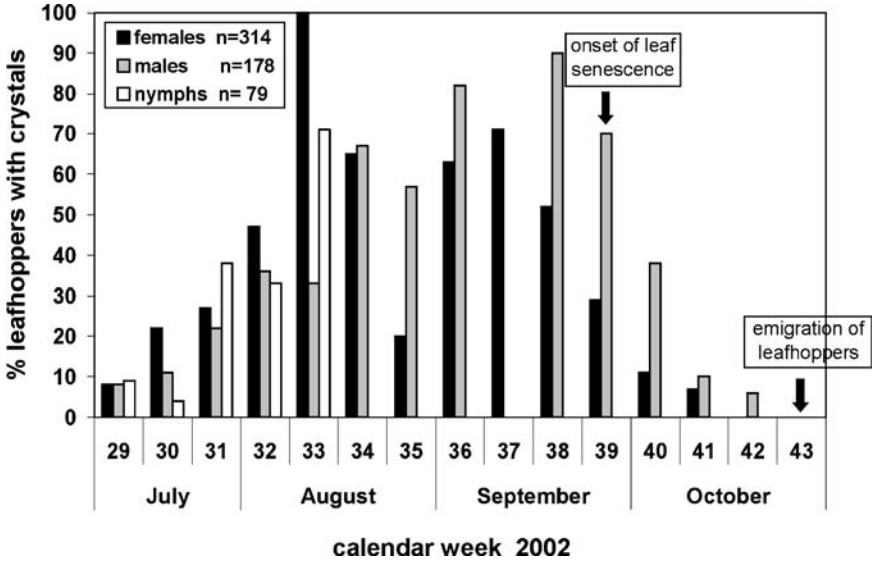

FIG. 4. Percentage of grape leafhoppers with calcium tartrate crystals during July–October 2002.

summer, the grape leafhopper left the vineyard earlier than usual at the end of August. Crystals were found in males as well as in females and nymphs (Figure 4). The percentage of leafhoppers showing crystals rose steadily during both vegetation periods, but dropped with the beginning senescence of vine leaves, and went to zero after the emigration of the leafhoppers (Figures 3 and 4). Following the same pattern, the number of crystals found in the gut per individual varied over the season and ranged from 1 to 150 crystals/individual. The size of the crystals varied greatly between and within grape leafhoppers (Table 1), with a total mean length of 153 ± SD = 87 μm and a mean width of 71 ± SD = 46 μm (range:

TABLE 1. CRYSTAL SIZE RANGE

Analyzed no. of crystals/ total no. of crystals/ individual	Length of crystal (μm), mean ± SD	Width of crystal (μm), mean ± SD
8/9	186 ± 144	72 ± 34
14/31	163 ± 40	83 ± 24
11/20	155 ± 69	71 ± 24
12/16	188 ± 81	74 ± 27
18/38	185 ± 100	68 ± 45
37/48	114 ± 74	56 ± 33

length 50–563 μm, width 25–225 μm, $N = 100$). In comparison, the abdomen of the grape leafhopper had a mean length of 1168 ± SD = 158 μm and a mean width of 568 ± SD = 62 μm ($N = 10$).

DISCUSSION

Translucent crystals occurring in the midgut of grape leafhoppers were identified as L-(+)-calcium tartrate. This is the first report of this compound in insects. Whereas other calcium salts such as calcium carbonate, calcium urate, or calcium oxalate that have been observed in a variety of insects usually occur in the Malpighian tubules, the calcium tartrate crystals were found in the midgut of the grape leafhopper. Only in rare cases, e.g., in *B. mori* (Waku and Sumimoto, 1971) and the fulgorid *Pyrops candelaria* (Ammar, 1985), has an accumulation of calcium salts been observed in the midgut of insects.

The functional significance of the temporary accumulation of large amounts of calcium tartrate crystals in the grape leafhopper has yet to be determined. As this species overwinters as an adult on evergreens exposed to harsh temperatures, calcium tartrate could serve as an antifreezing agent. However, this is unlikely as alcohols, such as glycerol, sorbitol, or trehalose, usually serve this purpose in insects (Dettner and Peters, 1999). In addition, no calcium tartrate was found in grape leafhoppers once they had left the vineyards to retreat to overwintering sites. Another possible function of the sequestration of calcium tartrate might be chemical defense. However, calcium tartrate is neither a particular toxic substance nor does it belong to the predominant compound groups that play a role in chemical defense in insects, such as terpenes, fatty acid derivates, aromatic compounds, or alkaloids (Dettner and Peters, 1999).

Alternatively, the observed calcium tartrate crystals in the grape leafhopper might simply result from passive accumulation of this compound, as it seems to follow the seasonal pattern of tartaric acid concentrations in the vine leaves. Calcium tartrate crystals were not observed in the midgut of grape leafhoppers until several weeks after migration to the vineyard, when tartrate concentrations in the vine leaves start to increase (Kliewer, 1966). With further increase of tartrate concentrations in the leaves during continued vegetation growth and ripening of the vine (Kliewer, 1966), the percentage of leafhoppers carrying crystals increased as well. This was accompanied by a continuous calcium tartrate accumulation in the midgut of the leafhoppers that can be densely packed with up to 150 crystals per leafhopper. The crystals, varying greatly in size even within individuals, can reach huge dimensions attaining lengths that are equivalent to the width of the abdomen of the leafhopper. At the onset of the senescence of the vine leaves, when tartrate concentrations in the leaves drop (Kliewer, 1966), the presence of crystals as well as the number of crystals per

leafhopper declined accordingly. During overwintering and spring migration, no crystals were found in the midgut of grape leafhoppers.

Occurrence of tartaric acid in higher plants is relatively rare; nevertheless, in the vine, this substance usually represents quantitatively the most important component within the acid pool of leaves and berries (Kliewer, 1966; Ruffner, 1982). The physiological function of the tartaric acid is not completely understood, although, at least in part, it serves as a calcium-binding agent. The vine has a high uptake of calcium that accumulates in the leaves during the vegetation period, particularly in the tips. Calcium tartrate is stored in crystalline form in specialized large cells, known as idioblasts, that are located in intercostal sections of the leaves with the highest density in the serrated tips (Ruffner, 1982).

In the grape leafhopper, the situation is rather the reverse. Generally considered as a pure phloem sucker as described by Vidano (1963, 1967), it has to cope with increasing tartrate concentrations in the leaves until the senescence of the leaves sets in. Calcium uptake poses a problem. As calcium is transported in the xylem, phloem-feeding hemiptera are usually in short supply of calcium (Raven, 1983). Thus, the grape leafhopper might not produce calcium tartrate crystals to bind an excess of calcium, as is the case with the vine, but rather to neutralize the relatively strong tartaric acid in the gut system. The stabilization of the pH in the midgut might be a prerequisite particularly for the nymphs of *E. vitis* to be able to feed exclusively on vine as a host plant.

The question then remains how *E. vitis* accomplishes a sufficient intake of calcium. Mixed feeding of closely related leafhopper species is known. Hunter and Backus (1989) have shown that the closely related sister species *Empoasca fabae*, which was also thought to be a pure phloem sucker, feeds as well on the mesophyll. Furthermore, mixed phloem and mesophyll feeding has been found in *Empoasca decipiens* (Günthardt and Wanner, 1981). All *Empoasca* species so far studied that cause hopperburn on the host plant employ the same feeding behavior of multiple cell laceration, which leads to a mixed diet (Backus et al., 2005). Likewise, *E. vitis*, a typical hopperburn species, might not be a pure phloem sucker as hitherto thought. On the contrary, the presence of calcium tartrate crystals in the midgut during the summer as well as the observed greenish color of the midgut (Figure 1) suggests strongly that the grape leafhopper employs a mixed feeding strategy that enables it to satisfy its calcium demands for neutralizing tartaric acid accumulations in the gut system. However, to determine the exact nature of the feeding behavior of *E. vitis*, electronic penetration graph monitoring should be employed (Backus et al., 2005).

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CHEMICALLY MEDIATED HOST-PLANT SELECTION BY THE MILFOIL WEEVIL: A FRESHWATER INSECT–PLANT INTERACTION

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Abstract—The milfoil weevil *Euhrychiopsis lecontei* is a specialist aquatic herbivore that feeds, oviposits, and mates on the invasive freshwater macrophyte *Myriophyllum spicatum*. We characterized the weevil's preference for *M. spicatum*, and through bioassay-driven fractionation, isolated and identified two chemicals released by *M. spicatum* that attract *E. lecontei*. Mass spectrometry and nuclear magnetic resonance spectroscopy were used to identify the attractive compounds as glycerol and uracil. Dose-response curves for glycerol and uracil indicated that weevil preference increased as sample concentration increased. Weevils were attracted to a crude sample of *M. spicatum*-released chemicals from 0.17 to 17 mg/l, to glycerol from 18 to 1800 μM (0.0017–0.17 mg/l), and to uracil from 0.015 to 15 μM (0.00014–1.4 mg/l). Although glycerol and uracil are ubiquitous, weevils are likely responding to high concentrations that are released as a result of the rapid growth of *M. spicatum*. Uracil concentration was greater in the exudates of *M. spicatum* than other *Myriophyllum* spp. *E. lecontei* was attracted to glycerol at a concentration similar to that at which terrestrial insects are attracted to sugar alcohols. This is the first example of a freshwater specialist insect being attracted to chemicals released by its host plant. Analysis of the water milfoil–weevil interaction provides further understanding as to how insects locate their host plants in aquatic systems.

Key Words—Freshwater macrophyte, host location, *Myriophyllum spicatum*, *Euhrychiopsis lecontei*, specialist herbivore, chemical attractant, aquatic plant–herbivore interactions.

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INTRODUCTION

Plants produce and release chemicals that can be used by herbivores to locate mates, food sources, and oviposition sites. There is a great variety of compounds that attract terrestrial insects, including ubiquitous chemicals such as ethanol, hexanal, and sugars, as well as species-specific chemicals, such as polyphenols and isothiocyanates (Metcalf and Metcalf, 1992; Bernays and Chapman, 1994). Some ubiquitous chemicals, such as sugars, are nutrients that act as phagostimulants (Chapman, 2003). Common volatiles, such as ethanol and six-carbon alcohols, can be used as long-range attractants (Bruce et al., 2005). However, in aquatic systems, natural products are transported through water rather than air. Therefore, their mobility and persistence in the environment can be different. Simple sugars and amino acids can be rapidly degraded by microorganisms. Nonvolatile chemicals located on leaf surfaces, which typically have low mobility in the air, may become mobile in water. The dynamics of plant–insect interactions will undoubtedly be similar in both aquatic and terrestrial systems, but the mechanism of the interactions and the chemicals involved in those interactions may be different (for a review, see Hay and Steinberg, 1992).

In aquatic systems, studies of animal response to chemical stimuli have focused primarily on fish and noninsect invertebrates. Feeding stimulants are often common metabolites of low-molecular weight. For example, amino acids, such as glycine, attract lobster, shrimp, and several species of fish (Carr, 1988). Amino acids, nucleotides, simple sugars, and organic acids have been used to assess the attraction of marine invertebrates (primarily crustaceans) to their prey (see review by Weissburg et al., 2002).

Recent studies on the functional ecology of natural products in freshwater macrophytes have demonstrated the role they play in defense against generalist herbivores (Kubanek et al., 2001; Choi et al., 2002; Cronin et al., 2002; Walenciak et al., 2002). Furthermore, several weevil species have a specialist or close association with freshwater macrophytes (Buckingham and Bennett, 1995; Cronin et al., 1998; Solarz and Newman, 2001; Center et al., 2002). However, the role of chemistry in these specialist–macrophyte interactions is unknown.

The sole example of a specialist insect being attracted to a submersed freshwater macrophyte is the *Myriophyllum spicatum* L. (Haloragaceae)–*Euhrychiopsis lecontei* Dietz (Coleoptera: Curculionidae) system (Solarz and Newman, 1996, 2001). Eurasian water milfoil *M. spicatum* is an aquatic macrophyte exotic to North America that is typically abundant in the littoral zone of mesotrophic to moderately eutrophic lakes (Smith and Barko, 1990). It may remain unnoticed for years, then suddenly grow, forming a dense canopy that shades out other plants, impedes navigation, and adversely affects recreational activities (Smith and Barko, 1990; Boylen et al., 1999). Reductions

in *M. spicatum* populations have occurred in lakes where *E. lecontei* was present and in controlled experiments with *E. lecontei* (Creed and Sheldon, 1995; Sheldon and Creed, 1995; Newman, 2004).

Water milfoils native to North America, such as the northern milfoil *Myriophyllum sibiricum* Komarov, are the presumed original hosts of *E. lecontei*, which has expanded its host range to include *M. spicatum* (Creed and Sheldon, 1994; Solarz and Newman, 1996). *E. lecontei* has shorter development times, greater mass, and higher survival on the invasive Eurasian water milfoil than on its native host plants (Newman et al., 1997; Solarz and Newman, 2001). Based on field observations, oviposition studies, and behavioral bioassays, Solarz and Newman (1996) hypothesized that there may be chemical cues that attract weevils to *M. spicatum*. In this study, we tested their hypothesis that the weevils are attracted to *M. spicatum*, and we isolated two chemicals involved in that interaction.

METHODS AND MATERIALS

Plant and Insect Material. Plants were obtained from various lakes in Minnesota, USA. *M. spicatum* was collected from Lake Auburn (Carver County), Cedar Lake, Lake of the Isles, and Smith's Bay in Lake Minnetonka (all in Hennepin County), *M. sibiricum* from Christmas Lake (Hennepin County) and Smith's Bay, *M. spicatum* × *M. sibiricum* hybrid from Otter Lake (Anoka County) and White Bear Lake (Washington County), *Myriophyllum alterniflorum* DC from Lake Sissabagamah (Aitkin County), *Myriophyllum tenellum* Bigel from Graham Lake (Carlton County) and West Twin Lake (St. Louis County), and *Ceratophyllum demersum* L. (Ceratophyllaceae) from Lake Auburn. The identities of *M. spicatum* × *M. sibiricum* hybrid, *M. alterniflorum*, and *M. tenellum* were confirmed by sequence comparison of nuclear and chloroplast markers (analysis performed by M. Moody, University of Connecticut; see Moody and Les, 2002 for methodology).

Preparation of Exudates. All plants were collected from stands with no apparent damage from the aquatic weevil *E. lecontei*. Stems were held in lake water until laboratory processing, which occurred within 24 hr of collection. The top 20 cm of the plants was inspected for herbivore damage and cleaned of algae and detritus. Plants were then placed in well water (characterized by Oseid and Smith, 1974) to avoid contamination with chlorine and other additives in tap water. Plants were incubated for 2–4 d in direct sunlight or under constant illumination in a laboratory light bank that delivered 1065 μmol m^{-2} sec^{-1} photosynthetically active radiation. Temperature was at or below 30°C. After incubation, plants were removed and spun in a salad spinner for 15 sec to remove excess water. Plant material was weighed and frozen for future

use. The water containing the milfoil exudates was filtered through cheesecloth, stored in plastic bags (Ziploc™), and frozen at −20°C until processing. Adult *E. lecontei* were collected biweekly from several of the same area lakes and held in a 0.545-m^3 stock tanks (61 × 183 × 76 cm) located in an outdoor setting or in 76-l aquaria on fresh *M. spicatum*.

Chemicals and Other Materials. Ultrafiltration membranes used to concentrate extracts were obtained from Millipore, Bedford, MA, USA, and the ultrafiltration cell was obtained from Diaflo Amicon, W. R. Grace & Co., Beverly, MA, USA. Diethylaminoethyl (DEAE) ion exchange and Sephadex G-10 resins and the Superdex Peptide HR10/30 column used in the isolation of attractants were purchased from Amersham Biosciences, Piscataway, NJ, USA. Several additional high-performance liquid chromatography (HPLC) columns, C18, C8, and Source columns were obtained from the same manufacturer. The Magic C-18 column for liquid chromatography–mass spectrometry was from Michrom BioResources, Auburn, CA, USA. Uracil, used as a standard, was from Aldrich Chemical Company, Milwaukee, WI, USA. Glycerol was purchased from Spectrum Chemical Manufacturer, Gardena, CA, USA. The milfoil polyphenol, tellimagrandin II [β-1,2,3-tri-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-D-glucose], was a gift from E. Gross, Limnological Institute, University of Konstanz, Germany.

Behavioral Bioassays. Adult *E. lecontei* preferences were determined using a two-way choice test performed in Y-tubes according to published procedures described in Solarz and Newman (1996, 2001). Assays were designed to maximize the behavioral analysis with small amounts of material. The experimental apparatus consisted of a glass Y-tube, 1.2-cm diam with 14-cm-long arms and stem. Each end of the tube was sealed with a cork, and the Y-tube was filled with well water. The control material was randomly placed into one arm of the Y-tube. Control material was either a plastic plant or a blank tube when fresh milfoil was used or a 6-mm diam filter paper saturated with well water or solvent for the chemical choice tests. Sample material, similarly prepared, was introduced into the second arm, and a weevil into the stem of the Y-tube. The Y-tube stem was covered with an opaque cloth and placed at a 10° incline (Solarz and Newman, 1996). A choice was indicated by the weevil swimming to or within 1 cm of either the control or sample material within a 10-min period. Choice and the time required to make that choice were recorded. No choice weevils were excluded from analyses. Weevils usually made a choice within 5 min. There was no difference in weevil choice between a plastic plant and a blank tube ($N = 20$, $P = 0.37$); therefore, either one was used as a control. As an alternative to the plastic plant or blank tube, exudates from a common co-occurring aquatic plant, *C. demersum*, were prepared as above and also tested against *M. spicatum* exudates and isolated fractions. Y-tubes were rinsed with 95% ethanol, then rinsed three times with well water after every three to six

trials to ensure that previous test weevils did not affect the behavior of subsequent ones, and that chemicals did not diffuse into both arms of the Y-tube (Solarz and Newman, 2001). Throughout the isolation, Y-tube choice tests were used to determine whether weevils were attracted to the fractions.

For Y-tube bioassays, an aliquot from a known concentration of sample was infiltrated into filter paper disks, then used in the choice test. For dose–response curves, a range of concentrations of *M. spicatum* exudates and each isolated chemical was infiltrated. An estimate of the dose perceived by the weevils is the applied amount (in mg or mol) divided by the volume of one arm of the Y-tube (12 ml). This value in mg/l or molar amounts is indicated in the appropriate figure captions, and can be considered an average concentration that the weevils perceived during a trial. Likely, weevils were able to detect lower concentrations as the material dispersed throughout the Y-tube. Approximately 50 (range 20–127) randomly selected weevils were tested at each concentration level.

Chemical Isolation and Identification. Plant exudates and partially purified fractions were kept frozen at −20°C. Exudates were thawed, filtered through Whatman No. 1 cellulose filter paper, and lyophilized. The powder was resuspended in Milli-Q water and used in the Y-tube bioassay to confirm attraction. The resuspended exudate was fractionated into large and small molecules by using ultrafiltration. Ultrafiltration was performed with a 76-mm YM1 membrane (MWCO 1000) in a model 8400 Diaflo Amicon ultrafiltration cell under a 70-psi N_2 atmosphere. Both the retentate (mw > 1000) and the filtrate (mw < 1000) were tested vs. water in the Y-tube bioassay.

Active filtrate was loaded onto a DEAE-anion exchange column (3.5 × 10 cm column) that had been previously equilibrated with Milli-Q water. The column was eluted with Milli-Q water at a flow rate of at 1.7 ml/min. The effluent was monitored at 254 nm, and fractions were collected. A single UV-absorbing peak was observed. Fractions from this peak were pooled, concentrated by lyophilization, and tested in the Y-tube bioassay. Material that was eluted with 0.5 M NaCl was also collected, but showed no activity in the bioassay.

The DEAE pool was further purified by size exclusion chromatography on a G-10 Sephadex column (30 × 2.5 cm) that had been previously equilibrated with Milli-Q water. The column was eluted with water at a flow rate of 0.3 ml/hr, the effluent monitored at 254 nm, and fractions collected. Four peaks were eluted, and fractions from each were pooled separately, concentrated by lyophilization, and tested in Y-tube bioassays.

The active peak, with an absorbance maximum at 259 nm, was analyzed by atmospheric pressure chemical ionization–mass spectrometry (APCI-MS) to assess its purity and obtain information about the components. The active pooled fraction from the G-10 column was loaded onto a Superdex Peptide HR

10/30 size-fractionating column (30 × 1 cm) that had been equilibrated with Milli-Q water. The column was eluted with water at a flow rate of 0.3 ml/min. One major peak with an absorbance maximum at 259 nm showed a very broad shoulder. The active pooled fraction was concentrated by lyophilization, and the components were identified by a combination of positive ion APCI and electron impact mass spectrometry and NMR spectroscopy. The purification procedure is summarized in Table 1.

APCI analyses were performed on a ThermoElectron LCQ Classic Ion Trap mass spectrometer (Waltham, MA, USA) fitted with an APCI ionization source. The APCI heated capillary temperature was set at 450°C and controlled at 10 μA. Xcaliber™ (ThermoFinnigan Corp.) was used to acquire the data. Several LC-MS runs with C18, C8, and Source columns correlated the absorption spectrum to the molecular weight, but did not further separate components of the active fraction.

Samples for NMR spectroscopy were dissolved in D_2O. NMR spectra were performed on Varian Inova 500, 600, and 800 spectrometers (University of Minnesota) using standard pulse sequences and water suppression. Identity of the active compounds was confirmed by comparison to standards using ^{1}H, ^{13}C, ^{1}H–^{1}H correlated spectroscopy (COSY), ^{1}H–^{13}C COSY, MQ COSY, heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple-bond correlation (HMBC) NMR spectra.

Uracil content of partially purified plant exudates (DEAE pool) was measured with a QTrap LC/MS/MS hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Inc., ABI, Foster City, CA, USA). A 5-μl sample was withdrawn with a Agilent 1100 Micro Autosampler and transferred by an Agilent 1100 Capillary Pump to a Magic C18 column (150 × 0.2 mm) with 5-μm, 200-Å pore size particles. Components were sequentially eluted with a gradient. Solvent A was 98:2 water/acetonitrile containing 0.1% formic acid. Solvent B was 5:95 water/acetonitrile containing 0.1% formic acid. Using a flow rate of 5 μl/min, the gradient went from 100% A to 75% A in 4 min, was held for 3 min at 75% A, then ramped over 3 min to 25% A. The LC system was in line with the QTrap equipped with a TurboIonSpray ion source. The electrospray voltage was set at 5500 V. Samples were analyzed using a multiple reaction monitoring scan mode. Ions with a m/z of 113.10 in positive polarity were detected in quadrupole 1, autofragmented in quadrupole 2, and a m/z of 69.90 (a fragment of uracil) was monitored in quadrupole 3. Analyzst 1.3.1 (ABI) was used to acquire and analyze the data. A standard solution of 1.0 mg/ml of material that eluted from the DEAE column was spiked with uracil to a final concentration ranging from 0.09 to 900 μM to generate a standard curve. These samples were used to estimate the concentration of uracil present in DEAE pool samples from *M. spicatum*, *M. sibiricum*, *M. spicatum* × *M. sibiricum* hybrid,

M. alterniflorum, *M. tenellum*, and *C. demersum*. Peak areas from standards were used to determine uracil concentrations.

Statistical Analyses. Y-tube data were tested with a χ^2 analysis. Dose–response curves for exudates and each isolated attractant were developed using logistic regression based on the amount of sample infiltrated into filter disks used in Y-tube bioassays. Comparisons among models were made using χ^2 analyses of model deviances. Weevil preference was determined in the Y-tube bioassay. Glycerol and uracil were combined in Y-tube bioassays to test for possible synergistic effects. Differences in uracil content between *M. spicatum*, *M. sibiricum*, and *M. spicatum* × *M. sibiricum* hybrid were determined with an analysis of variance.

RESULTS

Behavioral Bioassay. E. lecontei adults were attracted to *M. spicatum* in preference to control ($P < 0.001$), *M. sibiricum* ($P < 0.001$), and *C. demersum* ($P = 0.003$; Table 1A). Weevils did not show this level of preference for their native host plant *M. sibiricum*; they chose a control nearly as often as they selected *M. sibiricum* ($P = 0.131$). The *M. spicatum* × *M. sibiricum* hybrid attracted weevils, but different hybrid populations elicited different reactions. Weevils were attracted to hybrid milfoil from White Bear Lake ($P < 0.001$) and only moderately to hybrid milfoil from Otter Lake ($N = 179$, $P = 0.059$). Neither of the other milfoil species, *M. alterniflorum* nor *M. tenellum*, nor the non-milfoil plant *C. demersum* were attractive ($P = 0.78$, $P = 0.13$, and $P = 0.45$, respectively).

To determine the chemical identity of the weevil attractant(s), exudates of *M. spicatum* were tested for weevil preference, lyophilized, and retested for weevil preference (Table 1B). Weevils were attracted to certain *M. spicatum* exudates ($P < 0.001$) but not to *M. sibiricum* exudates ($P = 0.62$) or *C. demersum* exudates ($P = 0.41$). The *M. spicatum* exudates were separated into low-molecular weight (<1000 amu) and high-molecular weight (>1000 amu) compounds. Weevils were attracted to the low-molecular weight fraction ($P < 0.001$) and preferred it to *C. demersum* exudates ($P = 0.017$; Table 1C). The low-molecular weight fraction was purified using an anion exchange column, and weevils were attracted to the one major peak that passed through this column ($P < 0.001$) and preferred that peak to *M. sibiricum* exudates ($P = 0.028$; Table 1D). The anion exchange column pool was purified by size exclusion chromatography, and weevils were attracted to the third ($P = 0.001$) of the four peaks that eluted from the column (Table 1E). The active G-10 pool contained several components as determined by MS, and was further purified by

TABLE 1. BIOASSAY-GUIDED FRACTIONATION OF *M. spicatum* EXUDATES FROM TWO-WAY Y-TUBE CHOICE TESTS

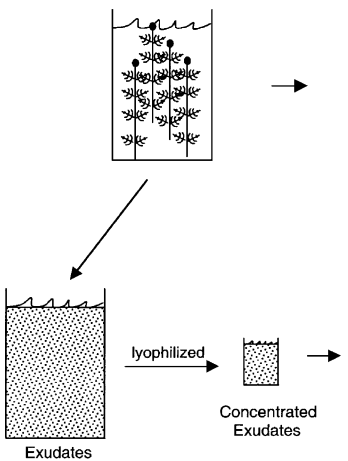

TABLE 1A. Y-TUBE CHOICE TESTS OF *Myriophyllum* spp. AND *Ceratophyllum demersum*

Choice				
A	vs.	B	*N*	% A
M. spicatum		control	652	66**
M. sibiricum		control	101	57
M. sp. × *M. sib.* hybrid		control	229	61**
M. spicatum		*M. sibirium*	110	65**
M. alterniflorum		control	64	41
M. tenellum		control	50	48
C. demersum		control	143	53
M. spicatum		*C. demersum*	49	69*
M. sibiricum		*C. demersum*	64	44

Significance for χ^2 analysis at: $^*P < 0.01$, $^{**}P < 0.001$.
Data are combined results from several years.

TABLE 1B. Y-TUBE CHOICE TESTS OF *Myriophyllum* spp. AND *Ceratophyllum demersum* EXUDATES

Choice				
A	vs.	B	*N*	% A
M. spicatum exudates		control	35	74*
M. sibiricum exudates		control	25	52
M. spicatum concentrated exudates		control	275	66**
M. sibiricum concentrated exudes		control	144	48
C. demersum exudates		control	54	44

Significance for χ^2 analysis at: $^*P < 0.01$, $^{**}P < 0.001$.
Data are combined results from several years.

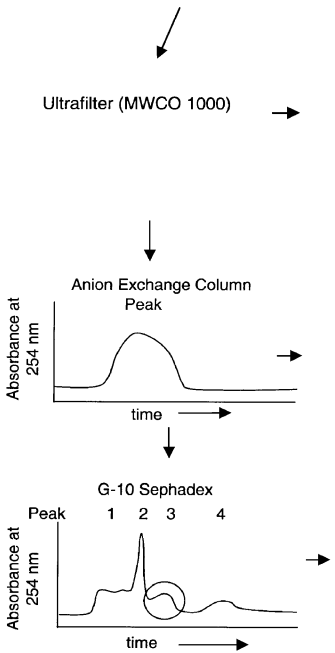

TABLE 1C. Y-TUBE CHOICE TESTS OF *Myriophyllum spicatum* AND *Ceratophyllum demersum* HIGH AND LOW MOLECULAR WEIGHT SAMPLES

Choice				
A	vs.	B	*N*	% A
<1000 mw		control	106	69**
>1000 mw		control	36	42
<1000 mw		*C. demersum* exudates	22	73^{+}

Significance for χ^2 analysis at: $^{+}P < 0.05$, $^{**}P < 0.001$.
Data are combined results from several years.

TABLE 1D. Y-TUBE CHOICE TESTS OF EXUDATES OF *Myriophyllum spicatum* AFTER CHROMATOGRAPHY ON AN ANION EXCHANGE COLUMN

Choice				
A	vs.	B	*N*	% A
DEAE fraction vs.		control	178	68**
M. spicatum DEAE pool		*M. sibiricum* exudates	34	68^{+}

Significance for χ^2 analysis at: $^{+}P < 0.05$, $^{**}P < 0.001$.
Data are combined results from several years.

TABLE 1E. Y-TUBE CHOICE TESTS OF FRACTIONS OF *Myriophyllum spicatum* AFTER CHROMATOGRAPHY ON A SIZE-FRACTIONATING COLUMN

Choice				
A	vs.	B	*N*	% A
M. spicatum G-10 fraction 1		control	50	48
M. spicatum G-10 fraction 2		control	51	59
M. spicatum G-10 fraction 3		control	55	71^{+}
M. spicatum G-10 fraction 4		control	48	52

Significance for χ^2 analysis at: $^{+}P < 0.05$.
Data are combined results from several years.

The two choices are listed as A vs. B in the treatment column. Number of Y-tube trials is indicated in the column '*N*.' '% *A*' is the percent of weevils that selected choice A.

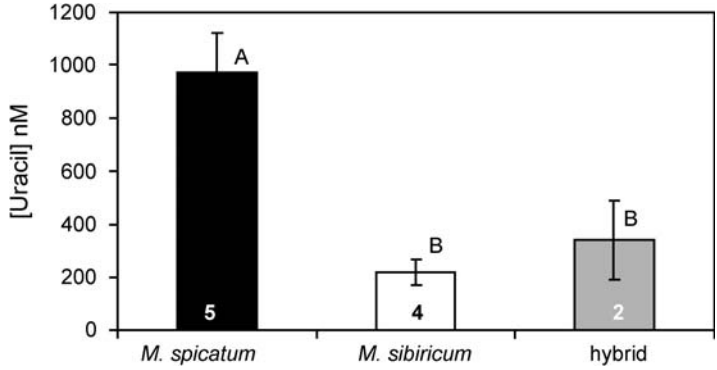

FIG. 1. Uracil concentration (nM) in exudates of *M. spicatum*, *M. sibiricum*, and hybrid (*M. spicatum* × *M. sibiricum* hybrid) after chromatography on anion-exchange column. Mean ± 1 SE are presented. Quantitation of uracil content is described in Methods and Materials. Number of samples analyzed is indicated within the bar for each species. Letters above each bar indicate Tukey's honestly significant difference between treatments. Uracil was also found in the anion-exchange column pool from *M. alterniflorum*, *M. tenellum*, and *C. demersum*, but only one sample of each was analyzed.

TABLE 2. Y-TUBE CHOICE TESTS OF ISOLATED ATTRACTANTS AND THE POLYPHENOL, TELLIMAGRANDIN II, FOUND IN *M. spicatum*

Choice[a]			
A	B	*N*	% *A*
Glycerol[b]	control	155	63**
Uracil[c]	control	202	73**
Glycerol	*C. demersum* exudates	24	75*
Glycerol and uracil[d]	control	110	70**
Tellimagrandin II[e]	control	102	55

*Significance for χ^2 analysis at $P < 0.01$.
**Significance for χ^2 analysis at $P < 0.001$.
[a]The two choices are listed as A vs. B in the treatment column. Number of Y-tube trials is indicated in the column '*N*.' '% *A*' is the percent of weevils that selected choice A.
[b]Glycerol at 22 or 220 μmol, combined results, amount applied to filter disk.
[c]Uracil at 18 or 180 nmol, combined results, amount applied to filter disk.
[d]Glycerol 220 μmol + uracil 180 nmol and glycerol 22 μmol + uracil 18 nmol, combined results, amount applied to filter disk.
[e]Tellimagrandin II at 2.1 or 21 nmol, combined results, amount applied to filter disk.

HPLC. Mass spectral analysis indicated that the attractant consisted of two major components.

Chemical Isolation and Identification. Uracil and glycerol were identified as active components of the *M. spicatum*. Monitoring of column effluents by UV absorption led to the isolation of uracil, which has an UV absorption maximum at 259 nm. Glycerol does not absorb in the ultraviolet spectrum; however, its presence in the attractive fraction of the *M. spicatum* exudates was apparent in NMR spectra of the final fraction. Uracil was identified by low-resolution positive ion APCI and by high-resolution chemical ionization mass spectral analysis. Direct infusion APCI of the isolated fraction showed a base peak of *m*/*z* 113.1 $[M + H]^+$, which, upon fragmentation, yielded peaks at 95.9 and 69.9 *m*/*z*. Under high-resolution CI, the molecular ion of 113.0346 $[M + H]^+$ was observed consistent with a molecular formula of $C_4H_4N_2O_2$. Electron ionization MS also showed the fragmentation pattern characteristic of glycerol (61.0, 43.0, and 31.0 *m*/*z* values).

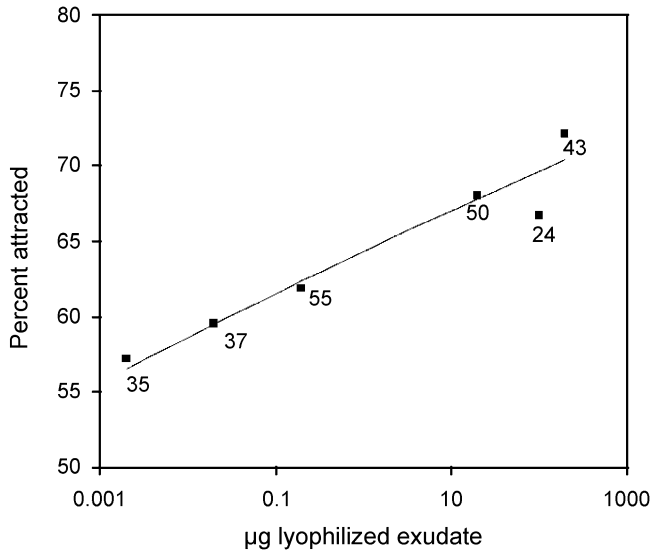

FIG. 2. Weevil response to increasing amounts of *M. spicatum* exudates. Exudates from *M. spicatum* were lyophilized, weighed, and resuspended in water. An exudate amount (amt) of 0.002–200 µg was infiltrated into filter paper discs. The estimated concentrations of exudates in one arm of the Y-tube ranged from 0.00017 to 17 mg/l. The Y-tube bioassays were performed as described in Methods and Materials. The number of weevils used in each sample is indicated next to each point. The logistic regression line is defined as percent attracted $= 1/\left(1 + e^{-(0.5890 + 0.1207 \times \log_{10}(\text{amt}))}\right)$ and is significant as a first-order curve ($P = 0.10$).

The presence of uracil and glycerol was confirmed by NMR spectroscopy. ^{1}H-NMR spectra showed an aromatic region with peaks at 5.4 and 7.4 ppm corresponding to uracil and a multiplet at 3.6 ppm and two doublet of doublets at 3.4 and 3.5 ppm corresponding to glycerol. The ^{13}C-NMR spectra revealed peaks at 165.5, 153.4, 143.7, and 101.2 ppm corresponding to uracil and 72.2 and 62.6 ppm corresponding to glycerol. The COSY ^{1}H-NMR spectra showed that the aromatic protons coupled at 5.6 and 7.4 ppm were consistent with the spectral properties of uracil. The MQ COSY spectra confirmed the coupling of the 3.4- and the 3.5-ppm doublet of doublets with each other and the multiplet consistent with the spectrum of glycerol. HMQC and HMBC spectra permitted the correlation of protons and carbons. The identities of uracil and glycerol were confirmed by comparisons to standards. A third compound was also present in some samples, but could not be separated from other components or identified.

The presence of uracil in the exudates of *M. spicatum*, *M. sibiricum*, *M. alterniflorum*, *M. tenellum*, and *C. demersum* was determined using a Qtrap LC-MS. Uracil was present in the exudates of all of the aquatic plants tested (Figure 1).

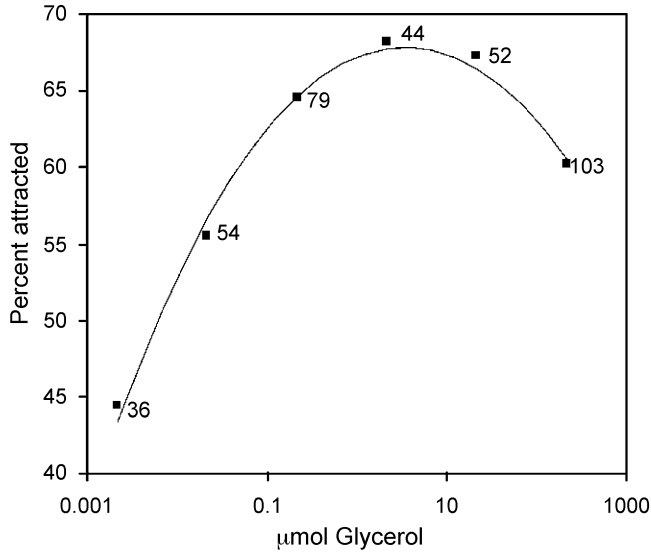

FIG. 3. Weevil response to an increasing amount of glycerol. An aliquot of glycerol was infiltrated into filter paper discs for a final amount (amt) of 0.00217–217 μmol glycerol. The estimated concentration in one arm of the Y-tube is 0.00018–18 mM glycerol. The Y-tube bioassays were performed as described in Methods and Materials. The number of weevils used in each sample is indicated next to each point. The logistic regression line is defined as percent attracted $= 1/(1 + e^{-(0.7180 + 0.1061 \times \log_{10}(\text{amt}) - 0.988 \times \log_{10}(\text{amt}) \times \log_{10}(\text{amt}))})$ and is significant as a second-order curve ($P = 0.026$).

Concentration of uracil was higher in *M. spicatum* than in *M. sibiricum* or the *M. spicatum* × *M. sibiricum* hybrid (ANOVA, $F_{2,10} = 11.63$, $P = 0.004$).

Weevils were attracted to standards of glycerol ($P < 0.001$), uracil ($P < 0.001$), and the combination of glycerol + uracil ($P < 0.001$; Table 2). Glycerol was preferred to *C. demersum* exudates ($P = 0.005$). Weevils were attracted to glycerol + uracil (70%) at a percentage intermediate to the levels of attraction to uracil or glycerol. They were not attracted to a characteristic milfoil polyphenol, tellimagrandin II (55%, $P = 0.32$; Table 2; Gross et al., 1996).

A dose response curve was determined for exudates, glycerol, and uracil. Weevil preference for exudates increased as the amount of exudates used in each test increased (Figure 2); a similar response was seen for glycerol (Figure 3) and uracil (Figure 4). A minimum amount of 20 μg significantly attracted *E. lecontei* to exudates vs. a control; 68% of weevils selected the exudates (Figure 2). Based on extrapolation of the regression curve, the minimum threshold that attracts weevils is probably closer to 2 μg (0.17 mg/l), which would attract 65% of the weevils. The minimum dose of 0.217 μmol (0.018 mM) significantly attracted *E. lecontei* to glycerol vs. a control; 64% of the weevils were attracted

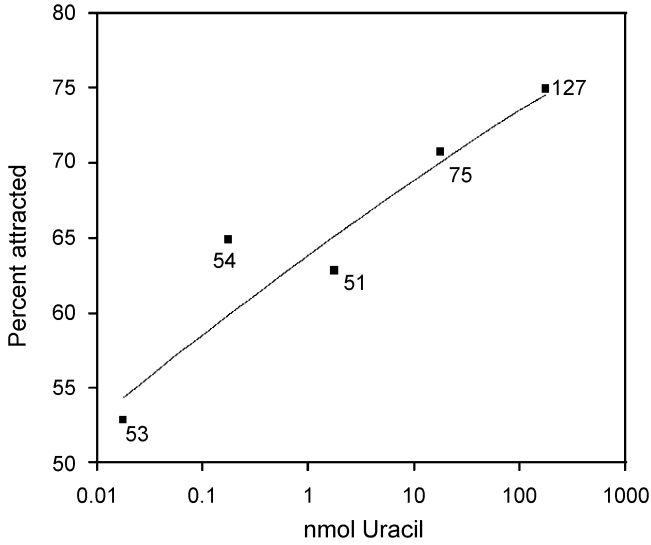

FIG. 4. Weevil response to increasing amount of uracil. An aliquot of uracil was infiltrated into filter paper discs for a final amount (amt) of 0.0178–178 nmol. The estimated concentration in one arm of the Y-tube is 0.0015–15 μM. The Y-tube bioassays were performed as described in Methods and Materials. The number of weevils tested in each sample is indicated next to each point. The logistic regression line is defined as percent attracted $= 1/\left(1 + e^{-(0.5675 + 0.2254 \times \log_{10}(\text{amt}))}\right)$ and is significant as a first-order curve ($P = 0.003$).

(Figure 3). At 217 μmol glycerol (18 mM), the highest amount of glycerol tested, there was a decrease in weevil preference. Sixty-five percent of *E. lecontei* were attracted to uracil at a minimum amount of 0.178 nmol uracil (0.015 μM; Figure 4).

The slopes of the curves in Figures 2–4 can be used to indicate whether the weevil is responding differently to increasing doses of the different chemicals. Glycerol and uracil curves are significantly different (χ^2 goodness of fit test, $\chi^2 = 12.1$, $df = 2$, $P = 0.002$), but the curve of the exudates is not significantly different from that of either uracil or glycerol. This suggests that the exudates attract the weevil at a level between that of glycerol and uracil.

DISCUSSION

This study together with the previous work by Solarz and Newman (1996) provides evidence that a fully aquatic specialist insect is attracted to plant-released chemicals. Solarz and Newman (1996) demonstrated that *E. lecontei* was attracted to *M. spicatum* in the dark as well as in the light. Using bioassay-driven fractionation, we identified uracil and glycerol as two compounds released from *M. spicatum* that attract *E. lecontei*. Although herbivore attraction to plant-released chemicals is a well-known phenomenon in terrestrial systems, this interaction has not previously been shown for a submersed freshwater plant and insect herbivore.

In two-way choice tests, *E. lecontei* preferred *M. spicatum* to a plastic plant (control), its native host *M. sibiricum*, and the co-occurring unrelated macrophyte *C. demersum*. Although historically associated with *M. sibiricum* (Creed and Sheldon, 1994), the weevils used in this experiment did not prefer *M. sibiricum* or its exudates to either a control or to *C. demersum*. This lack of preference for *M. sibiricum* may be a response to the host plant on which weevils developed (collected from *M. spicatum* at Lake Auburn). Solarz and Newman (2001) found that larvae reared on or adults exposed to *M. spicatum* preferred *M. spicatum*, but larvae reared on *M. sibiricum* had no preference. Alternatively, *M. sibiricum* and *E. lecontei* are presumed to have coevolved, and, therefore, *M. sibiricum* may exude deterrents or lack a specific attractant. The weevil's response to one co-occurring species, *C. demersum*, and three additional milfoils, the *M. spicatum* × *M. sibiricum* hybrid, the morphologically similar *M. alterniflorum*, and the morphologically dissimilar *M. tenellum*, all of which have some uracil in their exudates, was variable. Only the *M. spicatum* × *M. sibiricum* hybrid attracted weevils.

Attraction of *E. lecontei* adults to *M. spicatum* is a result of chemical compounds in exudates that were released by *M. spicatum* (Table 1B). *Myriophyllum* spp. release chemicals, including sugars, proteins, cyanogens, tannic substances,

and polyphenols, into the water (Lyr and Streitberg, 1955; Godmaire and Nalewajko, 1990; Nakai et al., 2000; Gross, 2003). By testing *M. spicatum* exudates, we avoided weevil exposure to chemicals that may be present in the plant tissue, but are not released into the water (Hay et al., 1998).

Like terrestrial plants that release volatiles as a result of the oxidative degradation of leaf lipids (Bernays and Chapman, 1994; Paré and Tumlinson, 1999), aquatic plants release hydrophilic metabolites as a by-product of rapid growth (Nalewajko and Godmaire, 1996). Peptides with water-soluble groups were suggested by Decho et al. (1998) to be good attractants in aquatic systems. Glycerol and uracil are two ubiquitous, low-molecular weight compounds that fit this profile.

Glycerol is a common metabolite and can also protect against abiotic stresses. The identification of glycerol-specific transporters in cell membranes of *Arabidopsis thaliana* indicates that it plays a role as a general osmolyte (Weig and Jakob, 2000; Eastmond, 2004). Glycerol content in the leaves of several agriculturally important plants such as maize and alfalfa typically ranges from roughly 0.1–0.4 mM (10–39 μg/g wet weight) to as high as 6 mM (554 μg/g wet weight) in *Zea mays* after a rain event, which may increase the demand for intracellular osmolytes (Gerber et al., 1988). Carbohydrates are also found on leaf surfaces of terrestrial plants at concentrations up to 0.01 mM at which point they act as feeding stimulants for insects (Bernays and Chapman, 1994). Carbohydrates located on leaf surfaces of aquatic plants will dissolve in water and may then function as foraging kairomones that attract herbivores (Ruther et al., 2002). Glycerol produced by *M. spicatum* attracts *E. lecontei* at 0.018- to 1.8-mM range, which is similar to the range that attracts insects to similar compounds in terrestrial systems (Bernays and Chapman, 1994).

Uracil is effective at a much lower concentration. Weevils were significantly attracted to uracil at 0.015–15 μM. At these concentrations, *E. lecontei* adults are responding at a concentration more indicative of a plant-specific attractant than a nutrient. For example, the terrestrial weevil *Ceutorhynchus assimilis* adults were attracted to a 120 μM phenylacetonitrile, a metabolite common in their host plant *Brassica napus* (Bartlet et al., 1997). In marine systems, shrimp *Palaemonetes pugio* were attracted to amino acids and nucleotides at a concentration of 0.1–1000 μM, a range that Carr (1988) suggests is intermediate between background noise and the concentration in prey tissue. Although it is not a species-specific attractant, the presence of uracil in the water may indicate either a damaged plant or an actively growing one suitable to *E. lecontei* for feeding and oviposition.

Like glycerol, uracil is also ubiquitous in plants. We found it in exudates of all plants tested, including all *Myriophyllum* spp. and *C. demersum*. However, its presence in exudates raises the question as to why a plant would release an expensive metabolite. In plants, uridine nucleosides and nucleotides are the second most common in cells after adenosine. In *Arabidopsis*, ureide permease transporters have been identified as the main transporters of uracil in phloem

(Schmidt et al., 2004). Uracil in exudates may be a function of its abundance and ubiquity in rapidly growing cells, such as the apical meristems, where pyrimidine salvage is needed to support the high demand for nucleotides. Of the pyrimidines, plants can only salvage uracil (Wagner and Backer, 1992).

An alternative use for uracil in plants is the production of alkaloids and pseudoalkaloids (*sensu* Waterman, 1998). Plants that use uracil in this capacity have pools of the pyrimidine for rapid synthesis (Brown and Turan, 1995). Ostrofsky and Zettler (1986) reported that alkaloids were present in *M. spicatum*. However, no alkaloids or pseudoalkaloids were detected in our collections (Stermitz, Colorado State University, unpublished data, see Marko and Stermitz, 1997 for methods), suggesting that uracil-based natural products are not produced in these aquatic plants, or that they are found in low concentrations.

The concentration of uracil was highest in *M. spicatum* exudates, intermediate in *M. spicatum* × *M. sibiricum* hybrid, and lowest in *M. sibiricum*. *M. spicatum* grows faster than *M. sibiricum* and starts growing earlier in the season (Madsen et al., 1991). Therefore, it would produce and release glycerol and uracil sooner than other co-occurring plants.

The presence of uracil in aquatic plant exudates may also be a result of damage because of abiotic stressors such as UV radiation that can damage nucleic acids. The sun-exposed meristem tips of *M. spicatum* form dense mats at the water surface. This greater surface exposure may lead to more UV stress than that experienced by other milfoils located below the surface, potentially resulting in the loss of cell contents, including uracil. Ultraviolet radiation can also induce the production of polyphenols in aquatic plants (Meijkamp et al., 1999; Rozema et al., 1999). The higher concentration of polyphenols found in *M. spicatum* compared to *M. sibiricum* (Spencer and Ksander, 1999; Marko et al., unpublished results) suggests that it is under greater UV stress.

Polyphenols in *M. spicatum* deter feeding by herbivores (Leu et al., 2002; Li et al., 2004). Li et al. (2004) found that *M. spicatum* had higher phenolic content than co-occurring aquatic plants, and that in two-way choice tests, the snail *Radix swinhoei* ate less *M. spicatum* than five of the seven tested plants. One polyphenol in *M. spicatum*, tellimagrandin II, is known to inhibit the growth of periphyton and deter the growth of the lepidopteran herbivore *Acentria ephemerella* (Gross et al., 1996; Choi et al., 2002). However, tellimagrandin II was neither attractive nor deterrent to *E. lecontei* at the concentrations tested. This suggests that the weevil does not respond to all *M. spicatum*-released chemicals, but rather that it is specifically attracted to glycerol and uracil.

Host plant attraction often involves a mixture of chemicals, and the effective concentration of one attractant can be modified by a specific mixture (Roseland et al., 1992; Bartlet et al., 1997). For example, *C. assimilis* adults were more attracted to phenylacetonitrile plus a mixture of isothiocyanates than to phenylacetonitrile alone (Bartlet et al., 1997). Synergism between glycerol

and uracil was not detected in the *M. spicatum–E. lecontei* system. Similar percentages of *E. lecontei* adults were attracted to *M. spicatum* exudates, uracil, and a combined fraction of uracil + glycerol.

In both aquatic and terrestrial systems, there is considerable variation in herbivore response to chemical attractants, and sometimes, it is the presence of a trace chemical that drives the plant–insect interaction. For example, in terrestrial systems, *Hylobius pales* (Curculionidae) adults show a dose-dependent response to some chemicals, but not others (Salom et al., 1994). Furthermore, weevil response can depend on whether the study is conducted in the field or in the lab. In field studies, *C. assimilis* exhibited a dose response to known attractants (Smart et al., 1997), but not under laboratory conditions (Bartlet et al., 1997). Bartlet et al. (1997) suggest that the ratio of chemicals and the presence of trace compounds may ultimately affect behavior in the field. Variation in *E. lecontei* behavior to *M. spicatum* populations was observed throughout these experiments. Certain populations of *M. spicatum* did not attract the weevils, and environmental variables such as weather also affected weevil response. Because *M. spicatum* is a nonindigenous plant, it may release chemicals that attract *E. lecontei*, whereas native milfoils may have been selected to release fewer attractant chemicals. Undoubtedly, the attraction of *E. lecontei* to *M. spicatum* is a complex process that involves a variety of factors.

In terrestrial systems, ubiquitous plant volatiles, such as phenylpropanoids, fatty acid derivatives, and isoprenoids, are involved in the attraction and deterrence of insects to their host plants (Bruce et al., 2005). In aquatic systems, common low-molecular weight compounds, such as amino acids, sugars, and nucleotides, perform the same function (Weissburg et al., 2002). These water-soluble compounds may be reversibly bound to leaf surfaces or released from trichomes. Aquatic herbivores probably rely on taste receptors rather than odor for host location (Spänhoff et al., 2005). Additionally, because many of these chemicals are readily taken up by bacteria, they may act as short-term signals. Alternatively, compounds released by plants may be degraded by epiphytes, and the degradation products may serve as semiochemicals. Comparisons of chemical mobility and persistence in different environments will provide an understanding of the ecological and evolutionary relationships among aquatic insects and their host plants.

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UNRAVELING THE NATURE OF INDIVIDUAL RECOGNITION BY ODOR IN HERMIT CRABS

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Abstract—Individual recognition is a key element in the social life of many invertebrates. However, most studies conducted so far document that several species are capable of a "binary" discrimination among conspecifics, but not of a "true individual recognition." Our objective was to learn more about the mechanisms that underlie individual recognition by odor in hermit crabs by individuating some of its properties. Using *Pagurus longicarpus* Say 1817 as a model species, we conducted four series of experiments in which the response of every test crab (the "receiver") to the different odor treatments (emitted by a "sender") was evaluated from its investigative behavior toward an empty, high-quality shell. After having excluded the possibility that crabs chemically recognize familiar/unfamiliar shells and/or shells of high/low quality, we explored whether the receivers discriminate odors from two familiar senders and whether this discrimination also occurs with unfamiliar crabs. We also asked whether crabs form an association between the odor of a familiar sender and some of its relevant attributes, i.e., rank, size, and shell quality. Finally, the shells inhabited by familiar individuals were manipulated to modify the association between odor and shell quality. Results showed that: (1) there is no odor specific of a rank; (2) individual crabs discriminate their own odor from the odor of other individuals; (3) they can chemically discriminate between larger crabs inhabiting higher-quality shells and smaller crabs inhabiting lower-quality shells, provided that these crabs are familiar to them; (4) they associate the odor of an individual crab with the quality of the shell it inhabits; and (5) this association quickly changes when social partners switch to shells of different quality. These results indicate that the nature of chemical recognition in *P. longicarpus* is more refined than a simple binary

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system. The receiver appears able to associate a type of information from the sender with memories of past experiences, therefore suggesting the hermit crab's potential for relatively high-order knowledge about conspecifics.

Key Words—Individual recognition, odors, dominance hierarchies, hermit crabs, *Pagurus longicarpus*.

INTRODUCTION

Individual recognition is a key element in the social life of many organisms, where it can play an essential role in the structure and stability of a number of behavioral networks, such as dominance hierarchies, territorial defense, competitive aggression, pair bonds, mate selection, and kin favoritism (reviewed in Zayan, 1994). The most detailed information available in the literature concerns the recognition of vocal signals by birds (e.g., Falls, 1982). Many studies have demonstrated the ability of nonhuman mammals to discriminate individuals by the use of chemicals (e.g., Halpin, 1980, 1986; Brown et al., 1990; Hurst et al., 2001). Particularly in the last few years, considerable effort has been directed at defining in vertebrates the processes of identification and recognition (Beecher, 1989): at the cognitive analysis level, questions have been addressed on the nature of individual representation exhibited by a handful of vertebrate taxa and on the evolutionary pathways leading to high-order knowledge about individuals (e.g., Johnston and Bullock, 2001).

To date, a relatively small body of literature exists that analyzes these issues in invertebrates (see, e.g., Leonard et al., 1974 in *Drosophila* spp.; Barrows et al., 1975 in halictid sweat bees *Lasioglossum zephyrum*; Liechti and Bell, 1975 in the cockroach *Byrsotria fumigata*; Linsenmair and Linsenmair, 1971 in the desert wood louse *Hemilepistus reaumuri*). Even fewer studies exist that have advocated pheromones as the basis of individual recognition in aquatic invertebrate species (Wickler and Seibt, 1970 in the clown shrimp *Hymenocera picta*; Johnson, 1977 in the banded shrimp *Stenopus hispidus*; Caldwell, 1985 in the mantis shrimp *Gonodactylus festae*; Karavanich and Atema, 1998 in the American lobster *Homarus americanus*).

Among other aquatic invertebrates, hermit crabs are optimal model organisms to investigate the mechanisms of chemically mediated individual recognition. The ability to recognize individuals in *Pagurus bernhardus* (Hazlett, 1969) or to discriminate familiar from unfamiliar conspecifics in *P. longicarpus* (Gherardi and Tiedemann, 2004a) was assumed to be a means to maintain stable hierarchical relationships. In fact, dominance hierarchies (Allee and Douglis, 1945; Winston and Jacobson, 1978) seem not to be laboratory artifacts, but they may develop in the small temporary aggregations of hermit

crabs that often form in tide pools (Scully, 1978), mostly around gastropod predation sites (Rittschof, 1980a).

A number of behavioral studies have shown that olfaction in hermit crabs can mediate a form of chemical recognition. Several species display adaptive behaviors when exposed to odors that signal shell availability (e.g., Rittschof, 1980a; Rittschof et al., 1992; Rittschof and Hazlett, 1997; Gherardi and Atema, 2005a), and chemical cues in the medium affect investigatory responses toward shells occupied by conspecifics (Hazlett, 1996a,b; Rittschof and Hazlett, 1997; Hazlett, 2000). Recently, Gherardi and Tiedemann (2004b) showed that *P. longicarpus* spends more time investigating an empty shell in the presence of odors released by unfamiliar, rather than familiar, conspecifics.

Based on these premises, our objective here was to learn more about the mechanisms that underlie chemical individual recognition in hermit crabs by using *P. longicarpus* as a study species. After having excluded the ability of crabs to recognize shells by odor, we explored whether they discriminate between two familiar individuals of different rank, size, and shell quality and whether this ability is expressed also toward unfamiliar crabs. Experiments have documented a binary discrimination between familiar and unfamiliar individuals, but have failed in demonstrating a recognition of one out of many, known individuals (Gherardi and Tiedemann, 2004a), even if provided suggestions of its potential (Gherardi and Atema, 2005b). Our experimental design here showed that the number of the individuals *P. longicarpus* can recognize is wider than previously thought. Then, two general questions were raised. The first was whether hermit crabs form an association between the odor of a familiar conspecific and one of its relevant attributes (rank, size, or shell quality). In other words, what does the odor of a crab mean to another individual? Finally, because the odor appeared to be associated with the high/low quality of the shell occupied by the "sender" crab (here defined as the crab releasing the odor, without any implication of signal selection for communication), we investigated the plasticity of this association by experimentally altering shell quality.

METHODS AND MATERIALS

Subjects, Collection, and Housing Conditions. The long-clawed hermit crab, *P. longicarpus* Say 1817, is common in shallow waters along the western Atlantic coasts of North America, from Nova Scotia south to eastern Florida, and in the northern Gulf of Mexico from the west coast of Florida to Texas (Williams, 1984).

Between July and August 2003, we hand-collected around 400 hermit crabs with the major chela width (CW) of 0.1–0.4 mm (corresponding to individuals

with shield length of about 4–6 mm) from Little Sippewissett salt marsh (Massachusetts, USA) during diurnal low tides. Immediately after capture, crabs were separated into small groups and transferred to the Marine Biological Laboratory in Woods Hole, where they were maintained in groups of up to 25 individuals in a temperature-controlled room (22°C) and under a natural 14-h light–10-h dark cycle. They were kept in separate 20-l holding aquaria containing constantly aerated seawater and fed with a diet of commercial shrimp pellets every third day. Water was changed weekly. After being used in experiments, crabs were released at the collection site.

Experimental Design. We conducted four series (A–D) of one to three related experiments (conditions). Each condition consisted of a 24-hr familiarization phase immediately followed by a test phase in which every test crab (the "receiver") was subjected to two to three different odor treatments in a random sequence. A preliminary experiment (experiment A) aimed at exploring whether hermit crabs chemically recognize familiar/unfamiliar shells (condition A1) and/or shells of high/low quality (condition A2). Then, we aimed at understanding in Experiment B whether crabs discriminate between two familiar conspecifics of different rank, size, and shell quality (condition B1) and whether this discrimination occurs also toward unfamiliar conspecifics with similar attributes (condition B2). In experiment C (with conditions C1, C2, and C3), we asked whether receiver crabs form an association between this odor and relevant attributes of the sender, i.e., its rank, size, and shell quality. Finally, experiment D was a continuation of C3, in which the shell inhabited by the sender was manipulated to modify the association between odor and shell quality. The logic behind our study is shown in the flowchart of Figure 1, and details of each experiment are given in Table 1.

Experimental Methods. All experiments and conditions were staged in opaque plastic bowls (10-cm diam), containing 160-ml unfiltered seawater at 22°C, illuminated during observations by a 75-W incandescent light, 50 cm above the water level. Observations were always conducted between 0900 and 1600 hr.

At least 10 d from the collection and 2 d before the beginning of experiments A–C, each crab was randomly assigned to one of the two or three conditions. We always used intact individuals (no missing limbs), and, for experiments B and C, we formed groups composed of three crabs ("trios"), using animals taken from separate holding aquaria to ensure that they had no memory of one another (in fact, crabs forget a familiar conspecific after 5 d of separation; Gherardi and Atema, 2005b). According to the major chela width, crab size was categorized as large, L (CW > 0.33 mm), medium, M (CW 0.23–0.33 mm), or small, S (CW < 0.23 mm). Sex was not noted because sex has been shown to exert no effect on agonistic interactions in this and other hermit crab species (Hazlett, 1966; Winston and Jacobson, 1978), at least during the

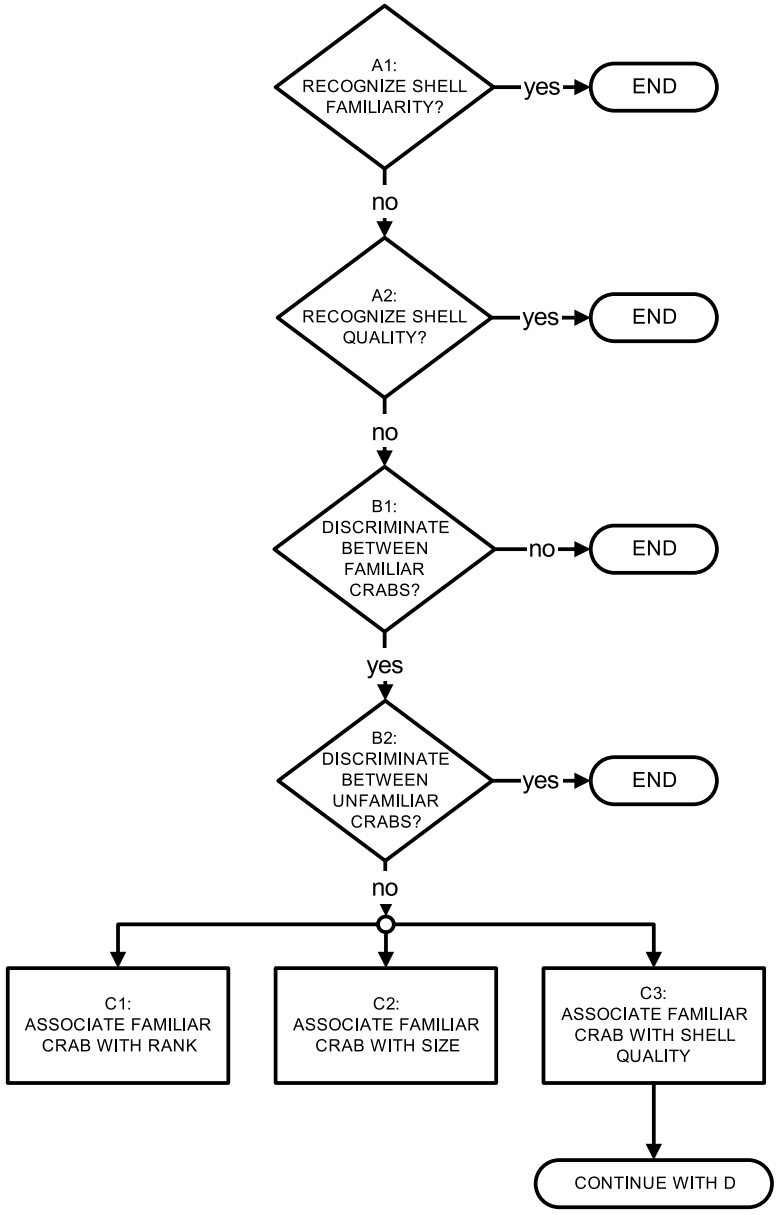

FIG. 1. A flowchart showing the sequence of questions addressed in this study. A1, A2, etc. denote experiments/conditions. Experiment D consisted of altering the association between familiar crab and shell quality.

TABLE 1. DETAILS OF THE EXPERIMENTS

Experiment/ condition	Test for	Receiver's attributes	Sender's attributes (A: shells; B–D: crabs)					
			Familiarity	Crab rank	Crab size	Shell size	Shell quality for the receiver	*n*
A1	Shell odor	Any crab	Yes/no				LTO, LTO	18
A2	Shell odor	Any crab	Yes				STO, LTO	17
B1	Familiarity	β (rank/size/shell quality)	Yes	α, γ	L, S	L, S	LTO, STO	26
B2	Familiarity	β (rank/size/shell quality)	No	α, γ	L, S	L, S	LTO, STO	21
C1	Rank	β (rank)	Yes	α, γ	M, M	M, M	OPT, OPT	15
C2	Size/rank	β (size/rank)	Yes	α, γ	L, S	M, M	OPT, OPT	13
C3	Shell quality/rank	β (shell quality/rank)	Yes	α, γ	M, M	L, S	LTO, STO	31
D	Plasticity	β (shell quality/rank)	Yes	α, γ*	M, M		STO, LTO	11

Experiment A evaluated the effect exerted on receiver crabs by the odor of shells with different familiarity and quality, and experiments B–D by the odor of crabs with different attributes (familiarity, dominance rank, size, and shell quality). The effect of the sender's odor was assessed from the investigatory behavior on a target shell performed by receiver crabs (any crab in experiment A and β crabs in experiments B–D). All the receivers were medium in their size and occupied medium-sized shells that were optimal for them. Asterisk denotes that individuals in D were the same individuals used in C3 but in shells of opposed quality, i.e., α and γ crabs of C3 occupying a STO and a LTO shell, respectively.
OPT: optimal shell size; STO: smaller-than-optimal shell size; LTO: larger-than-optimal shell size; L: large crab size and shell size; M: medium crab size and shell size; S: small crab size and shell size; α, β, and γ: dominance rank; *n*: sample size.

nonreproductive period (this species reproduces between October and May with a peak in autumn; Wilber, 1989). When crabs were tested in groups (experiments B, C, and D), the shells of every trio were marked by 0, 1, or 2 dots of permanent black ink to permit their identification. Individual hermit crabs were recognized by the length of their antennae and by slight differences in cheliped and pereopod color.

To make test animals as homogeneous as possible (for experiment A) and to obtain individuals that were similar (in C1 and C3) or different (in experiment B and in C2) for the quality of the occupied shell, crabs were extracted from their original shell by gently breaking it with a vise. Then, they were allowed to enter a new shell from a collection of five empty, undamaged, similarly sized shells that were prepared by collecting live periwinkle *Littorina littorea* (the dominant shell type used by the study population), boiling and removing the flesh, rinsing the shells several times in seawater, and air-drying them to remove odors of previous occupants (snail, epiphytes, etc.). Crabs were allowed 48-hr access to new shells, at which time they had ceased exploring and moving into new shells.

The size of the offered shells were changed in each experiment depending on the experimental protocol (see Table 1 and below). Shells were classified according to their length (measured from the base–apex axis in millimeters, SL) and to their adequacy for the body size of the inhabiting crabs. In fact, although hermit crabs can have preferences for various characteristics of shells, particularly in the case of the study species (Wilber, 1990), size is the most important determinant of shell selection.

Shells were categorized as L (SL > 18 mm), M (SL: 15–18 mm), and S (SL < 15 mm). The size of the optimal shell for a crab of a given size (OPT) was computed by regressing the equation $y = 37.9x + 7.3$, where y is SL and x is crab size (CW, in millimeters). This equation was obtained from a preliminary free-choice experiment in which every crab (of a total of 192) was allowed to choose among five empty shells of different size. Then, we defined as "larger-than-optimal" (LTO; and "smaller-than-optimal," STO) for a crab of a given size a shell whose size was about 10% longer (and shorter) than the size of the shell optimal for it.

Behavioral Assay: Crab Responses to a High-Quality Shell. The behavioral assay we used in the test phase was the investigative behavior shown by hermit crabs toward a novel shell that was about 10% longer than the optimal shell for the test crab's size (Gherardi and Tiedemann, 2004b). Notwithstanding its relatively large volume and heavy weight, this shell is highly attractive to *P. longicarpus*, as shown in a previous study (Gherardi, 2005). The cost of wearing a too large shell (e.g., the energetic costs of locomotion) seems to be overweighed in this species by a number of possible benefits. For instance, by accepting or even selecting an oversized shell, crabs may delay the need to find

larger shells to assure their growth and reproduction and gain some fighting advantages (Gherardi, 2005).

Tests were run in bowls containing 160-ml seawater that had been conditioned for an hour with the odor released by either shells (experiment A) or conspecifics (experiments B, C, and D). The experimental bowl was provided with an empty periwinkle shell placed with its apex upward, functioning as the target shell. This shell was prepared as described above for shell choice experiments; however, here, its aperture was blocked with a resin to avoid its occupation by the crab. Preliminary observations had shown that the resin and its odor had no effect on shell attractiveness.

Tests started by placing an individual (the receiver crab) into the bowl about 8 cm from the shell. Each receiver was subjected to two or three subsequent odor treatments, the sequence of which was varied systematically per crab. All hermit crabs of experiment A were used as receivers. In the other experiments in which we worked with trios, tests were conducted only on those crabs that had an intermediate score (hereafter defined β crabs) for dominance rank in experiments B and C1, for size (and rank, see below) in C2, and for shell quality (and rank, see below) in C3 and D.

For every odor treatment, the events occurring during 5-min observation and time were recorded on a voice tape and later analyzed to obtain: (1) latency in seconds (time until first shell investigation; when the test crab never investigated the shell, we arbitrarily assigned a time equal to 305 sec); (2) number of bouts of shell investigation; (3) total duration of shell investigation in seconds; and (4) total time spent in locomotion in seconds.

Experiment A: Recognizing the Odor of Empty Shells. Details for experiment A are given in Table 1. During shell choice, crabs were offered with shells optimal for their size. Then, they were kept isolated for 24 hr in bowls to become familiar with either one (LTO, A1) or two (one LTO and one STO, A2) empty, resin-blocked periwinkle shells. Treatments in the test phase were the odor from the familiar shell, as opposed to the odor from a novel shell (A1), and the odor from the LTO shell, as opposed to the odor from the STO shell (A2).

Experiment B: Discriminating the Odor between Familiar/Unfamiliar Conspecifics with Different Attributes. In this experiment, we investigated whether hermit crabs were able to discriminate by odor between two familiar (B1) or two unfamiliar (B2) conspecifics with different rank, size, and shell quality. Our purpose was to understand if crabs could chemically recognize more than one familiar individual while excluding the effects of rank odor. Trios were composed of α (large body size and large shells, OPT for the senders but LTO for the receivers), β (medium body size and medium shells, OPT for them), and γ (small body size and small shells, OPT for the senders but STO for the receivers) crabs. Other details are given in Table 1.

Familiarization started by placing three crabs in the experimental bowl and lasted 24 hr. The day after, we checked for shell switches that might have occurred overnight (none), and then we recorded the events taking place during 10-min observation; from these records, we evaluated the agonistic level of every trio from the number and the duration of fights. We defined as fights those interactions that started when one crab approached one or two rivals and ended when one or two opponents retreated to a distance greater than 3 cm and for at least 10 sec.

The dominance rank of an individual was defined from the relative number of wins. The winner was the opponent that did not retreat at the end of the interaction or that retreated after the other(s) withdrew into the shell. In the few instances in which the three crabs won the same number of interactions, we defined the ranks from the intensity of crab locomotion (i.e., the time spent exploring the experimental bowl). We never observed dominance reversal during any 10-min observation. Based on Winston and Jacobson's (1978) data, 24 hr were sufficient for the formation of a dominance hierarchy. In all groups analyzed, crabs that were classified as α, β, and γ for size and shell quality were also α, β, and γ, respectively, for rank. In the test phase, β crab behavior was analyzed in the presence of the odor released (1) by themselves (in both B1 and B2) or (2) by familiar (in B1) or unfamiliar (in B2) α and γ crabs.

Experiment C: Associating the Odor of Familiar Conspecifics with One Attribute. Because crabs seemed to discriminate between odors emitted by α and γ familiar conspecifics with different attributes, our aim was to investigate whether hermit crabs can associate the odor of the sender with one of its attributes. To this end, we analyzed crab behavior in the presence of odors from familiar conspecifics that differed for a single attribute (size in C2 and shell quality in C3), the other being equal. The results obtained from C2 and C3 were compared with C1, in which crabs differed for rank but had equal size and shell quality.

The familiarization phase was conducted as in experiment B. With only one exception in C3, crabs intermediate by size and by shell quality (i.e., β crabs in C2 and C3, respectively) were also β by rank. In the test phase, β crabs (the receivers) were presented with the odor of familiar α and γ crabs, which had the same size and shell quality as the receiver in C1, a different size (being either larger or smaller) but the same shell quality in C2, and the same size but either a LTO or a STO shell in C3 (see Table 1 for details).

Experiment D: Plasticity of the Association between Individual Odor and Shell Quality. We tested if crabs that had formed an association between the odor of a familiar conspecific and its shell quality would form a new association when the conspecific switched to a new shell of a different quality. In this experiment, we therefore investigated crab behavior when the familiar con-

specifics had been forced to occupy shells of poorer quality with respect to the shells they occupied previously.

We analyzed the results of 11 out of the 31 trios of C3, since five crabs died overnight and 15 shell switches occurred. Immediately after the test phase of C3, the shells occupied by α and γ crabs were gently broken with a vise, and crabs were forced to enter a novel shell that was STO for the former α crab and LTO for the former γ crab. After an hour of separation (not sufficient to forget former opponents; Gherardi and Atema, 2005b), we reconstituted the trios with the same individuals as in C3 but now with α in a low-quality shell and γ in a high-quality shell (see Table 1 for details). The new trios were then subjected to the familiarization phase and to the test phase as in experiment C.

Data Analyses. We applied nonparametric tests (Sokal and Rohlf, 1969; Siegel and Castellan, 1988) because the assumptions of normality of data and homogeneity of variance were not always met, and some measures taken represented ordinal data. We used Mann–Whitney *U* tests (statistics: *U* and *z* for samples >20) and Kruskal–Wallis one-way analyses of variance (statistic: *H*) to examine differences in the agonistic level reached by trios during the familiarization phase between/among the conditions of experiments B and C, respectively. We compared conditions for latency and for the other measures taken during the test phase using Wilcoxon matched-pairs signed-ranks tests (statistics: *T* and *z* for samples >25; experiments A, B, and D) and Friedman two-way analyses of variance (statistic: Fr; experiment C). When the null hypothesis was rejected after Friedman two-way analysis of variance, a multiple comparisons test (Siegel and Castellan, 1988) was used to determine which pairs of samples differed significantly. The test takes into account the correction of the α level for multiple comparisons (Siegel and Castellan, 1988). Text and figures provide medians and interquartile ranges (first–third quartiles). *P* values of less than 0.05 were considered statistically significant.

RESULTS

Experiment A: Recognizing the Odor of Empty Shells. Crab behavior in the test phase did not differ when water was conditioned with odor from either familiar or unfamiliar shells (A1) or from familiar shells of LTO/STO quality (A2; Table 2). Thus, crabs did not recognize familiar or high-quality shell attributes alone.

Experiment B: Discriminating Odor between Familiar/Unfamiliar Conspecifics with Different Attributes. In the familiarization phase, trios used for B1 and B2 displayed the same agonistic level (number of fights: $z = 0.86$, $P = 0.39$; duration of fights: $z = 1.44$, $P = 0.66$). This established that the results obtained

TABLE 2. STATISTICAL OUTPUTS OF EACH EXPERIMENT AND CONDITION FOR LATENCY, BOUTS OF SHELL INVESTIGATION, DURATION OF INVESTIGATION, AND TIME OF LOCOMOTION

Experiment/ condition	Latency		Shell investigation		Duration of investigation		Time of locomotion	
	T/*z*/Fr	*P*	*T*/*z*/Fr	*P*	*T*/*z*/Fr	*P*	*T*/*z*/Fr	*P*
A1	61.5 (17)	>0.05	52.5 (15)	>0.05	72 (18)	>0.05	78 (18)	>0.05
A2	65.5 (16)	>0.05	71 (16)	>0.05	89 (17)	>0.05	68 (17)	>0.05
B1	0.602 (26)	>0.05	8.551 (26)	**<0.02**	10.431 (26)	**<0.01**	0.857 (21)	>0.05
B2	2.571 (21)	>0.05	15.311 (21)	**<0.01**	7.238 (21)	**<0.05**	0.857 (21)	>0.05
C1	60 (15)	>0.05	30 (14)	>0.05	70 (15)	>0.05	44.5 (15)	>0.05
C2	37 (13)	>0.05	13 (10)	>0.05	43 (13)	>0.05	26 (13)	>0.05
C3	−2.2732 (31)	**<0.01**	69 (25)	**<0.02**	−3.5078 (31)	**<0.001**	−1.8100 (30)	>0.05
C3	10 (11)	**<0.05**	0 (7)	**<0.02**	9 (11)	**<0.05**	22 (11)	>0.05
D	7 (10)	**<0.05**	4 (11)	**<0.01**	11 (11)	**<0.05**	27 (10)	>0.05

Sample size is shown in parentheses. The analyses were done by Wilcoxon matched-pairs signed-ranks tests (statistic: *T* and *z* for samples >25) except for B1 and B2, in which we used Friedman two-way analyses of variance (statistic: Fr). Significant differences in bold.

in test phases were not the effect of an inherent between-group difference in crab behavior.

In the test phase of experiment B1, β crabs investigated the target shell more often in the presence of odor from familiar conspecifics than with their own odor (Figure 2B, after multiple comparisons test: α crab odor = γ crab odor > self-odor). Shell investigation was also longer in the presence of α crab odor (Figure 2C, after multiple comparisons test: α odor > γ odor = self; Table 2). Different odors had no apparent effect on latency (Figure 2A) or on the duration of locomotion (Figure 2D). In experiment B2, β crabs discriminated between their own odor and the odor released by conspecifics, but they did not differentiate between unfamiliar α and γ crabs (Figure 2B, after multiple

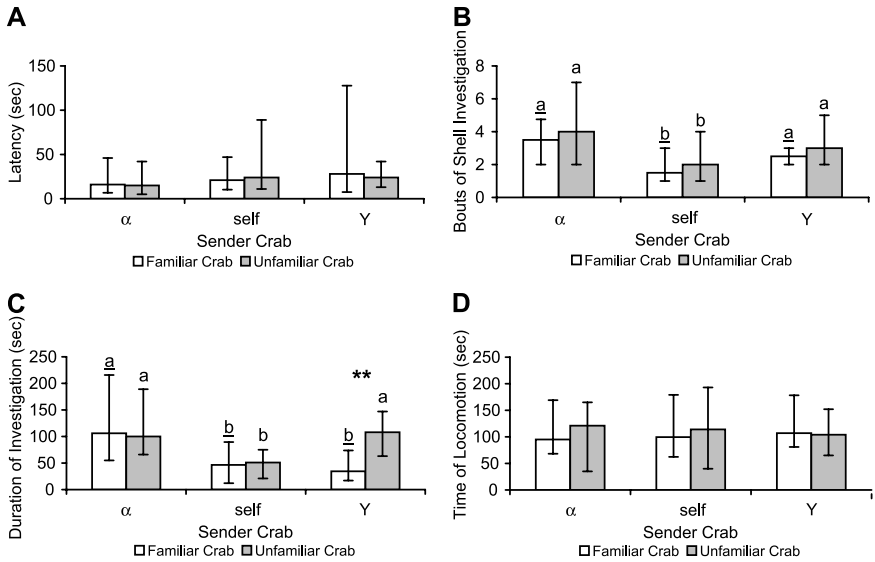

FIG. 2. Results from experiment B: discriminating odor between familiar/unfamiliar conspecifics with different attributes. Median values (and interquartile ranges) of latency (A), bouts of shell investigation (B), duration of shell investigation (C), and time of locomotion (D), compared between familiar (experiment B1) and unfamiliar (experiment B2) crabs, for three odor sources: α crabs, the test crabs (self, β crabs), and γ crabs. Alpha (and γ) crabs were the bigger (and smaller) crabs occupying a larger (and a smaller) shell compared to the β test crabs. See Table 2 for test statistics. Letters above bars (underlined for familiar crabs and not underlined for unfamiliar crabs) indicate differences among odor treatments (multiple comparisons test after Friedman two-way analysis of variance); ** denotes $P < 0.01$.

comparisons test: α odor = γ odor > self-odor; Figure 2C, after multiple comparisons test: α odor = γ odor > self-odor; Table 2).

Experiment C: Associating Odor of Familiar Conspecifics with One of Its Attributes. As in experiment B, trios used in C1, C2, and C3 showed the same agonistic level during the familiarization phase (number of fights: $H = 1.1614$, $df = 2$, $P > 0.05$; $H = 5.3524$, $df = 2$, $P > 0.05$; duration of fights: $H = 1.2573$, $df = 2$, $P > 0.05$; $H = 3.2290$, $df = 2$, $P > 0.05$), thus excluding inherent between-group differences in crab behavior.

In the test phase, crabs reacted in the same fashion to the target shell when the attributes of the senders differed for their rank only (experiment C1) or for their size (and rank) (experiment C2; Figure 3). However, in experiment C3, β

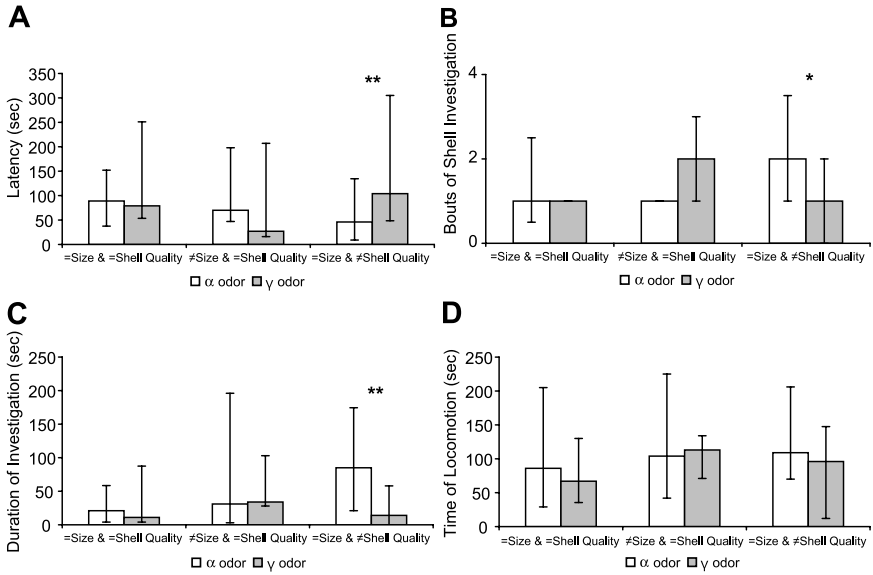

FIG. 3. Results from experiment C: associating odor of familiar conspecifics with one of its attributes. Median values (and interquartile ranges) of latency (A), bouts of shell investigation (B), duration of shell investigation (C), and time of locomotion (D), comparing treatments with odor from α and γ crabs in three conditions (same body size and same shell quality, C1, different body size and same shell quality, C2, and same body size and different shell quality, C3). Alpha (and γ) were the dominant (and subordinate) crabs in condition C1, the bigger (and the smaller) crabs in condition C2, and the crabs occupying a larger-than-optimal (and a smaller-than-optimal) shell in condition C3. See Table 2 for test statistics; * and ** denote $P < 0.05$ and $P < 0.01$, respectively.

crabs showed a quicker response to the target shell (Figure 3A), and they investigated it more often (Figure 3B) and for a longer time (Figure 3C) when the odor was emitted by crabs that occupied LTO (i.e., α crabs), rather than STO (i.e., γ crabs), shells (Table 2). No difference was found for the duration of locomotion (Figure 3D).

Experiment D: Plasticity of the Association Between Individual Odor and Shell Quality. Altering the quality of the shells occupied by α and γ crabs modified the behavior of β crabs. The quality of the shell, but not the identity of the sender crab inhabiting it, affected shell investigation by β crabs. Receivers (β crabs) reacted quicker (Figure 4A), with greater frequency (Figure 4B), and for a longer time (Figure 4C) in the presence of odor from senders in LTO shells regardless of their identity. The duration of locomotion (Figure 4D) did not differ significantly between experiments C3 and D (Table 2).

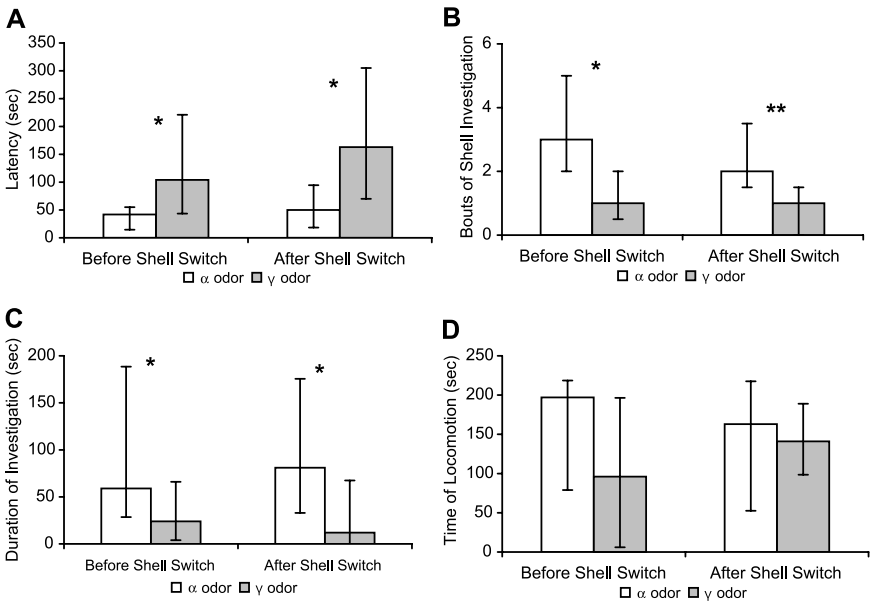

FIG. 4. Results from experiment D: plasticity of the association between individual odor and shell quality. Median values (and interquartile ranges) of latency (A), bouts of shell investigation (B), duration of shell investigation (C), and time of locomotion (D), comparing treatments with odor from α and γ crabs before (experiment C3) and after (experiment D) shell switch. Alpha (and γ) were the crabs occupying a larger-than-optimal (and a smaller-than-optimal) shell. Alpha (and γ) crabs in experiment D are the individuals that were ranked γ (and α) crabs in experiment C3. See Table 2 for test statistics; * and ** denote $P < 0.05$ and $P < 0.01$, respectively.

DISCUSSION

Our study revealed a number of properties that characterize the nature of individual recognition by odor in hermit crabs. First, it proved that *P. longicarpus* is unable to chemically recognize familiar and high-quality empty shells. Then, it showed that (1) there is no odor specific of a rank; (2) individual crabs discriminate their own odor from the odor of other individuals; (3) they can chemically discriminate between larger crabs inhabiting higher-quality shells and smaller crabs inhabiting lower-quality shells, provided that these crabs are familiar to them; (4) they associate the odor of an individual crab with the quality of the shell it inhabits; and (5) this association quickly changes when the social partners switch to shells of different qualities.

By excluding that individual recognition by hermit crabs could be simply a consequence of their ability to learn shell odors, this study confirms Gherardi and Tiedemann's (2004a) and Gherardi and Atema's (2005b) findings in the same species. To the contrary, Jackson and Elwood (1989) showed that *P. bernhardus* is able to discriminate, at least by sight, between familiar and novel empty shells, even if seemingly identical, by remembering certain subtle features of already investigated shells. Our results are also consistent in part with Gherardi and Tiedemann (2004b), who showed that *P. longicarpus* identifies its own odor from the odor of other individuals and is capable of chemically discriminating between familiar and unfamiliar conspecifics.

However, in Gherardi and Tiedemann's (2004b) study and in the majority of other studies on individual recognition in invertebrates (see Caldwell, 1985 for an exception), the tasks employed measured, at best, differences in responses to two "heterogeneous subgroups" of conspecifics (Barrows et al., 1975), i.e., familiar and unfamiliar subgroups. That is, results of such experiments simply arrived at documenting that the study animals were capable of a "binary" discrimination among opponents (Boal, 1996), but not of "true individual recognition" (i.e., the ability to discriminate one individual of a group from every other individual on the basis of "a unique set of cues defining that individual"; Beecher, 1989).

Our study has partly overcome this methodological limitation. By investigating graded animal responses to a target shell in the presence of odors of different provenience, we found that hermit crabs can chemically discriminate (1) between themselves and others and (2) between at least two familiar individuals with different attributes. As a consequence, *P. longicarpus* seems to rely on a form of recognition that is more complex than a simple binary system. Because crabs seemed not to discriminate among unfamiliar crabs with the same attribute differences as the familiar crabs, the ability to chemically recognize at least two different familiar individuals was not because of odors proper of a rank, of a size class, or of a shell type. To the contrary, our results

might be explained by hypothesizing that during the familiarization phase, the odor from a social partner is associated by the receiver with the rank, the size, or the shell quality of the sender (or with a combination of these three attributes).

Experiment C illustrated that *P. longicarpus* can associate odor from a conspecific with the quality of the shell it occupies and reacts accordingly in the presence of a target shell. It is likely that during the familiarization phase, individual odors became labels of shell quality; if these labels indicate a high shell quality, their detection evokes an intense shell investigation when the receivers are presented with a high-quality shell; otherwise, shell investigation would have been scarce or absent. This view was supported by experiment D that also showed the plasticity of individual odor–shell quality association. Once an individual crab had switched to a shell of a different quality, responses to the offered shell were consistent with the changed association. Shell investigation was strong in the presence of odor of former crab γ in a high-quality shell and weak in the presence of former crab α in a low-quality shell.

An explanation for these results might be provided by the model of classical conditioning. This has been advocated to describe the dynamics of odor learning involved in food location by honeybees (e.g., Menzel, 1999) and in host detection by parasitoid hymenopterans (e.g., Kaiser et al., 2003). In our case, hermit crabs may memorize a stimulus (conditioning stimulus, i.e., the odor of a social partner) when it is associated with an unconditioned stimulus (i.e., the high quality of the shell occupied by the sender). The conditioned stimulus then becomes predictive of the reward (i.e., the potential acquisition of a high-quality shell) and elicits a conditioned response identical to the response normally elicited by the unconditioned stimulus (unconditioned response, i.e., investigating the offered shell). However, a difficulty in applying this model to odor learning in hermit crabs arises when we examine the nature of the reward. Associative learning is usually highly sensitive to unrewarded presentations of the conditioned stimulus, and in our experiments, we tested only individuals that had not been successful in acquiring crab α shell. As a consequence, if associative learning is the mechanism underlying acquisition and retention of memory of individual odors in hermit crabs, the reward for these organisms should be regarded as neither immediate nor certain, but rather as prospective and likely.

The question remains why this hermit crab species relies on individual odors of conspecifics to identify the quality of a shell. The answer remains speculative and thus provisional without any systematic fieldwork. Previous studies (Rittschof, 1980a,b; Rittschof et al., 1992) have shown that *P. longicarpus*, and particularly individuals inhabiting badly fitting shells (e.g., Rittschof, 1980b; Gherardi and Atema, 2005b), are chemically attracted to gastropod predation sites by fluids from partly digested snail tissue. At these sites, the attracted crabs form temporary and relatively small aggregations (Scully, 1978),

in which they agonistically interact to establish dominance hierarchies (Winston and Jacobson, 1978). The dominant crab obtains the first opportunity to occupy the empty shell as it is released by the predator (McLean, 1975); afterwards, other individuals exchange shells down the hierarchy. As a consequence, these aggregations function as "shell markets" and benefit a large number of the predation site attendants (Rittschof et al., 1992). Having obtained a high-quality shell, a crab generally leaves the predation site (Rittschof, 1980a; Tricarico and Gherardi, unpublished data), thus subtracting the shell from the market. It would be advantageous for an individual to rapidly classify the quality of the shells inhabited by other attendants and to spend time combating or negotiating (Hazlett, 1978) for a "really good shell." Because of water turbidity typical of many salt marsh habitats, chemical cues signaling shell quality might provide more reliable information than visual stimuli emitted by the shell itself; on the other hand, the exclusive use of tactile information from the shell would require time and energy consumption in repeated investigatory acts. Previous studies have shown that *P. longicarpus* quickly learns the chemical identity of a social partner (Gherardi and Tiedemann, 2004b; Gherardi and Atema, 2005b), is inaccurate in discriminating shells by sight (Gherardi and Tiedemann, 2004a), and often switches shells without prior investigation (Scully, 1986). However, we cannot exclude that sight and touch might integrate olfaction and lead to the improved detectability and discriminability of signals (Gherardi and Tiedemann, 2004b).

In this scenario, the plastic nature of the association between the individual odor of a conspecific and the quality of its shell has a clear adaptive value. Obviously, any shell exchange breaks the link between a given hermit crab and a given shell. The aggregations of hermit crabs around gastropod predation sites are characterized by a cascade of shell switches as the effect of a vacancy chain process (Chase et al., 1988). Therefore, the plastic response to the cues associated with high-quality shells is a key factor to optimize shell acquisition and to reduce errors. Indeed, *P. longicarpus* learned the odor of a conspecific after less than 30-min exposure to a stimulus animal (Gherardi and Atema, 2005b).

A final intriguing result of our study is an outline of the kind of representation of social partners that hermit crabs may have. We found that the sender, labeled by its individual odor, was classified by the receiver in function of the quality of its shell; on the basis of this classification, the receiver seemed to modulate the intensity of its investigatory acts toward the offered shell. The mechanism needed for this kind of recognition is one that involves in the receiver the association of a type of information from the sender (e.g., chemical cues) with memories of past experiences with it (e.g., exploration of its shell; for a general discussion, see Johnston and Bullock, 2001). This representation could be thought of as hermit crabs having a "concept" of other individuals, and hints at potential for relatively high-order knowledge about conspecifics.

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PHEROMONE COMPONENTS FROM BODY SCALES OF FEMALE *Anarsia lineatella* INDUCE CONTACTS BY CONSPECIFIC MALES

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Abstract—Pheromonal communication of adult peach twig borers, *Anarsia lineatella* Zeller (Lepidoptera: Gelechiidae), was reinvestigated based on recent findings that virgin female-baited traps were more attractive to mate-seeking males than a two-component synthetic sex pheromone consisting of (*E*)-5-decen-1-yl acetate (1000 μg) and (*E*)-5-decen-1-ol (100 μg), suggesting that females use additional pheromone components. Hypothesizing that these additional components may be released from body parts other than abdominal sex pheromone glands, we extracted female body scales and analyzed aliquots by coupled gas chromatographic–electroantennographic detection (GC-EAD) and GC–mass spectrometry. Eight straight-chain and four methylated aliphatic hydrocarbons, as well as two acetates, all elicited responses from excised male antennae. In laboratory experiments with synthetic candidate pheromone components, a combination of octadecyl acetate, (*R*)-11-methyltricosane, and (*S*)-11-methyltricosane in the presence of gland-derived sex pheromone components were shown to elicit contact of female decoys by males. However, body pheromone components did not enhance attractiveness of sex pheromone components in field trapping experiments, suggesting that they are effective only at close range and that other stimuli are responsible for superior attractiveness of female-baited traps.

Key Words—*Anarsia lineatella*, peach twig borer, sex pheromone, body pheromone, scales, contact, close-range communication, enantiomer, octadecyl acetate, (*R*)-11-methyltricosane, (*S*)-11-methyltricosane.

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INTRODUCTION

Roelofs et al. (1975) identified a two-component sex pheromone consisting of (*E*)-5-decen-1-yl acetate (*E*5-10:OAc; 87%) and (*E*)-5-decen-1-ol (*E*5-10:OH; 13%) in pheromone gland extracts of female *Anarsia lineatella* Zeller (Lepidoptera: Gelechiidae) that attracted conspecific males. Synthetic pheromones released from various dispensers and traps were tested as tools for monitoring populations of *A. lineatella* in commercial fruit orchards (Rice and Jones, 1975; Hathaway, 1981; Kehat et al., 1994). Deployment of synthetic pheromone for control of *A. lineatella* by pheromone-based mating disruption yielded unsatisfactory results (Rice, personal observation, cited in Millar and Rice, 1992). Re-analysis of the pheromone of *A. lineatella* led to the identification of several candidate pheromone components [decyl acetate, (*E*)- and (*Z*)-4-decenyl acetate, and (*E*,*E*)-3,5- and (*Z*,*E*)-3,5-decadienyl acetates; Millar and Rice, 1992], but none enhanced long-range attractiveness of the previously identified two-component blend (Roelofs et al., 1975). Traps baited with the two-component blend of *E*5-10:OAc (1000 μg) and *E*5-10:OH (100 μg) remained significantly less attractive than those baited with virgin female *A. lineatella* (Schlamp, 2005). These results suggested that if additional pheromonal communication signals existed, they probably were present in, or released from, body parts other than abdominal pheromone glands.

Contact- or copulation-inducing pheromones are typically present on the body surface of (signaling) insects. Although they appear effective only at short range, they often complement attractiveness of long-range sex or aggregation pheromones. Close-range pheromones have been noted and/or identified, in several orders of the Insecta, including Diptera (Stoffolano et al., 1997), Hymenoptera (Kimani and Overholt, 1995), Coleoptera (Ginzel et al., 2003), Isoptera (Clement, 1982), and Lepidoptera (Grant et al., 1987). Conceivably, similar pheromone components may exist in *A. lineatella* and play a role in short-range communication among males and females.

Our objective was to test the hypothesis that pheromone components derived from body scales of females are part of the sexual communication system in *A. lineatella*.

METHODS AND MATERIALS

Rearing of Insects. Insects were collected from peach orchards in Keremeos, British Columbia, and reared according to protocols developed and modified, respectively, by McElfresh and Millar (1993) and Sidney (2005).

Body Extraction of Moths. Separate groups of 10, 3- to 6-d-old males or females were submerged in pentane. After 5 min, the supernatant was withdrawn

and pipetted into a new vial. This procedure was repeated twice with the same group of moths, and extracts were then combined and concentrated such that 12.5 μl equaled one body-extract equivalent. All extracts were prepared during the photophase, well separated from the (pre)dawn calling period of females, thus minimizing potential extraction of sex pheromone components from abdominal pheromone glands.

Analyses of Extracts. Aliquots of body extracts were analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD; Arn et al., 1975; Gries et al., 2002), employing a Hewlett-Packard 5890 gas chromatograph fitted with a GC column (30 m × 0.25 or 0.32 mm ID) coated with DB-5, DB-23, DB-210 (J&W Scientific, Folsom, CA, USA), or SP-1000 (Supelco, Bellefonte, PA, USA). For GC-EAD recordings, an antenna was gently pulled from an insect's head, the distal segment removed, and then suspended between glass capillary electrodes filled with Ringer's solution [NaCl (6.5 g/l), KCl (1.4 g/l), $CaCl_2$ (0.12 g/l), Na_2CO_3 (0.1 g/l), Na_2HPO_4 (0.01 g/l)] in distilled water. Coupled GC–mass spectrometric (MS) analyses of pheromone extract [300 female equivalents (FE)] and of synthetic standards employed a Varian Saturn 2000 Ion Trap GC-MS fitted with the above-referenced DB-5 column.

General Instrumentation and Syntheses. Nuclear magnetic resonance (NMR) spectroscopy of synthetic compounds was conducted on a Varian AS500 (at 499.77 MHz for ^1H and 125.68 MHz for ^{13}C) spectrometer with chemical shifts reported in ppm relative to TMS (^1H, $\delta = 0.00$) and $CDCl_3$ (^{13}C, $\delta = 77.00$). Elemental analyses were performed using a Carlo-Erba model 1106 elemental analyzer. Optical rotations were measured with a Perkin-Elmer 341 polarimeter.

(*Z*)-11-Eicosenyl acetate (Sugawara et al., 1978) was obtained by reduction of (*Z*)-11-eicosanoic acid (Aldrich) with lithium aluminum hydride in tetrahydrofuran (THF) to (*Z*)-11-eicosen-1-ol and acetylation of this alcohol (Pederson et al., 2003) with acetic anhydride in the presence of pyridine.

Previously reported methylated hydrocarbons 11-methyltricosane (11me-23Hy), 2-methyltetracosane (2me-24Hy), 11-methylpentadecane (11me-25Hy), and 13-methylheptacosane (13me-27Hy) (Jackson, 1970; Tarvita and Jackson, 1970; Howard et al., 1978; Tsuda et al., 1981; Lange, 1993; Szafranek et al., 1994; Finidori-Logli et al., 1996; Wagner et al., 1998; Haverty et al., 2000) were synthesized from corresponding carbonyl precursors and ylids by Wittig reactions and by subsequent hydrogenation of the resulting olefins in the presence of platinum oxide.

*(S)-and (R)-11-Methyltricosanes (**8** and **12**, Figure 1). tert*-Butyldimethylsilylchloride (3.50 g; 1.1 equiv.) and 1.60 g (1.1 equiv.) of imidazole were added to 2.50 g of methyl (*R*)-3-hydroxy-2-methyl propanoate **1** (21.2 mmol; Aldrich) dissolved in 10 ml dimethylformamide. After stirring overnight at room temperature (RT), methyl (*R*)-3-*tert*-butyldimethylsilyloxy-2-methyl-

propanoate **2** was obtained in quantitative yield. Borane reduction of silyl ether **2** with 45 ml of a 1.0 M solution of BH_3 in a THF matrix under argon yielded known (*S*)-2-methyl-3-*tert*-butyldimethylsilyloxy-1-propanol (**3**) (King et al., 1995) after 48 hr. Ether **3** (quantitative yield) was isolated by quenching the reaction mixture with concentrated aq. $NaHCO_3$. The product was extracted with a 1:1 mixture of ether/hexane (3 × 50 ml), dried ($MgSO_4$), and the solvent was removed *in vacuo*.

All of monosilyl ether **3** (>99% pure, GC) was converted to (*R*)-mesylate **4** (King et al., 1995) at 0°C in dichloromethane with 1.1 equiv. of methanesulfonyl chloride and 1.5 equivalent of triethylamine. After 30 min of vigorous stirring at 0°C, the mixture was allowed to warm to RT and quenched with water. The organic layer was extracted with hexane, washed with 0.5 M HCl, concentrated aq. $NaHCO_3$ and brine, and dried ($MgSO_4$). After removal of excess solvents at 15 mm Hg, 10 ml of dry THF were added to the sulfonate. The mixture was transferred slowly via cannula under argon pressure to a stirred suspension of Grignard reagent [freshly prepared from 10.5 ml (55 mmol) of *n*-nonyl bromide and 2.7 g (111 mmol) of Mg] and CuI (0.84 g, 4.4 mmol) in 100 ml of THF at −23°C. After 1 hr, the reaction mixture was warmed to RT and quenched with a concentrated aq. NH_4Cl solution. The organic layer was extracted with hexane (2 × 75 ml), washed with water and brine, and dried (Na_2SO_4). The product was concentrated *in vacuo* and filtered through 10 g of silica to yield crude (*S*)-2-methyl-1-(*tert*-butyldimethylsilyloxy)-dodecane (**5**). Without any further purification, the silyl protective group was removed by stirring **5** with an excess of tetrabutylammonium fluoride in THF/H_2O

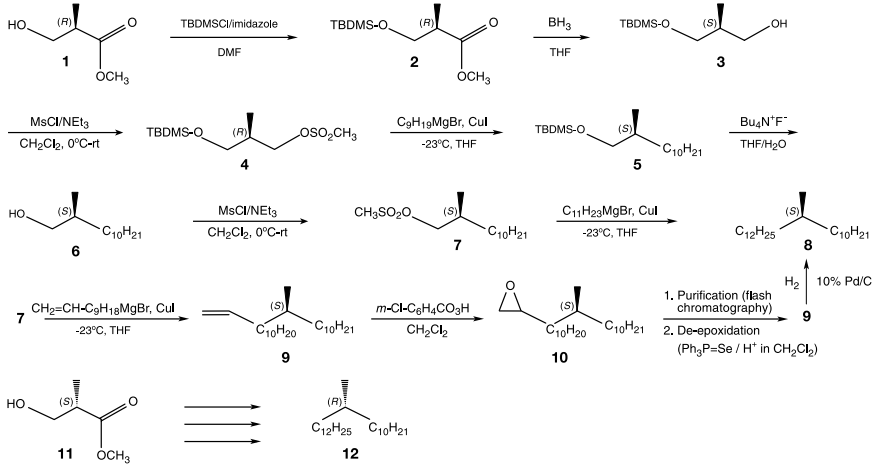

FIG. 1. Scheme for the syntheses of (*R*)- and (*S*)-11-methyltricosane.

overnight. Alcohol **6** was extracted from the reaction mixture with 100 ml of ether/hexane (1:1) and washed with water and brine. The organic layer was then dried ($MgSO_4$) and concentrated *in vacuo*. Flash column chromatography [50 g of silica, hexane/ether as eluent with gradual increase (5–15%) of the ether content] afforded 2.30 g (11.5 mmol, 54% yield based on propanoate **1**) of 96% pure (*S*)-2-methyl-1-dodecanol (**6**), $[\alpha]_D^{23} = -8.4°C$ (c 1.0; $CHCl_3$). Anal. calculated for $C_{13}H_{28}O$ (%): C 77.93, H 14.09; found: C 77.80, H 14.01. ^1H NMR ($CDCl_3$), δ (ppm): 0.87 (t, 3H, $J = 7.0$ Hz), 0.90 (d, 3H, $J = 6.7$ Hz), 1.22–1.40 (m, 17H), 1.59 (m, 2H), 3.39 (dd, 1H, $J = 6.2$, 10.5 Hz), 3.49 (dd, 1H, $J = 6.2$, 10.5 Hz). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.08, 16.54, 22.66, 26.96, 29.32, 29.62, 29.63, 29.65, 29.93, 31.90, 33.13, 35.73, 68.35.

Mesylation of alcohol **6** (2.00 g, 10.0 mmol; conditions, reagent ratio, and workup as described for conversion of alcohol **3** to mesylate **4**) and immediate Grignard coupling of methanesulfonate **7** with 10-undecen-1-ylmagnesium bromide in the presence of CuI [7.60 ml (35.0 mmol) of 11-bromo-undec-1-ene (Aldrich), 1.70 g (70 mmol) of Mg, and 0.57 g (3.0 mmol) of CuI; reaction conditions and workup as described for the synthesis of ether **5**] yielded (*S*)-13-methyl-1-tridecene **9** (28%) with the following impurities in the mixture: 1,9-undecadiene (7%), 1-undecene (52%), 10-undecen-1-ol (2%), 1,21-docosadiene (7%), alcohol **6** (2%), and mesylate **7** (1%). Polar impurities were removed by filtering the mixture through 10 g of silica with hexane. Filtrates containing hydrocarbons were concentrated *in vacuo* and added to a cold solution of 11.2 g (77% pure, 50 mmol) of *m*-chloroperbenzoic acid (Aldrich) in 20 ml of CH_2Cl_2. The mixture was stirred for 3 hr at 0°C, allowed to warm to RT, and then quenched with 100 ml of 1 N NaOH. The organic layer was extracted with ether (2×50 ml), washed twice with water and brine, dried ($MgSO_4$), and concentrated *in vacuo*, yielding a mixture of mono- and di-epoxides. Flash column purification (50 g of silica, 2% ether in hexane as eluent) of this mixture gave 3.10 g of epoxide **10** (61% pure by GC) with 1,2-epoxyundecane as the main impurity (30%). No di-epoxides were present as impurities. De-epoxidation of the mixture containing **10** was carried out with freshly prepared triphenylphosphonium selenide [obtained by stirring 8.26 g (31.5 mmol) of TPP and 2.49 g (31.5 mmol) of Se for 30 min] in 50 ml of CH_2Cl_2 with 1 ml of trifluoroacetic acid (Clive, 1978). After 1 hr of stirring at RT, solvents were removed *in vacuo*. The mixture was filtered through 20 g of silica with 150 ml of hexane. Olefin **9** (65% pure by GC) was then hydrogenated in hexane with 10% Pd/C (3 hr). The catalyst was eliminated by filtering through 5 g of silica, and the solvent was removed *in vacuo* at 15 mm Hg. Undecane and other low-boiling impurities were removed at 2–3 mm Hg (70°C, 2 hr), yielding >98% pure (*S*)-11-methyltricosane (**8**) (1.68 g, 4.96 mmol, 50% yield based on alcohol **6**, overall yield 26.5%). Anal. calculated for $C_{24}H_{50}$ (%): C 85.12, H 14.88; found: C 85.06, H 15.08. ^1H NMR (in $CDCl_3$), δ (ppm): 0.83 (d, 3H, $J = 6.6$

Hz), 0.88 (t, 6H, J = 6.9 Hz), 1.18–1.37 (m, 41 H); ^{13}C NMR (in $CDCl_3$), δ (ppm): 14.09, 19.70, 22.67, 27.06, 29.34, 29.63–29.68 (several unresolved peaks), 30.00, 31.90, 32.72, 37.07.

Coupling of the mesylate **7** with 1-undecylmagnesium bromide leads directly to hydrocarbon **8**, which was impossible to separate from by-product docosane. In the reaction mixture, **8** comprised 20%; after the removal of low-boiling and polar impurities, it was ~60% pure.

(*R*)-11-Methyltricosane (**12**) was synthesized through the same route, starting with methyl (*S*)-3-hydroxy-2-methylpropanoate (**11**; overall yield 20%). GC retention times and NMR data matched those of (*S*)-11-methyltricosane (**8**). Optical rotation for intermediate (*R*)-2-methyl-1-dodecanol: $[\alpha]^{23}_D$ = +6.1°C (c 7.7; $CHCl_3$).

Laboratory Experiments with Pheromone Components. Candidate body pheromone (BP) components were tested in laboratory bioassays, employing a mesh (200 μm) cage (90 × 90 × 100 cm; BioEquip Products, Inc., Rancho Dominguez, CA, USA), with one of the two test stimuli randomly assigned to opposite corners of the cage. A test stimulus consisted of a white Teflon® decoy (0.25 × 0.75 cm) pinned to the center of an inverted Petri dish (10 × 2 cm) and impregnated with gland pheromone (GP) components or GP plus synthetic candidate BP components at 10 FE. For each replicate, 10 3- to 6-d-old males were introduced into each cage and acclimatized for 12 hr to environmental conditions (23°C; >70% RH; 16-hr light–8-hr dark) prior to testing. Bioassays were initiated by introducing test stimuli, starting a custom-designed computer program (Raymond G. Holland, Electronic Supervisor, Science Technical Centre, SFU, unpublished data) that increased the intensity of the light source (60-W Phillips incandescent light bulb) from 0 to 600 lx within 15 min, and by manually turning on a desk swing fan (Windmere, Miramar, FL, USA) behind the bioassay cage, which delivered intermittent pulses of air (0.3 m/sec). For each bioassay, numbers of contacts with test stimuli were recorded for 15 min. Repeated contacts by the same male were recorded, if that male was more than one body length apart from the stimulus between consecutive contacts. Each of 15 replicates per experiment employed a new set of 10 males and test stimuli.

Ten instead of 1 FE of candidate BP components were bioassayed taking into account that body scales may be better pheromone dispensers than Teflon® decoys, or that live female *A. lineatella* may replenish their pheromone components over time, whereas we administered only a single application of test stimulus at the beginning of each 15-min bioassay. The experimental protocol did not allow more than three replicates per day, so group bioassays instead of single-insect bioassays were conducted. This ensured that some males responded to test stimuli in each bioassay despite the lack of sonic signals females emit in response to sonic signals from males (Hart et al., Gries laboratory, unpublished data).

Two synthetic GP components [*E*5-10:OAc (100 ng) and *E*5-10:OH (10 ng)] were tested alone or in combination with the following: (1) body extract of females at 10 FE (experiment 1); (2) a complete synthetic blend of candidate BP components, consisting of two acetates [octadecyl acetate (18:OAc), (*Z*)-11-eicosenyl acetate (*Z*11-20:OAc)], four methylated hydrocarbons [11-methyltricosane (11me-23Hy), 2-methyltetracosane (2me-24Hy), 11-methylpentadecane (11me-25Hy), 13-methylheptacosane (13me-27Hy)], and eight straight-chain hydrocarbons [docosane (22Hy), tricosane (23Hy), tetracosane (24Hy), pentacosane (25Hy), hexacosane (26Hy), octacosane (28Hy), nonacosane (29Hy), and tricontane (30Hy)] (experiment 2); (3–6) BP *minus* the two acetates 18:OAc and *Z*11-20:OAc (experiment 3), BP *minus* all hydrocarbons (experiment 4), BP *minus* methylated hydrocarbons (experiment 5), or BP *minus* straight-chain hydrocarbons (experiment 6).

BP blends lacking acetates (experiment 3) or methylated hydrocarbons (experiment 5) were not effective in increasing the number of body contacts, so follow-up experiments explored which acetate (experiments 7 and 8) or methylated hydrocarbon(s) (experiments 9–12) contributed to behavioral activity of the BP blend. 18:OAc appeared more effective than *Z*11-20:OAc (experiments 7 and 8), and 11me-23Hy was the single-most effective methylated hydrocarbon (experiment 11), so additional experiments were run to investigate which enantiomer of 11me-23Hy was behaviorally active by testing GP alone or in combination with 18:OAc plus (*S*)-11-methyltricosane [(*S*)-11me-23Hy] (experiment 13), (*R*)-11-methyltricosane [(*R*)-11me-23Hy] (experiment 14), or both (1:1; experiment 15). With the presence of both the *R*- and *S*-enantiomers of 11-methyltricosane needed for males to respond (experiment 15), experiment 16 tested GP plus female body extract vs. GP plus synthetic 18:OAc and (*R*)- and (*S*)-11me-23Hy at equivalent ratios and quantities. Paired mean contacts of paired stimuli by male moths were analyzed statistically using paired *t* tests (Zar, 1996). All statistical analyses were performed with JMP® Version 4 (SAS Institute, Cary, NC, USA).

RESULTS

Gas chromatographic–electroantennographic detection analyses of body extracts from female *A. lineatella* revealed small amounts (<0.2 ng) of the two sex pheromone components *E*5-10:OAc and *E*5-10:OH and numerous compounds that elicited responses from male antennae and several that did not (Figure 2). In GC-MS analyses, two of these EAD-active compounds with fragmentation ion *m*/*z* 61 (indicative of an acetate functionality) and with molecular ions *m*/*z* 312 and *m*/*z* 338 were identified as octadecyl acetate

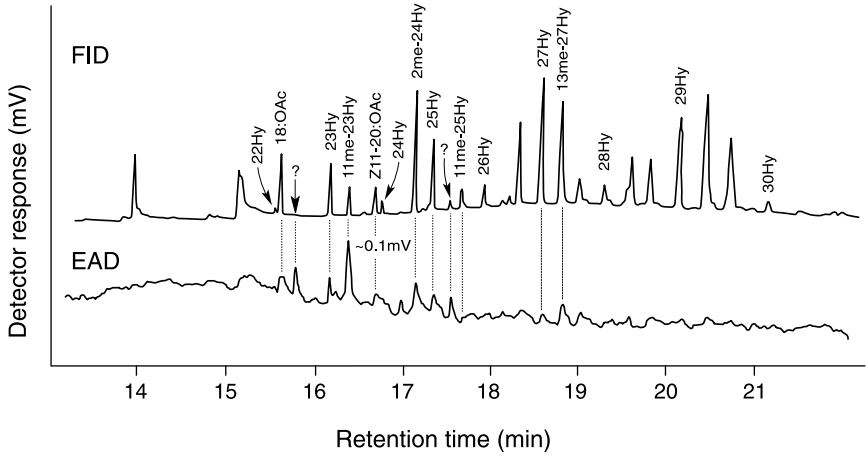

FIG. 2. Representative recording (N = 5) of flame ionization detector (FID) and electroantennographic detector (EAD: male *Anarsia lineatella* antenna) responses to 10 equivalents of body extract of female *A. lineatella*. Chromatography: splitless injection; injector and FID: 240°C, DB-5 column (30 m × 0.32 mm ID); temperature program: 50°C (2 min), then 15°C/min to 280°C (10 min). Compound abbreviation [with amounts per 1 female equivalent (FE) in parenthesis] as follows: 22Hy = docosane (7.4 ng); 18:OAc = octadecyl acetate (7.7 ng); 23Hy = tricosane (6.5 ng); 11me-23Hy = 11-methyltricosane (3.8 ng); *Z*11-20:OAc = (*Z*)-11-eicosenyl acetate (3.8 ng); 24Hy = tetracosane (1.8 ng); 2me-24Hy = 2-methyltetracosane (15.0 ng); 25Hy = pentacosane (9.1 ng); 11me-25Hy = 11-methylpentacosane (1.0 ng); 26Hy = hexacosane (3.1 ng); 27Hy = heptacosane (20 ng); 13me-27Hy = 13-methylheptacosane (19.0 ng); 28Hy = octacosane (3.6 ng); 29Hy = nonacosane (19.9 ng); 30Hy = triacontane (2.9 ng).

(18:OAc) and an eicosenyl acetate, respectively. Dimethyl disulfide treatment (Dunkelblum et al., 1985) of the latter without prior isolation yielded an adduct with GC-MS fragmentation ions *m*/*z* 173 and *m*/*z* 259, indicative of a double bond at C11. This compound was thus postulated and, through comparative GC-MS of an authentic standard, confirmed to be (*Z*)-11-eicosenyl acetate (*Z*11-20:OAc).

Mass spectra of other EAD-active compounds in female body extracts suggested that they were saturated hydrocarbons. Four of these had retention indices (Van den Dool and Kratz, 1963) indicative of methyl branches. Their mass spectra revealed fragmentation ions diagnostic of methyl branch positions (Pomonis et al., 1980; Francke et al., 1987, 1988; Gries et al., 1991, 1993, 1994) and suggested that they were 11me-23Hy, 2me-24Hy, 11me-25Hy, and 13me-27Hy, respectively. Comparative GC-MS of insect-produced and authentic standards confirmed the structural assignments.

Laboratory Experiments with Candidate Contact Pheromone Components. Teflon® decoys impregnated with body extracts from female *A. lineatella* at 10 FE plus GP components provoked more decoy contacts by male *A. lineatella* than GP components alone (Figure 3; experiment 1). These results could not be attributed to small (<0.2 ng) amounts of the sex pheromone components *E*5-10:OAc and *E*5-10:OH in body extracts of female moths because both treatment and control stimuli contained 100 and 10 ng, respectively, of synthetic *E*5-10:OAc and *E*5-10:OH.

A synthetic blend of all candidate BP components at ratios and concentrations equivalent to 10 FE plus GP provoked more contacts by males

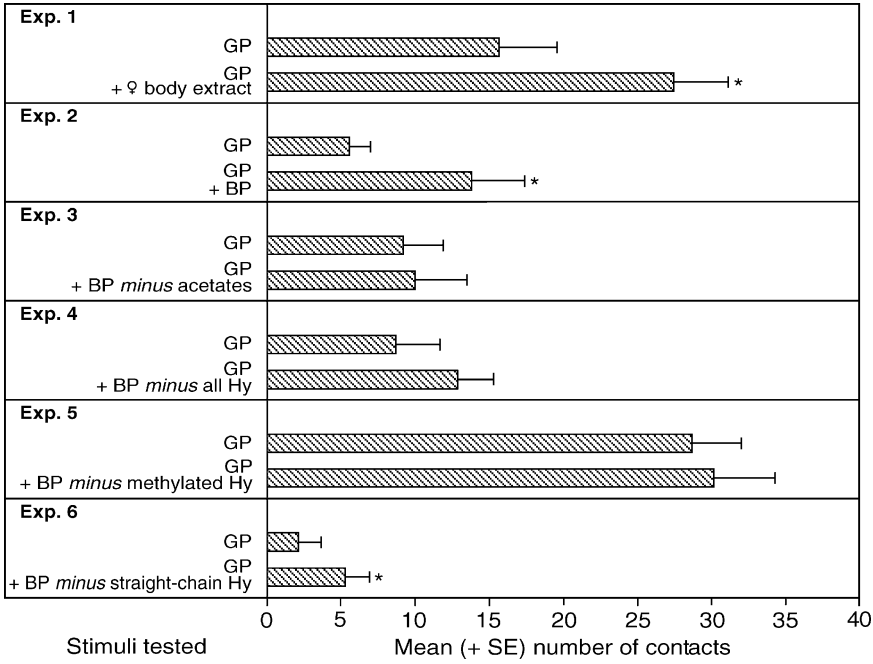

FIG. 3. Mean (+ SE) number of contacts made by male *Anarsia lineatella* in experiments 1–6 (15 replicates each) with a Teflon® decoy impregnated with various test stimuli. In each experiment, an asterisk (*) indicates a significant preference for a particular stimulus; paired *t* test, $P < 0.05$. Abbreviations as follows: GP = synthetic gland pheromone components [(*E*)-5-decen-1-yl acetate (100 ng) and (*E*)-5-decen-1-ol (10 ng)]; female body extract = body extract of female *A. lineatella* tested at 10 female equivalents; BP = synthetic body pheromone components consisting of two acetates [18:OAc, *Z*11-20:OAc], four methylated hydrocarbons (Hy) [11me-23Hy, 2me-24Hy, 11me-25Hy, 13me-27Hy], and eight straight-chain hydrocarbons [22Hy, 23Hy, 24Hy, 25Hy, 26Hy, 28Hy, 29Hy, 30Hy]. For full names of chemicals, see caption of Figure 2.

than GP alone (Figure 3; experiment 2). Synthetic BP blends lacking straight-chain hydrocarbons were still bioactive (Figure 3; experiment 6), but BP blends lacking acetates (Figure 3; experiment 3), all hydrocarbons (Figure 3; experiment 4), or all methylated hydrocarbons (Figure 3; experiment 5) were not. Neither *Z*11-20:OAc nor 18:OAc alone significantly enhanced the attractiveness of GP (Figure 4; experiments 7 and 8), but the opposite was true for 18:OAc combined with four methylated hydrocarbons (Figure 4; experiment 9).

Deleting a group of two or single methylated hydrocarbons from the BP blend determined that only 11me-23Hy, in addition to 18:OAc, is needed to retain the blend's behavioral activity (Figure 4; experiments 10–12).

The *R*- and *S*-enantiomers of 11me-23Hy in combination, but not singly, are BP pheromone components (Figure 5; experiment 15). A BP blend with the

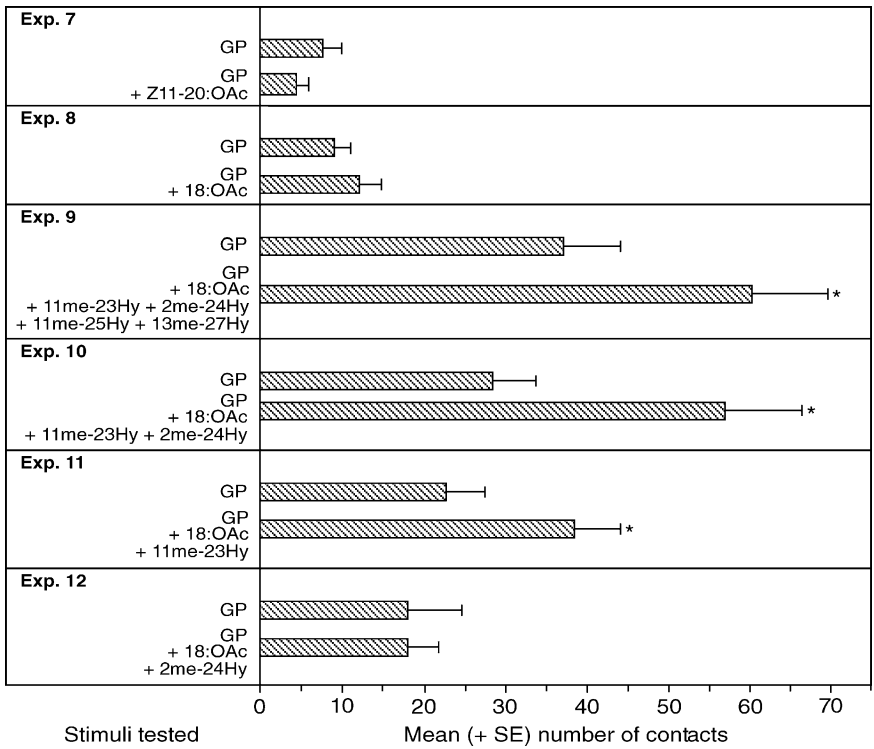

FIG. 4. Mean (+ SE) number of contacts made by male *Anarsia lineatella* in experiments (Exp.) 7–12 (15 replicates each) with a Teflon® decoy impregnated with various test stimuli. In each experiment, an asterisk (*) indicates a significant preference for a particular treatment; paired *t* test, $P < 0.05$. Abbreviations as in captions of Figures 2 and 3.

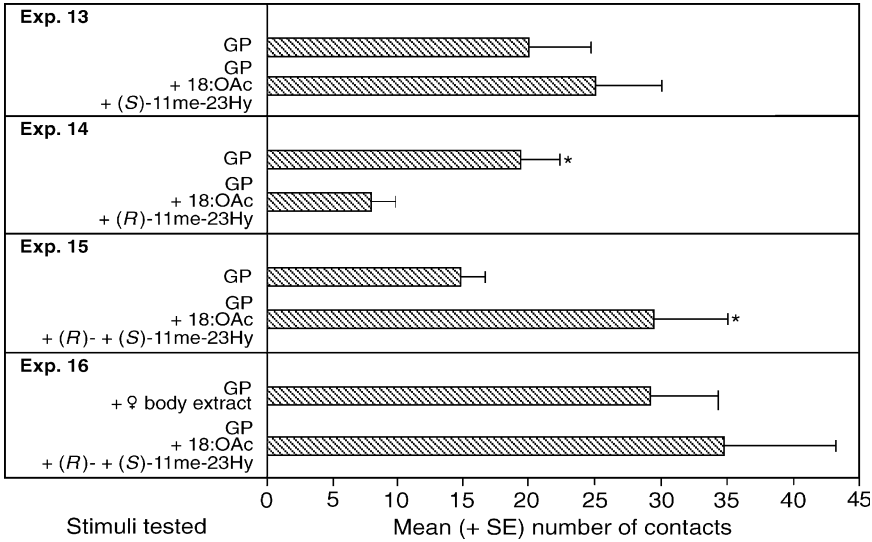

FIG. 5. Mean (+SE) number of contacts made by male *Anarsia lineatella* in experiments 13–16 (15 replicates each) with a Teflon® decoy impregnated with various test stimuli. In each experiment, an asterisk (*) indicates a significant preference for a particular treatment; paired *t* test, $P < 0.05$. (*R*)- and (*S*)-11me-23 = (*R*)- and (*S*)-11-methyltricosane, respectively. Other abbreviations as in captions of Figures 2 and 3.

S-enantiomer alone was benign (Figure 5; experiment 13) and was inhibitory with the *R*-enantiomer alone (Figure 5; experiment 14). The three-component BP blend consisting of 18:OAc, (*R*)-11me-23Hy, and (*S*)-11me-23Hy was as effective as a body extract in provoking contacts by male *A. lineatella* (Figure 5; experiment 16).

DISCUSSION

Significantly more captures of male *A. lineatella* in traps baited with conspecific virgin females than in those baited with a two-component synthetic sex pheromone (Schlamp, 2005) suggested that females use additional communication signals to attract mate-seeking males. Our data indicate that close-range communication signals include pheromone components from the females' body surface that may provoke contact by males.

Although numerous compounds were extractable from the females' body surface and elicited responses from male antennae (Figure 2), the body pheromone

(BP) seems to comprise only three components: 18:OAc, (*R*)-11me-23Hy, and (*S*)-11me-23Hy (Figures 4 and 5). Positive responses by males only to BP blends containing both the *R*- and *S*-enantiomers of 11me-23Hy (Figure 5; experiments 13–15), and even inhibition of response to blends containing only the *R*-enantiomer (Figure 5; experiment 14), indicate that (*R*)- and (*S*)-11me-23Hy are BP components of female *A. lineatella*.

Varying levels of responding insects to control stimuli from one experiment to another were likely because of the fact that we proceeded with experiments even at low atmospheric pressure, which is not conducive to high levels of response. Treatment stimuli also differed in their attractiveness, further modifying the overall level of response between experiments. However, with the same control stimulus retained in each experiment, we could assess the relative strength of a treatment stimulus within and between experiments.

Methyl (*R*)- and (*S*)-3-hydroxy-2-methylpropanoates (99% ee) were chosen as starting materials for the syntheses of the enantiomers of 11-methyltricosane by a route that did not affect the chiral center. When the Grignard coupling of mesylate **7** with *n*-undecylmagnesium bromide was performed, an inseparable mixture of desired hydrocarbon **8** and the Grignard reagent dimer by-product *n*-docosane formed. To obtain pure **8**, we coupled mesylate **7** with 10-undecen-1-ylmagnesium bromide. The unsaturated reaction products were converted into their respective epoxides, and mono-epoxide **10** was separated from the di-epoxide derived from the Grignard by-product. Regenerated (by de-epoxidation) olefin **9** was then hydrogenated to give the final compound **8**. Polarimetric studies of final products **8** and **12** did not yield measurable values of optical rotation.

Body pheromone components may serve as ultimate cues to confirm the proper species, and sex, of a prospective mate. The two acetates, 18:OAc and *Z*11-20:OAc, are indeed present only in body extracts of female but not male *A. lineatella* (data not shown), suggesting that they may help males recognize females. In contrast, 11me-23Hy is present in body extracts of both males and females (data not shown), suggesting that it is not suitable for mate recognition. However, considering that both enantiomers of 11me-23Hy were required to induce positive responses by males, the presence of only one (*R*) or both enantiomers may help reveal the signaler's sex. In field experiments (Schlamp, 2005), those contact pheromone components had no effect on long-range attraction of male *A. lineatella*, indicating that they play a role only at close range before or during courtship.

This type of close- and long-range communication system with components from sex pheromone glands and body scales has been reported in other species of moths. Live female gypsy moths *Lymantria dispar* or physical models of female *L. dispar* baited with sex pheromone and covered with female abdominal scales, elicited copulatory responses by males, whereas exposure of

males to sex pheromone alone did not (Charlton and Cardé, 1990). Similarly, males of the smaller tea tortrix moth *Adoxophyses orana* will attempt copulation only in the presence of female-produced sex pheromone and scales (Shimizu and Tamaki, 1980).

In summary, this study has revealed contact pheromone components derived from scales of female *A. lineatella*, which, together with gland-derived sex pheromone components, induce contacts by males. Contact pheromone components do not enhance the efficacy of sex pheromone in attracting males in the field (Schlamp, 2005). Thus, the superior attractiveness of virgin female *A. lineatella* as a trap bait compared with synthetic sex pheromone (Schlamp, 2005) must be due to other signals that need to be investigated.

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INTERSEXUAL DIFFERENCES IN CHEMICAL COMPOSITION OF PRECLOACAL GLAND SECRETIONS OF THE AMPHISBAENIAN *Blanus cinereus*

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Abstract—Pheromonal communication seems to be especially important in amphisbaenians, a group of reptiles morphologically and functionally adapted to fossorial life. Both male and female amphisbaenians (*Blanus cinereus*) produce copious amounts of a secretion from the precloacal glands. Analysis by gas chromatography–mass spectrometry revealed that these secretions contain 29 major lipophilic compounds, including several steroids (mainly cholesterol and cholesteryl methyl ether), *n*-C_9 to *n*-C_{18} carboxylic acids (more diverse in males), and methyl and long-chain (waxy)-type esters of carboxylic acids, along with squalene (mainly in males) and tocopherol (only in females). There were clear intersexual differences in the presence/absence of some compounds, such as some fatty acids, steroids, and tocopherol, and in the relative proportions of some shared compounds, such as squalene. These differences may explain how the sexes of this amphisbaenian discriminate one another based on scent alone. The abundance of steroids and waxy esters in secretions of both male and female amphisbaenians may be useful to scent mark underground tunnels to facilitate orientation by these organisms.

Key Words—Amphisbaenians, *Blanus cinereus*, precloacal glands, fatty acids, steroids, mass spectrometry.

INTRODUCTION

Intraspecific communication by reptiles is often based on chemical cues (Halpern, 1992; Mason, 1992). Several studies have demonstrated the existence

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of pheromones in different species of lizards, snakes, and amphisbaenians (e.g., Mason et al., 1989; Alberts, 1993; Cooper et al., 1994; Aragón et al., 2001a). Semiochemicals used for communication are frequently produced in precloacal or femoral holocrine glands (Mason, 1992; Alberts, 1993). In lizards, the secretory activity of these glands is greatest during the breeding season when males produce more secretions than females, and androgens can influence secretory development and maintenance of activity (Cole, 1966; Van Wyk, 1990; Alberts, 1993). The presence and relative concentration of pheromone components may convey information on the identity of an individual (Alberts, 1990, 1992, 1993) and serve a variety of functions, such as advertising residence in a home range to other males (Aragón et al., 2001b) or mate choice by females (Martín and López, 2000).

Amphisbaenians are a group of reptiles that are morphologically and functionally adapted to fossorial life (Gans, 1978). Morphological adaptations to burrowing include trunk elongation, head modification, reduced vision, and loss of limbs in most species (Gans, 1978). These adaptations constrain amphisbaenians to meet ecological demands with a suite of responses that differ from those of epigean reptiles (e.g., Martín et al., 1991; López et al., 1998). For a blind fossorial reptile, chemical discrimination may be the only means of detecting the presence of conspecifics and discriminating between sexes. Thus, previous experiments have shown that pheromonal communication seems to be especially important in amphisbaenians (Cooper et al., 1994; López et al., 1997).

Amphisbaenians have several precloacal pores connected to precloacal glands that produce copious holocrine secretions, especially during the breeding season. The structures of precloacal glands have been studied in some (Gabe and Saint-Girons, 1965; Whiting, 1967; Antoniazzi et al., 1993, 1994; Jared et al., 1999), suggesting that they might be similar to the precloacal or femoral glands of lizards (Gabe and Saint-Girons, 1965; Whiting, 1967, 1969). Morphological and microscopic examination of secretions suggested that as an amphisbaenian moves inside tunnels, the secretion plugs are abraded against the substrate, releasing a secretion trail (Jared et al., 1999). This trail may contain semiochemicals that might be important in intraspecific communication inside tunnels. In fact, behavioral studies have shown that males of the amphisbaenian, *Blanus cinereus*, are able to detect and discriminate between chemical cues of males and females (Cooper et al., 1994) and between their own scent and those of other males (López et al., 1997). The ability to discriminate sexes was greater from precloacal gland secretions than from skin chemicals (Cooper et al., 1994). Moreover, before starting to burrow in unfamiliar substrates, amphisbaenians emitted numerous tongue flicks and moved for significantly longer periods of time than when on familiar substrates, which suggest that they are able to recognize their own burrows by chemosensory cues (López et al., 2000).

In spite of the potential importance of chemical signals in intraspecific relationships of lizards and amphisbaenians, only a few studies have analyzed the chemicals found in the skin of lizards (Weldon and Bangall, 1987; Mason and Gutzke, 1990) or femoral or precloacal secretions of lizards (Chauhan, 1986; Alberts, 1990; Weldon et al., 1990; Alberts et al., 1992; Escobar et al., 2001, 2003). These studies have shown that gland secretions of lizards are composed of both lipids and proteins, with lipids being considered to be the main compounds involved in communication. In amphisbaenians, histochemical studies have detected the presence of mucopolysaccharides and protein, but not lipids, in the precloacal glands of *Amphisbaena alba* (Antoniazzi et al., 1993). However, because lipids are common in secretions of lizards and snakes (Mason, 1992), the potential presence of lipids in secretions of amphisbaenians bears further investigation.

B. cinereus is a fossorial amphisbaenian endemic to the Iberian Peninsula (Salvador, 1998). It is seldom seen above ground and is usually found under rocks located in areas with abundant leaf litter (Martín et al., 1991; López et al., 1998). Vision is reduced in amphisbaenians, but studies of tongue-flicking behavior showed that *B. cinereus* uses its vomeronasal system to detect and discriminate not only self and conspecific scents (Cooper et al., 1994; López et al., 1997) but also odors of prey (López and Salvador, 1992, 1994) and potential predators (López and Martín, 1994, 2001). To understand the role of semiochemicals produced by precloacal glands in intraspecific communication of *B. cinereus*, we report here a gas chromatography–mass spectrometry (GC-MS) analysis of the lipid fraction of precloacal secretions of males and females.

METHODS AND MATERIALS

Study Animals and Collection of Secretions. During May 2004, we captured 8 adult male and 12 adult female *B. cinereus* by lifting stones in various locations over a large area in Collado Mediano and Cercedilla (Madrid, Central Spain). This collection period coincided with the mating season of this species. Amphisbaenians were weighed [mass (mean ± SE) = 5.2 ± 0.6 g, range = 2.5–9.8 g], and their snout-vent length was measured (mean ± SE = 179 ± 5 mm, range = 148–203 mm). Amphisbaenians had a mean (±SE) number of precloacal pores of 3.1 ± 0.1 (range = 2–4). We extracted the secretion of the precloacal pores by gently pressing the area around pores with forceps and collected the secretion directly in glass vials with Teflon-lined stoppers. Vials were stored at −20°C until the analyses were performed. Amphisbaenians were released at the point of capture. To control for the introduction of contaminants from the handling procedure or from the environment where amphisbaenians were found, we also used the same procedure on each sampling occasion to

obtain blank control vials. These vials were treated in the same manner as those used to collect secretions, and the blank samples were also used to further examine the solvent for impurities in the laboratory.

Sample Analyses. Samples were analyzed using a Finnigan-ThermoQuest Trace 2000 gas chromatograph fitted with a poly (5% diphenyl/95%) dimethylsiloxane column (Supelco, Equity-5, 30-m length × 0.25 mm ID, 0.25-μm film thickness) and a Finnigan-ThermoQuest Trace mass spectrometer as a detector. The samples, 2 μl of each sample dissolved in 250 μl of *n*-hexane (Sigma, capillary GC grade), were injected in splitless mode with an inlet temperature of 250°C. The GC was programmed so that the oven temperature was kept at 50°C for 10 min, increased to a final temperature of 280°C at a rate of 5°C/min, and kept at this temperature for 30 min. The carrier gas was helium at 30 cm/sec. Ionization by electron impact (70 eV) was carried out at 280°C. Mass spectral fragments below $m/z = 39$ were not recorded. Impurities identified in the solvent and/or the control vial samples are not reported. Initial identification of secretion components was performed by comparison of sample mass spectra with those in the NIST/EPA/NIH 1998 computerized mass spectral library. When possible, identifications were confirmed by comparison of spectra and retention times with those of authentic standards. Authentic samples were purchased from Aldrich Chemical Co.

Statistical Analyses. Data in this paper are reported as mean ± standard error (SE). The relative amount of each component was determined as the percent of the total ion current. Major peaks present in all individuals and representing >1% relative peak area were selected, and together, these accounted for 96% of total area. The relative areas of the nine selected peaks were restandardized to 100% and transformed following Aitchison's (1986) formula: $Z_{ij} = \ln(Y_{ij}/g(Y_j))$, where Z_{ij} is the standardized peak area i for individual j, Y_{ij} is the peak area i for individual j, and $g(Y_j)$ is the geometric mean of all peaks for individual j. The homogeneity of variance of these variables was tested with Levene's test, and Bonferroni's correction was applied. The transformed areas were used as independent variables in a multivariate analysis of variance (MANOVA) to determine whether males and females differed in the abundances of shared compounds (Sokal and Rohlf, 1995). We used Statistica 6.0 software (StatSoft Inc., Tulsa, OK) for statistical analyses.

RESULTS AND DISCUSSION

Both male and female *B. cinereus* produced copious secretions from the precloacal pores during the mating season. The number of precloacal pores did

not differ between males (3.1 ± 0.1 pores/side) and females (3.1 ± 0.2; ANOVA, $F = 0.05$, $df = 1,18$, $P = 0.82$). In addition, the total amount of secretion, although not quantified, appeared similar in males and females.

Precloacal secretions of both male and female amphisbaenians have a great diversity of lipophilic compounds. A total of 29 major compounds were detected in these secretions (Table 1). Steroids (86.8%), squalene (4.6%, mainly in males), carboxylic acids, and one of their methyl esters (4.2%), and waxy esters (4.1%) were the main components, but tocopherol acetate (0.3%) was also uniquely present in females. There were clear intersexual differences in the presence/absence of some compounds (i.e., males had eight compounds that

TABLE 1. LIPIDS FOUND IN PRECLOACAL GLAND SECRETIONS OF MALE AND FEMALE *B. CINEREUS*[a]

Rt (min)	Compound	Males ($N = 8$)	Females ($N = 12$)
25.3	Nonanoic acid	0.61 ± 0.43	–
32.9	Dodecanoic acid	1.52 ± 0.30	4.72 ± 0.97
37.4	Tetradecanoic acid	0.02 ± 0.01	–
41.5	Hexadecanoic acid	0.48 ± 0.19	0.25 ± 0.03
44.7	9.12-Octadecadienoic acid	0.16 ± 0.01	–
44.8	Octadecenoic acid	0.22 ± 0.06	–
45.2	Octadecanoic acid	0.21 ± 0.06	0.18 ± 0.07
52.8	Octadecanoic acid, methyl ester	0.01 ± 0.01	0.12 ± 0.08
55.7	Squalene	8.52 ± 3.59	0.64 ± 0.08
56.5	Cholesta-4,6-dien-3-ol	–	0.28 ± 0.10
56.8	Cholesta-3,5-diene	0.26 ± 0.03	0.30 ± 0.09
57.2	Cholesta-5,7-dien-3-ol, acetate	3.35 ± 0.53	3.90 ± 0.58
57.4	Cholesta-5,7,9(11)-trien-3-ol	0.41 ± 0.04	0.16 ± 0.05
57.9	α-Tocopherol	–	0.64 ± 0.15
58.9	Cholesta-2,8-dien-6-ol, 14-methyl-, acetate	0.36 ± 0.16	–
59.3	Hexadecyl octadecenoate	0.20 ± 0.01	–
59.7	Cholesteryl methyl ether	44.71 ± 1.87	33.99 ± 4.77
59.9	Cholestane, 3-methoxy	0.40 ± 0.02	0.21 ± 0.07
60.9	Cholesterol	30.49 ± 0.33	41.07 ± 5.29
61.7	Cholesta-5,7-dien-3-ol	0.60 ± 0.33	–
61.8	Unidentified steroid 1	0.40 ± 0.11	2.13 ± 0.43
62.0	Unidentified steroid 2	0.95 ± 0.02	0.21 ± 0.10
63.4	Campesterol	1.42 ± 0.05	1.78 ± 0.35
63.9	Cholest-5-en-3-one	–	0.42 ± 0.09
65.2	Hexadecyl hexadecenoate	–	0.03 ± 0.02
65.9	γ-Sitosterol	0.63 ± 0.09	–
66.3	Octadecyl octadecenoate	2.15 ± 1.28	5.41 ± 1.80
69.1	Cholest-5-en-3-ol/tetradecanoate	1.74 ± 1.30	3.36 ± 2.24
70.3	Octadecyl hexadecenoate	0.17 ± 0.01	0.19 ± 0.14

[a]The relative amount of each component was determined as the percentage of the total ion current and reported as the mean ± SE.

were not found in females, and females had five compounds that were not found in males) and in the relative proportions of some shared compounds (Table 1). Thus, with respect to the 10 shared major compounds, males and females showed overall differences in relative proportions (MANOVA, Wilks' λ = 0.045, $F = 29.17$, $df = 8,11$, $P < 0.001$). Univariate tests showed that males had greater relative proportions of squalene (ANOVA, $F = 19.86$, $df = 1,18$, $P < 0.001$) and cholesteryl methyl ether ($F = 6.75$, $df = 1,18$, $P = 0.02$) and lower relative proportions of an unidentified steroid ($F = 24.45$, $df = 1,18$, $P = 0.001$; Table 1). There were no significant differences between sexes in proportions of the other six major shared compounds (i.e., dodecanoic acid, cholesta-5,7-dien-3-ol acetate, cholesterol, campesterol, octadecyl octadecenoate, and cholest-5-en-3-ol/tetradecanoate; $P > 0.15$ in all cases).

Lipids were not detected by histochemical methods in a previous morphological study of the amphisbaenian *A. alba* (Antoniazzi et al., 1993). This tropical species has a short mating season and shows reproductive characteristics only during the dry season (Colli and Zamboni, 1999). Thus, it is possible that amphisbaenians examined in that study, which had also been maintained in captivity, were not in a reproductive condition and did not produce precloacal secretions or produced secretions without lipids. We observed that precloacal secretions of *B. cinereus* were apparently absent outside of the mating season (unpublished data).

The presence of a similar quantity of precloacal secretion in male and female amphisbaenians is different from what has been observed in precloacal or femoral gland secretions of lizards (Mason, 1992). In lizard families, where these glands are present, both male and female lizards show femoral or precloacal pores. However, femoral or precloacal glands are larger in males, and significant secretory activity, which can be induced by androgenic hormones, is limited almost entirely to males (Cole, 1966; Mason, 1992). Nevertheless, secretions from the urodaeal gland of female broad-headed skinks *Eumeces laticeps* contain a biologically active lipid fraction, with cholesterol, cholesterol esters, and waxy esters, among other compounds (Cooper and Gratska, 1987), which seem to act as a sex pheromone in these skinks (Cooper et al., 1986). In addition, both male and female snakes have cloacal gland secretions (see references in Mason, 1992). Thus, precloacal glands of female amphisbaenians might have a similar function to urodaeal glands of female lizards or to the cloacal glands of snakes.

Cholesteryl methyl ether and cholesterol were the main lipids found in all secretions of male and female *B. cinereus*. Cholesteryl methyl ether was also found in the skin of a gecko (Mason and Gutzke, 1990), whereas cholesterol was also found in abundance in many lizards, both in the skin (Weldon and Bangall, 1987; Mason and Gutzke, 1990) and in femoral and precloacal secretions (Alberts et al., 1992; Escobar et al., 2001; López and Martín, 2005).

The abundance and ubiquity of cholesterol are thought to help to constitute an unreactive apolar matrix that delivers the compounds that are the true semiochemicals (Escobar et al., 2003). We also found other steroids in significant quantities. The abundance of steroids and waxy-type esters in secretions of both male and female amphisbaenians may be useful to scent mark underground tunnels and then to facilitate orientation of individuals or conspecifics within the tunnels (Jared et al., 1999; López et al., 2000). Interestingly, males and females differ in the presence of some steroids, which may contribute to sex identification (Cooper et al., 1994). Finally, it is interesting to find α-tocopherol, a form of vitamin E, only in females (Brigelius-Flohe and Traber, 1999). Tocopherol has not been reported from secretions of any lizard or snake, although it was found in secretions from the gular and paracloacal glands of the American alligator (Weldon et al., 1987, 1988).

One acyclic terpenoid, squalene, was found in secretions of *B. cinereus* and also in the femoral secretions of some lacertid lizards (López and Martín, 2005), skin of male and female leopard geckos (Mason and Gutzke, 1990), skin of male garter snakes (Mason et al., 1989), and paracloacal glands of crocodiles (García-Rubio et al., 2002), but not in femoral or precloacal gland secretions of iguanid lizards (Weldon et al., 1990; Escobar et al., 2001). Squalene was much more abundant in male than in female amphisbaenians, which is similar to the situation in the skin of garter snakes (Mason et al., 1989). Only male snakes present squalene, and courtship to females is partially inhibited if squalene is experimentally added to female skin (Mason et al., 1989). Thus, in amphisbaenians, squalene may be also part of the male sex recognition system.

Carboxylic acids found in precloacal secretions of *B. cinereus* ranged from *n*-C_9 to *n*-C_{18}, with dodecanoic and hexadecanoic acids being the most abundant in both males and females. Similar long-chain carboxylic acids have also been found in other lizards as common and major constituents of skin and femoral and precloacal glands (Mason and Gutzke, 1990; Weldon et al., 1990; Alberts et al., 1992; Escobar et al., 2001). Male amphisbaenians have a greater number of carboxylic acids than females, which, again, may explain how sex discrimination by tongue-flicking behavior is made in this amphisbaenian (Cooper et al., 1994). In addition, a greater diversity of carboxylic acids in males may be useful to produce scents with different individual signatures, which an individual can use to recognize his own scent from that of potential rivals, a capacity that has been shown in behavioral tests (López et al., 1997).

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NEW PHEROMONE COMPONENTS OF THE GRAPEVINE MOTH *Lobesia botrana*

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Abstract—Analysis of extracts of sex pheromone glands of grapevine moth females *Lobesia botrana* showed three previously unidentified compounds, (*E*)-7-dodecenyl acetate and the (*E*,*E*)- and (*Z*,*E*)-isomers of 7,9,11-dodecatrienyl acetate. This is the first account of a triply unsaturated pheromone component in a tortricid moth. The monoenic acetate (*E*)-7-dodecenyl acetate and the trienic acetate (7*Z*,9*E*,11)-dodecatrienyl acetate significantly enhanced responses of males to the main pheromone compound, (7*E*,9*Z*)-7,9-dodecadienyl acetate, in the wind tunnel. The identification of sex pheromone synergists in *L. botrana* may be of practical importance for the development of integrated pest management systems.

Key Words—Grapevine moth, *Lobesia botrana*, Tortricidae, (*E*)-7-dodecenyl acetate, (7*E*,9*E*,11)-7,9,11-dodecatrienyl acetate, (7*Z*,9*E*,11)-7,9,11-dodecatrienyl acetate, sex pheromone, synergist

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INTRODUCTION

The grapevine moth *Lobesia botrana* Denis and Schiffermüller (Lepidoptera, Tortricidae) is among the economically most important insects in Europe. It occurs throughout the wine-growing area of ca. 4 million hectares and completes two to four generations per year. The larvae feed on grapes, where *Botrytis cinerea* and other fungi rapidly develop, causing the entire grape cluster to rot (Fermaud and Giboulot, 1992). Pheromone-based control of *L. botrana* has been successfully adopted in wine-growing areas in Germany, Italy, and Switzerland. However, the cost effectiveness of the mating disruption technique needs to be improved (Arn and Louis, 1996).

The sex pheromone of *L. botrana*, which has been studied since the 1970s, consists of the main compound (7*E*,9*Z*)-7,9-dodecadienyl acetate (*E*7,*Z*9-12Ac) plus at least four other compounds: (7*E*,9*Z*)-7,9-dodecadien-1-ol (*E*7,*Z*9-12OH), (*Z*)-9-dodecenyl acetate (*Z*9-12Ac), (*E*)-9-dodecenyl acetate (*Z*9-12Ac), and 11-dodecenyl acetate (11-12Ac; Roelofs et al., 1973; Arn et al., 1988; El-Sayed et al., 1999). Here, we show that *L. botrana* pheromone glands contain further compounds, which are of potential interest for the development of integrated pest management systems.

METHODS AND MATERIALS

Insects. Insects were collected near Fribourg (Germany), San Michele (Italy), and La Rioja (Spain) and were reared in the laboratory on an artificial diet under a 16-hr light/8-hr dark photoperiod. Pupae were separated by sex, and adult moths were kept in 36-l Plexiglas cages.

Chemical Analysis. Excised glands of 2- to 3-d-old calling females were extracted for 1 min in 7 μl of redistilled hexane (Labscan, Malmö, Sweden). These extracts were analyzed on a Hewlett-Packard (HP) 5970B mass spectrometer (MS) with electron impact ionization, which was interfaced with a HP 5890 gas chromatograph (GC), using a polar DB-Wax column (30 m × 0.25 mm; J&W Scientific, Folsom, CA, USA). Gland extracts were further studied on an HP 6890 GC, using a DB-Wax column and a nonpolar SE-54 column (25 m × 0.32 mm; Kupper, Bonaduz, Switzerland). The oven temperature was programmed from 60°C/2 min, then at 10°C/min to 100°C, at 1.5°C/min to 150°C, and at 10°C/min to 230°C.

Gland extracts were also injected on an HP 6890 GC equipped with an HP-INNOWax capillary column (30 m × 0.25 mm), which was coupled to an electroantennographic detector (GC-EAD; Syntech, Hilversum, The Netherlands). The oven was programmed from 50°C (2 min hold) at 10°C/min to 230°C. The column outlet was split in a 1:1 ratio for simultaneous recordings by

EAD and the flame ionization detector of the GC. Excised antennae were mounted in a holder, which was 0.5 cm away from the GC outlet.

Synthesis. The syntheses of all compounds are described in detail in the online supplement (Appendix) (Electronic Supplementary Material is available for this article at http://dx.doi.org/10.1007/s10886-005-8404-1 and is accessible for authorized users). 3-Octyn-1-ol (Aldrich) was isomerized to 7-octyn-1-ol using the "acetylene zipper" protocol (Abrams and Shaw, 1988). The obtained octynol served as the starting material for the syntheses of (7*Z*,9*E*,11)-dodecatrienyl acetate (Scheme 1) as well as for the (7*E*,9*Z*,11) and the (7*E*,9*E*,11) isomers (Scheme 2). Preparation of the latter two acetates proceeded similarly to the synthesis of the corresponding 9,11,13-tetradecatrienyl acetates (Tellier, 1991; Tellier et al., 1991), whereas the approach to (7*Z*,9*E*,11)-dodecatrienyl acetate was essentially the same as the sequences described for (9*Z*,11*E*,13)-tetradecatrienal (Millar, 1990) and for (11*Z*,13*E*,15)-hexadecatrienyl acetate (Gries et al., 2004).

Hydrogenation of conjugated triple bonds with activated zinc in methanol produced (*Z*)-double bonds (Boland et al., 1987), whereas $LiAlH_4$ reduction yielded (*E*)-double bonds (Porter et al., 1985). All reactions were carried out under argon. Intermediates were purified by distillation *in vacuo* or by column chromatography on silica gel. In all three syntheses, the alcohols corresponding to the target acetates were carefully recrystallized from hexane/toluene at −25°C to furnish products with stereochemical purity >98%.

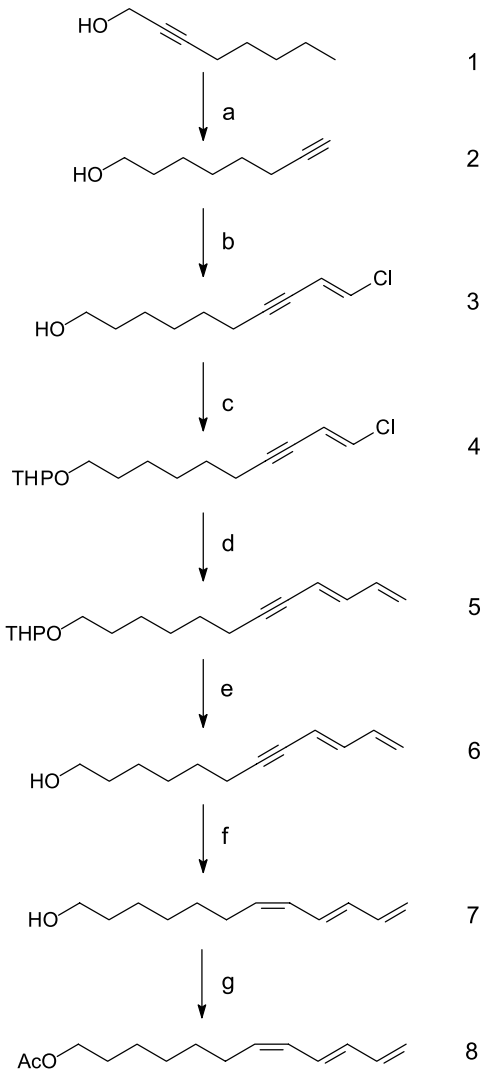
For the synthesis of (7*Z*,9*E*,11)-dodecatrienyl acetate, 7-octyn-1-ol was chain-elongated with (*E*)-1,2-dichloroethane (Sonogashira, 1991). The resulting ω-chloroalcohol was protected and, again, chain-elongated with vinyl magnesium bromide (Millar, 1990). The C_{14} chain obtained was easily transformed into the final product.

For the synthesis of the two (7*E*)-configured trienylacetates, 7-octyn-1-ol (**1**) was transformed in two steps into (*E*)-1-*t*-butoxy-8-iodo-7-octene (Alexakis et al., 1988; Alexakis and Duffault, 1988). Chain elongation of this key intermediate used Pd°-catalyzed alkynyl coupling (Sonogashira, 1991 and references cited therein). The syntheses of the target acetates were completed by conventional steps (Scheme 2).

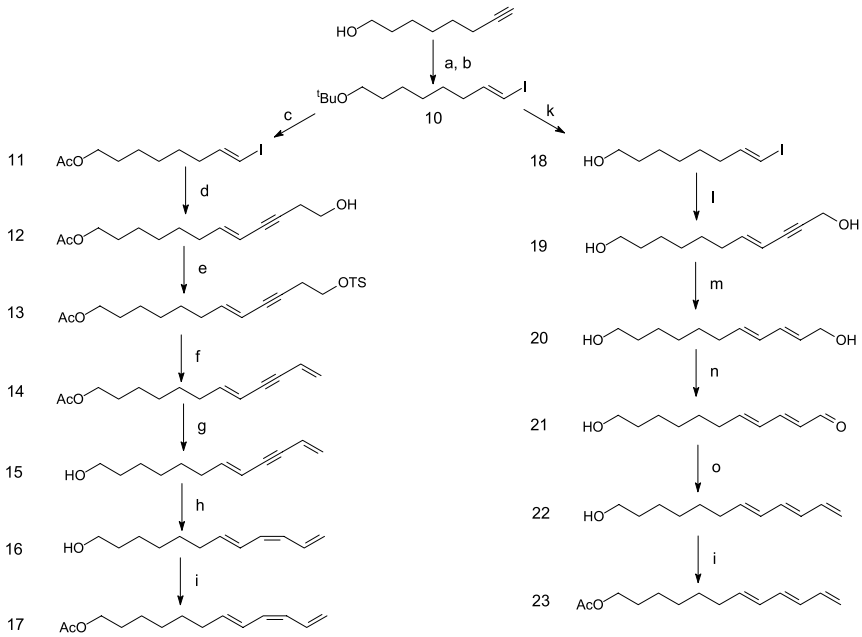
Details of the syntheses are provided in the online supplement.

Wind Tunnel. The wind tunnel hardware and the test protocol have been described (Witzgall et al., 2001). The wind tunnel with a flight section of 63 × 90 × 200 cm was lit diffusely at 6.5 lx. Charcoal-filtered air was blown by a horizontal fan through the tunnel at 30 cm/sec, temperature was 23 ± 2°C, and relative humidity was 40–60%. The outcoming air was aspired by another fan and cleaned by two sets of charcoal filters.

Synthetic pheromone components in redistilled ethanol were released from a piezoelectric sprayer (El-Sayed et al., 1999; Gödde et al., 1999). The



SCHEME 1. a) 1,3-Diaminopropane/Li; KO ^{t}Bu; b) (*E*)-1,2-dichloroethene, diisopropylamine, $PdCl_2$ $(PPh_3)_2$, CuI/THF; c) DHP, TsOH/Et_2O; d) vinylmagnesium bromide/THF; e) TsOH/MeOH; f) Zn/MeOH; g) Ac_2O/pyridine.



SCHEME 2. a) Amberlyst 15/isobutene–heptane → 9; b) DIBAH/I_2; c) $Ac_2O/FeCl_3/Et_2O$; d) 3-butyn-1-ol, Pd (PPh_3)$_4$, *n*-$C_3H_7NH_2$, CuI/toluene; e) TsCl/pyridine; f) K–O ^{t}Bu, 18-crown-6/toluene; g) KOH/MeOH; h) Zn/MeOH; i) Ac_2O/pyridine; k) i: TMS/(1.3 eq)/CCl_4 rt, ii: MeOH; l) as in (d) but using propynol; m) $LiAlH_4$/THF; n) MnO_2/CH_2Cl_2; o) Ph_3PCH_3Br, BuLi/THF, −78°C.

solution was delivered at a rate of 10 µl/min through Teflon tubing to a 20-µl glass capillary tube with a drawn-out tip. The capillary was vibrated by a piezo-ceramic disk at ca. 100 kHz to produce an aerosol, which evaporated within a few centimeters from its tip. This "sprayer" allows application of pheromone chemicals at a constant rate and known chemical purity. Isomeric purity of the test compounds was ≥99%, except for 7*E*,9*E*,11-12Ac (98%) and 7*E*,9*Z*,11-12Ac (95%). Single females were held in glass tubes (2.5 × 5 cm) covered with gauze on both sides. Females were kept in the wind tunnel for at least 30 min before experiments until they showed the typical calling behavior.

Wind tunnel tests were carried out from 1 to 4 hr after onset of the scotophase. Four batches of 15 2- to 3-d-old males (N = 60) were tested per pheromone blend on four different days. Single males were placed in glass tubes (2.5 × 12.5 cm), which were stoppered with gauze on both sides ca. 15 min before testing. Males were tested individually and were given 2 min to respond. The number of males landing at the source, following upwind-oriented flight, in

each batch of 15 males was transformed to $\log(x + 1)$ and submitted to a one-way ANOVA followed by Duncan's multiple range test ($P < 0.05$).

RESULTS AND DISCUSSION

Chemical analysis of sex pheromone gland extracts of *L. botrana* females confirmed the presence of previously identified compounds. The main pheromone compound is *E*7,*Z*9-12Ac. The analogous *E*7,*Z*9-12OH and two saturated alcohols were present, in addition to several saturated, mono-, and diunsaturated acetates (Table 1; Arn et al., 1988; El-Sayed et al., 1999).

GC-EAD recordings (Figure 1) showed an additional, active compound eluting after *E*7,*Z*9-12OH. GC-MS studies indicated a conjugated trienylacetate because of characteristic fragments and its retention time. Co-occurrence of Δ7, Δ9-12Ac and 11-12Ac in *L. botrana* pheromone glands made Δ7, Δ9,11-12Ac a likely candidate. Synthesis of 7,9,11-12Ac isomers confirmed the presence of *E*7,*E*9,11-12Ac in glands of Italian females (Table 1). The *EZ* and *ZE* isomers were found in smaller amounts. These three trienyl acetates also were present in gland extracts of females from Germany and Spain (not shown in Table 1). This is the first report of a triply unsaturated compound in a tortricid moth (Arn et al., 2000). The extremely unstable (7*Z*,9*Z*,11)-dodecatrienyl acetate or corresponding products of its rearrangements (Näf et al., 1975; Rockach et al., 1980) was not detected. No attempts were made to synthesize this isomer.

Another compound that elicited antennal responses, which could not be associated with known *L. botrana* pheromone compounds, eluted shortly before *E*- and *Z*9-12Ac. This new compound was shown to be *E*7-12Ac. It was present in gland extracts of females from Italy (Table 1; Figure 1) and from Spain. However, *E*7-12Ac was not detected in insects from Germany (this study, data not shown), which corroborates analysis of insects from Switzerland (Arn et al., 1988). This suggests the occurrence of *L. botrana* pheromone dialects north and south of the Alps.

Wind tunnel tests were performed with the newly identified compounds, *E*7-12Ac, and the *EE*, *EZ*, and *ZE* isomers of 7,9,11-12Ac (Table 2). These compounds were tested in two-component blends with the main compound, using males of a lab population originating from northern Italy. A 10% addition of *Z*7,*E*9,11-12Ac significantly increased attraction followed by landing at the source from 24% of the test males with the main compound alone to 65% ($N = 60$; Table 2). The two other isomers, *E*7,*E*9,11-12Ac and *E*7,*Z*9,11-12Ac, were less active. A four-component blend of the main compound plus 10% of each of the three triene isomers attracted 61% of males to the source. The trienic *E*7,*Z*9,11-12Ac may mimic the main pheromone compound *E*7,*Z*9-12Ac because some males were attracted to this compound as a single component.

TABLE 1. COMPONENTS OF THE SEX PHEROMONE GLAND OF *L. BOTRANA* FEMALES FROM NORTHERN ITALY

Compound	Short form	Identification[a]	Amount[b] (pg/Female)	Amount[b] (%)	GC-EAD[c] (mV/pg × 100)
Dodecyl acetate	12Ac	GC, MS	28 ± 12	3	–
(*E*)-7-Dodecenyl acetate[d]	*E*7-12Ac	GC, MS	106 ± 16	12	0.28 ± 0.24
(*E*)-9-Dodecenyl acetate	*E*9-12Ac	GC, MS	27 ± 30	3	0.43 ± 0.31
(*Z*)-9-Dodecenyl acetate	*Z*9-12Ac	GC, MS	113 ± 46	13	0.89 ± 0.33
11-Dodecenyl acetate	11-12Ac	GC	Trace[e]	–	–
(*E*,*E*)-7,9-Dodecadienyl acetate	*E*7,*E*9-12Ac	GC	28 ± 23	3	–[f]
(*E*,*Z*)-7,9-Dodecadienyl acetate	*E*7,*Z*9-12Ac	GC, MS	867 ± 472	100	0.46 ± 0.29
(*Z*,*E*)-7,9-Dodecadienyl acetate	*Z*7,*E*9-12Ac	GC, MS	25 ± 11	3	1.41 ± 0.64
(*Z*,*Z*)-7,9-Dodecadienyl acetate	*Z*7,*Z*9-12Ac	GC	Trace[e]	–	–[f]
(*E*,*Z*)-7,9-Dodecadien-1-ol	*E*7,*Z*9-12OH	GC, MS	88 ± 35	10	0.12 ± 0.18
(*E*,*E*)-7,9,11-Dodecatrienyl acetate[d]	*E*7,*E*9,11-12Ac	GC, MS	65 ± 27	7	0.73 ± 0.30
(*E*,*Z*)-7,9,11-Dodecatrienyl acetate[d]	*E*7,*Z*9,11-12Ac	GC	Trace[e]	–	–
(*Z*,*E*)-7,9,11-Dodecatrienyl acetate[d]	*Z*7,*E*9,11-12Ac	GC	1 ± 2	<1	–
Tetradecyl acetate	14Ac	GC	1 ± 2	<1	–
Octadecan-1-ol	18OH	GC, MS	73 ± 15	8	–
Eicosan-1-ol	20OH	GC, MS	109 ± 127	13	–

[a] Identification method: gas chromatography (GC) on two different columns, and mass spectrometry (MS).
[b] Absolute and relative amounts (mean ± SD) in batch extracts of 20–75 female pheromone glands ($N = 7$).
[c] Antennal response (mean ± SD) during GC–electroantennographic detection (EAD) recordings ($N = 5$).
[d] Newly identified compound.
[e] Trace amounts <1 pg/female.
[f] Recording not possible during antennal recovery phase after stimulation by preceding compound.

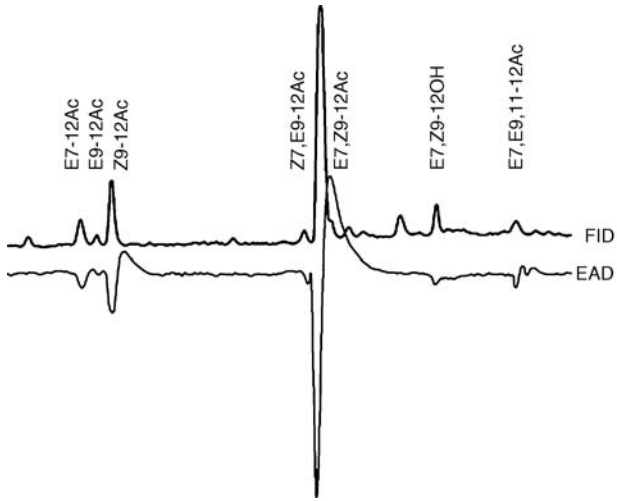


FIG. 1. Analysis of pheromone gland extracts of grapevine moth *L. botrana* females from Northern Italy ($N = 5$) by GC-EAD. FID: response of the flame ionization detector; EAD: response of a *L. botrana* male antenna.

*E*7-12Ac had a similar effect as *Z*7,*E*9,11-12Ac, with addition of 10% increasing landings to 67% ($N = 60$; Table 2). Male attraction to the best synthetic blends, at a release rate of 10 pg/min of the main compound, was not different from attraction to calling females. The female release rate is on the order of 10 pg/min (El-Sayed et al., 1999).

The monoene acetates *E*9-12Ac, *Z*9-12Ac, and 11-12Ac, and the alcohol corresponding to the main compound, *E*7,*Z*9-12OH, are known pheromone synergists of *L. botrana* (Arn et al., 1988; El-Sayed et al., 1999). Here, we show that *E*7-12Ac and *Z*7,*E*9,11-12Ac are additional, strong pheromone synergists. Partial blends are as attractive as calling females (Table 2; El-Sayed et al., 1999), and this may indicate redundancy in the pheromone signal of *L. botrana*, as described for the cabbage looper moth *Trichoplusia ni* (Linn et al., 1984).

Availability of new pheromone synergists is nonetheless of practical importance. Mating disruption of *L. botrana* by aerial dissemination of pheromone can be effective, but there are many reports of unexpected failures (Arn and Louis, 1996). For example, geographic pheromone races may contribute to inconsistent results. Studies of *L. botrana* sex pheromone have all relied on insects from Switzerland (Roelofs et al., 1973; Arn et al., 1988; El-Sayed et al., 1999), whereas pheromone-mediated mating disruption is used in vineyards both north and south of the Alps (Kast, 2001; Louis and Schirra, 2001; Varner

TABLE 2. WIND TUNNEL RESPONSE OF *L. Botrana* MALES FROM NORTHERN ITALY TO BLENDS OF THE MAIN PHEROMONE COMPOUND *E*7,*Z*9-12AC AND MINOR COMPOUNDS FROM FEMALE PHEROMONE GLANDS

Compound	Blend composition (pg/min)[a]							
*E*7,*Z*9-12Ac	10	10	10	10	10		10	
*Z*7,*E*9,11-12Ac		1			1			
*E*7,*Z*9,11-12Ac			1		1	10		
*E*7,*E*9,11-12Ac				1	1			
*E*7-12Ac							1	
Calling female[b]								1[b]
Attraction to source (%)[c]	24 c	65 a	48 b	38 bc	61 ab	11 d	67 a	71 a

[a] Release rate (pg/min).
[b] One live calling female.
[c] Percentage of males ($N = 60$) landing at the odor source. Numbers followed by different letters are significantly different according to ANOVA followed by Duncan's test ($P < 0.05$).

et al., 2001; Zingg, 2001). The availability of a behavioral synergist, *E*7-12Ac, which can be made economically, merits a reinvestigation of the effect of pheromone synergists on *L. botrana* mating disruption.

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(*S*,*S*)-2,12-, (*S*,*S*)-2,13-, AND (*S*,*S*)-2,14-DIACETOXYHEPTADECANES: SEX PHEROMONE COMPONENTS OF RED CEDAR CONE MIDGE, *Mayetiola thujae*

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Abstract—We identified, synthesized, and field-tested the sex pheromone of female red cedar cone midge *Mayetiola thujae* (Hedlin) (Diptera: Cecidomyiidae), a pest insect in red cedar *Thuja plicata* seed orchards. Coupled gas chromatographic (GC)–electroantennographic detection analyses of pheromone extract revealed three components (**A**, **B**, **C**) that elicited responses from antennae of males, all of which occurred below the detection threshold of the mass spectrometer and thus had to be identified without spectroscopic data. Taking into account (1) their retention indices (RI) on three GC columns (DB-5, DB-23, and DB-210), (2) intercolumn RI differentials, and (3) the molecular structures of known cecidomyiid pheromones, we synthesized seven candidate pheromone components: 2,10-, 2,11-, 2,12-, 2,13-, 2,14-, 2,15- and 2,16-diacetoxyheptadecanes. Of these, 2,12-, 2,13-, and 2,14-diacetoxyheptadecane had RIs on all columns consistent with those of **A**, **B**, and **C** and elicited strong antennal responses when tested at picogram levels. In field experiments with the twelve stereoselectively synthesized stereoisomers, only the *SS*-stereoisomers of 2,12-, 2,13-, and 2,14-diacetoxyheptadecane attracted male *M. thujae*. The three-component *SS*-stereoisomer blend was more attractive than the 12-component blend of all stereoisomers, suggesting that one or several nonnatural stereoisomers are inhibitory. One-, two-, and three-component lures of the *SS*-stereoisomers were equally effective in attracting male *M. thujae*, indicating redundancy in the phero-

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mone. Identification of the *M. thujae* sex pheromone will allow development of pheromone-based monitoring, and possibly control, of *M. thujae* populations in *T. plicata* seed orchards.

Key Words—Red cedar cone gall midge, *Mayetiola thujae*, Cecidomyiidae, sex pheromone, stereoisomers, 2,12-diacetoxyheptadecane, 2,13-diacetoxyheptadecane, 2,14-diacetoxyheptadecane.

INTRODUCTION

Increasing interest in western red cedar *Thuja plicata* Donn *ex* D. Don reforestation in recent decades has led to the planting of *T. plicata* seed orchards in British Columbia and more recently in Washington and Oregon. Throughout the range of *T. plicata*, the western red cedar cone midge *Mayetiola thujae* (Hedlin) (Diptera: Cecidomyiidae) is a serious pest of *T. plicata* seed production (Hedlin et al., 1980). In the absence of control measures, 100% of *T. plicata* cones may be infested by immature stages of *M. thujae* (Hedlin, 1964).

Mayetiola thujae and its detailed life history have been described by Hedlin (1959, 1964). Most of the life cycle is spent within *T. plicata* cones. Adults emerge from overwintering puparia in previous-year cones in late winter/early spring during the *T. plicata* pollination period. Adults are short-lived. Emergence, mating, oviposition, and death occur in less than 1 wk. Larvae hatch shortly after oviposition and spend the spring and early summer feeding upon scale and seed tissues within developing cones. In late summer, larvae spin individual silk cocoons between cone scales. Pupation occurs during the winter, although some larvae enter a period of extended diapause and apparently pupate the next or subsequent winters.

During the annual *T. plicata* pollination period, the British Columbia Ministry of Forests determines population levels of *M. thujae* through a tedious process of sampling and dissecting cones and counting eggs. When population levels exceed a variable economic threshold, they may be reduced by killing larvae within cones with systemic insecticides. In Washington and Oregon, populations are controlled with sprays of contact insecticides made on a calendar basis, targeting adults during the pollination period. Identification and deployment of the *M. thujae* sex pheromone may help pinpoint the adult flight period, facilitate assessment of population levels, and offer alternative means of population control.

METHODS AND MATERIALS

Experimental Insects. In early February 2002 and 2003, *T. plicata* cones infested with *M. thujae* larvae were collected near the north shore of Elk Lake, Victoria, British Columbia (48°32′N/123°23′W), and shipped to Simon Fraser

University. Cones were kept in a mesh cage (30 × 30 × 45 cm high) at room temperature and with a photoperiod of 14-hr light/10-hr dark, inducing the emergence of *M. thujae* within 1–3 wk.

Preparation and Analysis of Pheromone Extracts. Insects were separated by sex, and ovipositors with pheromone glands of (calling) females were removed and extracted with hexane for 5–50 min. Aliquots of 5–10 female equivalents (FE) of abdominal tip extracts were analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD; Arn et al., 1975), employing equipment and procedures previously described in detail (Gries et al., 2002). GC columns and temperature programs in this study were as follows—DB-5: 100°C (1 min), 15°C/min to 280°C; DB-23: 100°C (1 min), 10°C/min to 230°C; DB-210: 100°C (1 min), 20°C/min to 230°C. Nuclear magnetic resonance (NMR) spectroscopy of synthetic compounds was conducted on a Varian AS500 spectrometer at 499.77 MHz for ^{1}H and 125.68 MHz for ^{13}C spectra, with chemical shifts reported in parts per million relative to TMS (^{1}H, δ = 0.00) and $CDCl_3$ (^{13}C, δ = 77.00). Elemental analyses were performed using a Carlo-Erba model 1106 elemental analyzer. Compounds were purified for elemental analyses with a Waters semipreparative high-performance liquid chromatograph (HPLC; Delta 600 Pump, 600 Controller, 2487 Dual λ Absorbance Detector) equipped with a Nova Pak® HR C18 preparative column (19 × 300 mm; 6 μm, 60 Å) eluted with 10 ml/min of acetonitrile. Melting points of chemicals are reported uncorrected.

Syntheses. Racemic 2,10-, 2,11-, 2,12-, 2,13-, 2,14-, 2,15-, and 2,16-diacetoxyheptadecane (2,10-17; 2,11-17; 2,12-17; 2,13-17; 2,14-17; 2,15-17; 2,16-17) were obtained by coupling freshly prepared α,ω-di-Grignard reagents with equivalent amounts of equimolar mixtures of two epoxides (e.g., two equivalents of propylene oxide for 2,16-17), hydrolysis, separation of the resulting diols by flash column chromatography, and acetylation of asymmetric diols (Figure 1). Diols were well separated by flash chromatography (Still et al., 1978; Choi et al., 2004) on 50–60 g of silica gel 60 (230–400 mesh, E. Merck, Darmstadt) inside a glass column (30 × 600 mm), using a gradual increase (20–90%) of ether in the eluent (ether/hexane) mixture. Diols were also separated by thin-layer chromatography [DC-Alufolien, Aluminiumoxid 60 F_{254} neutral (EM Science, PO Box 70, Gibbstown, NJ, USA); ether (2)/hexane (1)] with the following Rf values: 0.28 (**1a**), 0.09 (**2a**), 0.45 (**3a**); 0.23 (**1b**), 0.11 (**2b**), 0.40 (**3b**); 0.21 (**1c**), 0.13 (**2c**), 0.32 (**3c**). Enantioselective syntheses of 2,12-17, 2,13-17, and 2,14-17 are described below. Compounds were synthesized from optically active propylene oxides and 1,2-epoxyheptanes, 1,2-epoxyhexanes, or 1,2-epoxypentanes. Separation of diols (**1**, **2**, and **3** in Figure 1) and acetylation of asymmetric diols led to the 12 target chiral diacetates (**4**).

(*S*)- and (*R*)-Propylene oxides [>99% enantiomeric excess (ee)] were purchased (Avocado Research Chemicals Ltd., Heysham, Lancs, UK), whereas

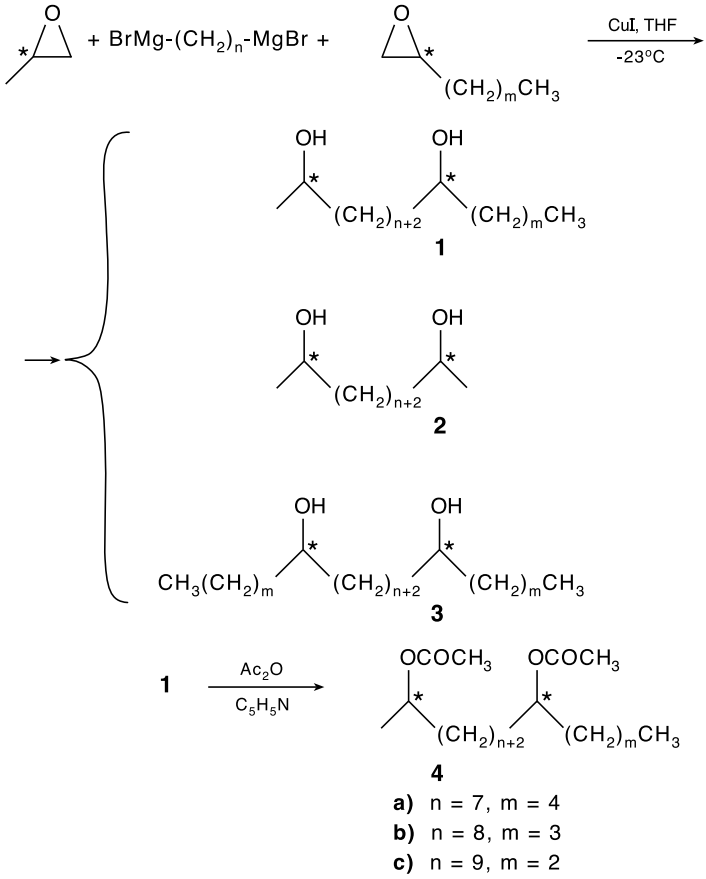

FIG. 1. Enantioselective syntheses of 2,12-, 2,13-, and 2,14-diacetoxyheptadecanes (**4a**, **4b**, and **4c**).

longer-chain (*S*)- and (*R*)-1,2-epoxides were synthesized according to methods previously reported (Tokunaga et al., 1997; Schaus et al., 1998). Enantiomeric excess of synthesized 1,2-epoxides (Table 1) was determined by ring opening with methylmagnesium chloride in the presence of CuI at −23°C, acetylation of the resulting alcohols with acetic anhydride in pyridine, and chromatography of resulting acetates on a custom-made chiral GC column coated with a 1:1 mixture of heptakis-(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin and OV-1701 (König et al., 1992; Pietruszka et al., 1992); temperature program: 80°C (10 min), then 5°C/min to 120°C; split injection. (*R*,*R*)-, (*S*,*S*)-, (*S*,*R*)-, and (*R*,*S*)-2,12-, 2,13-, and 2,14-diacetoxyheptadecanes prepared from the epoxides were

TABLE 1. ENANTIOMERIC EXCESS (ee) OF EPOXIDES AND DIACETOXYHEPTADECANES[a]

Compounds	ee (%)
(*R*)-1,2-Epoxypentane	89
(*S*)-1,2-Epoxypentane	99
(*R*)-1,2-Epoxyhexane	84
(*S*)-1,2-Epoxyhexane	94
(*R*)-1,2-Epoxyheptane	98
(*S*)-1,2-Epoxyheptane	99
(*S*,*S*)-2,12-Diacetoxyheptadecane	98
(*R*,*S*)-2,12-Diacetoxyheptadecane	98
(*R*,*R*)-2,12-Diacetoxyheptadecane	97
(*S*,*R*)-2,12-Diacetoxyheptadecane	97
(*S*,*S*)-2,13-Diacetoxyheptadecane	93
(*R*,*S*)-2,13-Diacetoxyheptadecane	93
(*R*,*R*)-2,13-Diacetoxyheptadecane	83
(*S*,*R*)-2,13-Diacetoxyheptadecane	83
(*S*,*S*)-2,14-Diacetoxyheptadecane	98
(*R*,*S*)-2,14-Diacetoxyheptadecane	98
(*R*,*R*)-2,14-Diacetoxyheptadecane	88
(*S*,*R*)-2,14-Diacetoxyheptadecane	88

[a]Chemical purities of diacetoxyheptadecane stereoisomers ranged between 91 and 98%.

assumed to have corresponding ee because chiral centers were not affected during the opening of epoxide rings and subsequent acetylation.

*(S,S)-2,12-Dihydroxyheptadecane (SS-**1a**, Figure 1).* At −23°C under argon and with vigorous stirring CuI (0.31 g, 1.63 mmol) was added to a freshly prepared solution of Grignard reagent [1,7-dibromoheptane (2.10 g, 8.14 mmol) and activated Mg (0.80 g, 32.9 mmol) were refluxed in tetrahydrofuran (THF; 50 ml) for 3 hr under argon excess of Mg was not removed]. After 5 min, a mixture of (*S*)-propylene oxide (0.63 ml, 8.95 mmol) and (*S*)-1,2-epoxyheptane (1.25 ml, 8.95 mmol, 98% by GC) in THF (5 ml) was added. The temperature was maintained at −23°C for 30 min. The reaction mixture was warmed to room temperature, quenched with saturated aqueous NH_4Cl, and extracted with ether (5 × 30 ml). Extracts were washed, dried, and concentrated. (*S*,*S*)-2,12-Heptadecanediol (*SS*-**1a**) was completely separated by silica (60 g) flash chromatography (35–45% ether in hexane as eluent) from the faster-eluting (*S*,*S*)-6,16-dihydroxyheneicosane (25% ether in hexane as eluent) and the slower-eluting (*S*,*S*)-2,12-dihydroxytridecane (50–80% ether in hexane as eluent). *SS*-**1a** was obtained in 72% yield (0.80 g, 2.94 mmol) as a white solid; m.p. 50–51°C; ^{1}H NMR: ($CDCl_3$): δ = 0.89 (t, 3H, J = 6.8 Hz), 1.18 (d, 3H, J = 6.4 Hz), 1.21–1.51 (m, 26H), 2.17 (s, 2H), 3.58 (m, 1H), 3.78 (m, 1H); ^{13}C NMR ($CDCl_3$): δ = 14.05, 23.49, 25.32, 25.63, 25.74, 29.49, 29.56, 29.57, 29.60, 29.63, 29.67, 30.93, 37.45, 37.46, 39.35, 68.19, 72.02. Anal. calcd. for

$C_{17}H_{36}O_2$ (%): C, 74.94; H, 13.32; found C, 75.30; H, 13.35. (*S*,*R*)-, (*R*,*S*)-, and (*R*,*R*)-2,12-Dihydroxyheptadecanes (*SR*-**1a**, *RS*-**1a**, and *RR*-**1a**, respectively) were obtained from 1,7-dibromoheptane, the corresponding (*R*)- and (*S*)-propylene oxides, and (*R*)- and (*S*)-1,2-epoxyheptanes (see above). Spectroscopic data for these alcohols matched those of *SS*-**1a**; m.p. of *SR*-**1a**, *RS*-**1a**, and *RR*-**1a** were 80–81, 80–81, and 50–51°C, respectively.

*(S,S)-2,13-Dihydroxyheptadecane (SS-**1b**).* This was obtained in 56% yield from 1,8-dibromooctane, (*S*)-propylene oxide, and (*S*)-1,2-epoxyhexane (see above). M.p. 56°C; ^{1}H NMR ($CDCl_3$): δ = 0.91 (t, 3H, J = 7.0 Hz), 1.18 (d, 3H, J = 6.4 Hz), 1.20–1.50 (m, 26H), 2.17 (s, 2H), 3.58 (m, 1H), 3.79 (m, 1H); ^{13}C NMR ($CDCl_3$): δ = 14.07, 22.76, 23.48, 25.63, 25.74, 27.83, 29.53, 29.57, 29.58, 29.60, 29.67, 30.93, 37.16, 37.46, 39.35, 68.20, 72.00. Anal. calcd. for $C_{17}H_{36}O_2$ (%): C, 74.94; H, 13.32; found C, 74.95; H, 13.10. (*S*,*R*)-, (*R*,*S*)-, and (*R*,*R*)-2,13-Dihydroxyheptadecanes (*SR*-**1b**, *RS*-**1b**, and *RR*-**1b**, respectively) were obtained from 1,8-dibromooctane, the corresponding (*R*)- and (*S*)-propylene oxides, and (*R*)- and (*S*)-1,2-epoxyhexanes. Spectroscopic data for these alcohols matched those of *SS*-**1b**; m.p. of *SR*-**1b**, *RS*-**1b**, and *RR*-**1b** were 80–81, 79, and 55–56°C, respectively.

*(S,S)-2,14-Dihydroxyheptadecane (SS-**1c**).* This was obtained in 40% yield from 1,9-dibromononane, (*S*)-propylene oxide, and (*S*)-1,2-epoxypentane (see above). M.p. 50°C; ^{1}H NMR ($CDCl_3$): δ = 0.92 (t, 3H, J = 6.8 Hz), 1.18 (d, 3H, J = 6.4 Hz), 1.24–1.51 (m, 26H), 2.17 (s, 2H), 3.60 (m, 1H), 3.80 (m, 1H); ^{13}C NMR ($CDCl_3$): δ = 14.11, 18.82, 23.47, 25.63, 25.75, 29.54, 29.55, 29.57, 29.59, 29.61, 29.64, 29.67, 37.48, 39.35, 39.65, 68.17, 71.72. Anal. calcd. for $C_{17}H_{36}O_2$ (%): C, 74.94; H, 13.32; found C, 75.25; H, 13.06. (*S*,*R*)-, (*R*,*S*)-, and (*R*,*R*)-2,14-Dihydroxyheptadecanes (*SR*-**1c**, *RS*-**1c**, and *RR*-**1c**, respectively) were obtained from 1,9-dibromononane, the corresponding (*R*)- and (*S*)-propylene oxides, and (*R*)- and (*S*)-1,2-epoxypentanes. Spectroscopic data of these alcohols matched those of *SS*-**1c**; m.p. of *SR*-**1c**, *RS*-**1c**, and *RR*-**1c** were 81, 79–80, and 50°C, respectively.

2,12-, 2,13-, and 2,14-Diacetoxyheptadecanes. The four stereoisomers each of 2,12-, 2,13-, and 2,14-diacetoxyheptadecane (**4a**–**c** in Figure 1) were obtained in 90–95% yields by stirring (12 hr) the corresponding diols **1a**–**c** (0.6–0.8 g) in pyridine (5 ml) and acetic anhydride (1.5 ml). Excess of the latter two reagents was eliminated *in vacuo*, and **4a**–**c** (all oils) were purified (94–98%) by flash chromatography [silica (15 g); 10% ether in hexane as eluent]. For NMR and elemental analyses, some material was further purified by HPLC.

*(S,S)-2,12-Diacetoxyheptadecane (SS-**4a**).* ^{1}H NMR ($CDCl_3$): δ = 0.87 (t, 3H, J = 6.9 Hz), 1.19 (d, 3H, J = 6.3 Hz), 1.21–1.34 (m, 20H), 1.42–1.52 (m, 6H), 2.02 (s, 3H), 2.03 (s, 3H), 4.86 (m, 2H); ^{13}C NMR ($CDCl_3$): δ = 14.00, 19.94, 21.29, 21.39, 22.53, 24.96, 25.30, 25.39, 29.42, 29.46, 29.48 (two),

29.50, 31.70, 34.05, 34.10, 35.90, 71.05, 74.41, 170.79, 170.93. Anal. calcd. for $C_{21}H_{40}O_4$ (%): C, 70.74; H, 11.31.; found C, 70.88; H, 11.20. $[\alpha]_D^{20} = -0.51°$ (c. 1.17, $CHCl_3$). MS: 43 (100), 55 (31), 67 (38), 81 (46), 83 (38), 95 (42), 109 (38), 123 (15), 138 (9), 152 (6), 165 (14), 183 (8), 225 (8), 236 (8), 253 (11). Spectroscopic data of *SR*-**4a**, *RS*-**4a**, and *RR*-**4a** matched those of *SS*-**4a**. For *SR*-**4a**, $[\alpha]_D^{20} = +1.32°$ (c. 1.50, $CHCl_3$), for *RS*-**4a**, $[\alpha]_D^{20} = -2.31°$ (c. 2.30, $CHCl_3$), and for *RR*-**4a**, $[\alpha]_D^{20} = +0.37°$ (c. 2.70, $CHCl_3$).

*(S,S)-2,13-Diacetoxyheptadecane (SS-**4b**).* ^{1}H NMR ($CDCl_3$): δ = 0.88 (t, 3H, J = 6.9 Hz), 1.20 (d, 3H, J = 6.3 Hz), 1.22–1.34 (m, 20H), 1.42–1.54 (m, 6H), 2.02 (s, 3H), 2.03 (s, 3H), 4.86 (m, 2H); ^{13}C NMR ($CDCl_3$): δ = 13.99, 19.95, 21.29, 21.39, 22.60, 25.30, 25.39, 27.47, 29.44, 29.50, 29.51 (unresolved), 29.52, 33.81, 34.11, 35.90, 71.06, 74.40, 170.78, 170.93. Anal. calcd. for $C_{21}H_{40}O_4$ (%): C, 70.74; H, 11.31; found C, 70.98; H, 11.37. $[\alpha]_D^{20} = -0.62°$ (c. 1.45, $CHCl_3$). MS: 43 (100), 55 (26), 67 (36), 81 (42), 95 (39), 109 (24), 110 (17), 123 (23), 138 (8), 152 (7), 179 (8), 197 (6), 236 (10), 239 (9), 253 (11). Spectroscopic data of *SR*-**4b**, *RS*-**4b**, and *RR*-**4b** matched those of *SS*-**4b**. For *SR*-**4b**, $[\alpha]_D^{20} = +1.47°$ (c. 2.25, $CHCl_3$), for *RS*-**4b**, $[\alpha]_D^{20} = -2.08°$ (c. 2.50, $CHCl_3$), and for *RR*-**4b**, $[\alpha]_D^{20} = +0.53°$ (c. 2.50, $CHCl_3$).

*(S,S)-2,14-Diacetoxyheptadecane (SS-**4c**).* ^{1}H NMR ($CDCl_3$): δ = 0.90 (t, 3H, J = 6.9 Hz), 1.19 (d, 3H, J = 6.3 Hz), 1.23–1.35 (m, 20H), 1.43–1.54 (m, 6H), 2.02 (s, 3H), 2.03 (s, 3H), 4.87 (m, 2H); ^{13}C NMR ($CDCl_3$): δ = 13.99, 18.56, 19.95, 21.28, 21.40, 25.30, 25.40, 29.44, 29.52 (unresolved), 29.53, 29.54, 29.59, 34.14, 35.91, 36.28, 71.06, 74.17, 170.79, 170.93. Anal. calcd. for $C_{21}H_{40}O_4$ (%): C, 70.74; H, 11.31.; found C, 70.47; H, 11.23. $[\alpha]_D^{20} = -2.60°$ (c. 1.65, $CHCl_3$). MS: 43 (100), 55 (26), 67 (38), 81 (40), 96 (34), 109 (21), 111 (22), 123 (19), 137 (11), 152 (5), 166 (6), 193 (4), 211 (3), 236 (9), 253 (17). Spectroscopic data of *SR*-**4c**, *RS*-**4c**, and *RR*-**4c** matched those of *SS*-**4c**. For *SR*-**4c**, $[\alpha]_D^{20} = +4.29°$ (c. 1.55, $CHCl_3$), for *RS*-**4c**, $[\alpha]_D^{20} = -3.58°$ (c. 1.35, $CHCl_3$), and for *RR*-**4c**, $[\alpha]_D^{20} = +2.97°$ (c. 3.37, $CHCl_3$).

Field Experiments. Field experiments were conducted in an abandoned *T. plicata* seed orchard immediately west of Blenkinsop Lake, Victoria, BC (48°29′N/123°23′W). Trees were 15–24 yr old and spaced 2.4 and 7.3 m within and between rows, respectively. At 10- to 15-m intervals, sticky 2-l milk carton traps (Gray et al., 1984) were suspended from trees 1.5 m above ground in complete randomized blocks. Traps were baited with a piece of dental cotton roll (10 × 15 mm; Richmond Dental, Charlotte, NC, USA) impregnated with candidate pheromone components in HPLC-grade hexane.

Experiment 1 was designed to determine which stereoisomers (*RR*, *RS*, *SR*, or *SS*) of 2,12-17, 2,13-17, and 2,14-17 are pheromone components of *M. thujae*. Four sets of three structural isomers, each with the same absolute configuration (*RR*, *RS*, *SR*, or *SS*), were tested singly and in quaternary combination. Taking into account that only the *SS*-stereoisomer blend attracted

male *M. thujae* in experiment 1, experiment 2 explored potential synergism between the three *SS*-stereoisomers by testing them singly and in ternary combination. Considering that the three *SS*-stereoisomer blends appeared more attractive than individual stereoisomers in experiment 2, experiment 3 tested the *SS*-stereoisomers in all possible binary and ternary combinations.

Trap catch data were analyzed by nonparametric analyses of variance (Friedman's test) followed by comparison of means by Scheffé (Dunn) *t*-test (Zar, 1984; SAS/STAT, 1988). In all analyses, $\alpha = 0.05$.

RESULTS AND DISCUSSION

Gas chromatographic–electroantennographic detection analyses of pheromone gland extracts of female *M. thujae* revealed three components (**A**, **B**, and **C** in Figure 2) that elicited responses from male antennae. All components occurred below detection threshold (~10 pg) of the flame ionization detector even in a concentrated (>100 FE) extract, requiring compound identifications without the benefit of mass spectrometric data.

Retention indices (RI; relative to alkane standards; Van Den Dool and Kratz, 1963) of the three components **A**, **B**, and **C** on each of the GC columns

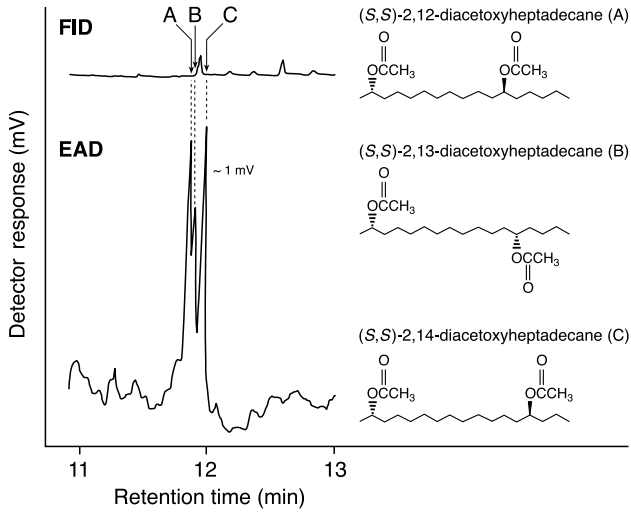

FIG. 2. Representative ($n = 7$) recording of flame ionization detector and electroantennographic detector (EAD = male *M. thujae* antenna) responses to one female equivalent of pheromone gland extract of female *M. thujae*. Splitless injection; injector and detector temperature: 240°C; DB-5 column, program 100°C (1 min), 15°C/min to 280°C.

(DB-5, DB-23, and DB-210) were similar (Table 2), suggesting that all three components had the same chemical functionality. Their RIs and antennal stimulatory activity remained unaltered in GC-EAD analyses of hydrogenated pheromone extract, indicating that all three components had no C–C multiple bonds. The RI intercolumn differentials of components **A**, **B**, and **C** were similar to those of 2,7-diacetoxytridecane (Table 2; Choi et al., 2004) and dissimilar to those of the acetoxy pheromone (2*S*,4*Z*,7*Z*)-2-acetoxy-4,7-tridecadiene (Table 2; Gries et al., 2002), suggesting that **A**, **B**, and **C** each had two acetate esters. The RIs of **A**, **B**, and **C** were 398–468 RI units higher than those of 2,7-diacetoxytridecane (Table 2), further suggesting that **A**, **B**, and **C** had four more carbons in the chain than 2,7-diacetoxytridecane. Taking into account (1) that most of the presently identified cecidomyiid pheromone components have an acetoxy group at C2 (Foster et al., 1991; Harris and Foster, 1991; Millar et al., 1991; Hillbur et al., 1999, 2000, 2001; Gries et al., 2002; Choi et al., 2004) and (2) that some cecidomyiid pheromone components have the second oxygenated group in ω-1 (2,12-diacetoxytridecane, Hillbur et al., 1999) or in ω-2 (2,11-diacetoxytridecane, Hillbur et al., 1999; 2,7-dibutyrox-

TABLE 2. RETENTION INDICES (RI) AND INTERCOLUMN RI DIFFERENTIALS OF PHEROMONE COMPONENTS **A**, **B**, AND **C** IN PHEROMONE GLAND EXTRACTS OF FEMALE *M. Thujae*, AND OF SYNTHETIC STANDARDS

Compounds	RI on GC columns			Intercolumn RI differentials		
	DB-23	DB-5	DB-210	DB-23 vs. DB-5	DB-5 vs. DB-210	DB-23 vs. DB-210
A[a] in Figure 2	2907	2273	2844	634	571	63
B[b] in Figure 2	2918	2279	2853	639	574	65
C[c] in Figure 2	2935	2291	2865	644	574	70
2,7-Diacetoxytridecane[d]	2487	1889	2422	598	533	65
(4*Z*,7*Z*)-2-Acetoxy-4,7-tridecadiene[e]	1932	1605	1872	327	267	60
2,10-Diacetoxyheptadecane	2898	2266	2831	632	565	67
2,11-Diacetoxyheptadecane	2902	2269	2839	633	570	63
2,12-Diacetoxyheptadecane	2907	2273	2844	634	571	63
2,13-Diacetoxyheptadecane	2918	2279	2853	639	574	65
2,14-Diacetoxyheptadecane	2935	2291	2865	644	574	70
2,15-Diacetoxyheptadecane	2973	2314	2899	659	585	74
2,16-Diacetoxyheptadecane	3001	2335	2924	666	589	77

[a]**A** in Figure 2 = 2,12-diacetoxyheptadecane (**4a** in Figure 1).
[b]**B** in Figure 2 = 2,13-diacetoxyheptadecane (**4b** in Figure 1).
[c]**C** in Figure 2 = 2,14-diacetoxyheptadecane (**4c** in Figure 1).
[d]Sex pheromone of *A. aphidimyza* (Choi et al., 2004).
[e]Sex pheromone of *C. oregonensis* (Gries et al., 2002).

ynonane, Gries et al., 2000), we synthesized 2,15- and 2,16-diacetoxyheptadecane. However, the RIs of these two compounds were too high on all columns (Table 2), suggesting that one of the acetate groups needed to be closer to the center of the molecule, thereby decreasing the RI of the molecule. We thus synthesized 2,10-, 2,11-, 2,12-, 2,13-, and 2,14-diacetoxyheptadecanes. The latter three (2,12-17, 2,13-17, and 2,14-17) had RIs on all columns (Table 2) consistent with those of antennal-stimulatory components **A**, **B**, and **C**, respectively, in pheromone gland extracts (Figure 2).

In field experiment 1, traps baited with the *SS*-stereoisomer blend of 2,12-17, 2,13-17, and 2,14-17 captured significant numbers of male *M. thujae*, whereas the *RR*-, *RS*-, and *SR*-stereoisomer blends were not attractive (Figure 3A). Reduced attractiveness of the treatment containing the four stereoisomers of each of the three structural isomers (Figure 3A) suggested that one or more sets of stereoisomers (*RR*, *RS*, or *SR*) inhibited the males' response to the *SS*-stereoisomers. Considering that nonnatural stereoisomers in experiment 1, when tested at a 1:1:1 ratio with the attractive *SS*-stereoisomers, reduced but did not completely inhibit the responses of males, it is not likely that trace amounts of nonnatural stereoisomers (Table 1) in synthetic *SS*-pheromone components had significant behavior-modifying activity.

In experiment 2, the *SS*-stereoisomers of 2,12-17, 2,13-17, and 2,14-17, when tested individually or in a three-component blend, attracted similar numbers of *M. thujae* and significantly more males than unbaited control traps (Figure 3B) except for *S*,*S*-2,14-17, which was not different than either the 3-component blend or the unbaited control. In experiment 3, the *SS*-stereoisomers of 2,12-17, 2,13-17, and 2,14-17, when tested in all possible binary combinations and the ternary combination, were equally effective in attracting male *M. thujae*.

Our data suggest that *M. thujae* produces three pheromone components, each of which is equally active, with no apparent synergism between components, at least not at the 1:1:1 component ratio that was tested in experiments 2 and 3. Other blend ratios are not likely to reveal synergistic activity of components because there was no evidence that any one component was more important than the others. On the contrary, each component was attractive by itself (Figure 3), and each elicited comparable antennal responses in GC-EAD analyses, both of pheromone gland extract (Figure 2) and synthetic standards (10 pg each; data not shown).

The apparent redundancy of pheromone components in *M. thujae* contrasts with the strong synergistic activity of pheromone components in the pea midge *Contarinia pisi* (Hillbur et al., 2000, 2001). Significant attraction of male *C. pisi* requires the presence of each of the three pheromone components (*S*,*S*)-2,11-diacetoxytridecane, (*S*,*S*)-2,12-diacetoxytridecane, and (as yet not determined) (*S*)- or (*R*)-2-acetoxytridecane (Hillbur et al., 2000, 2001). With *M. thujae*, as with

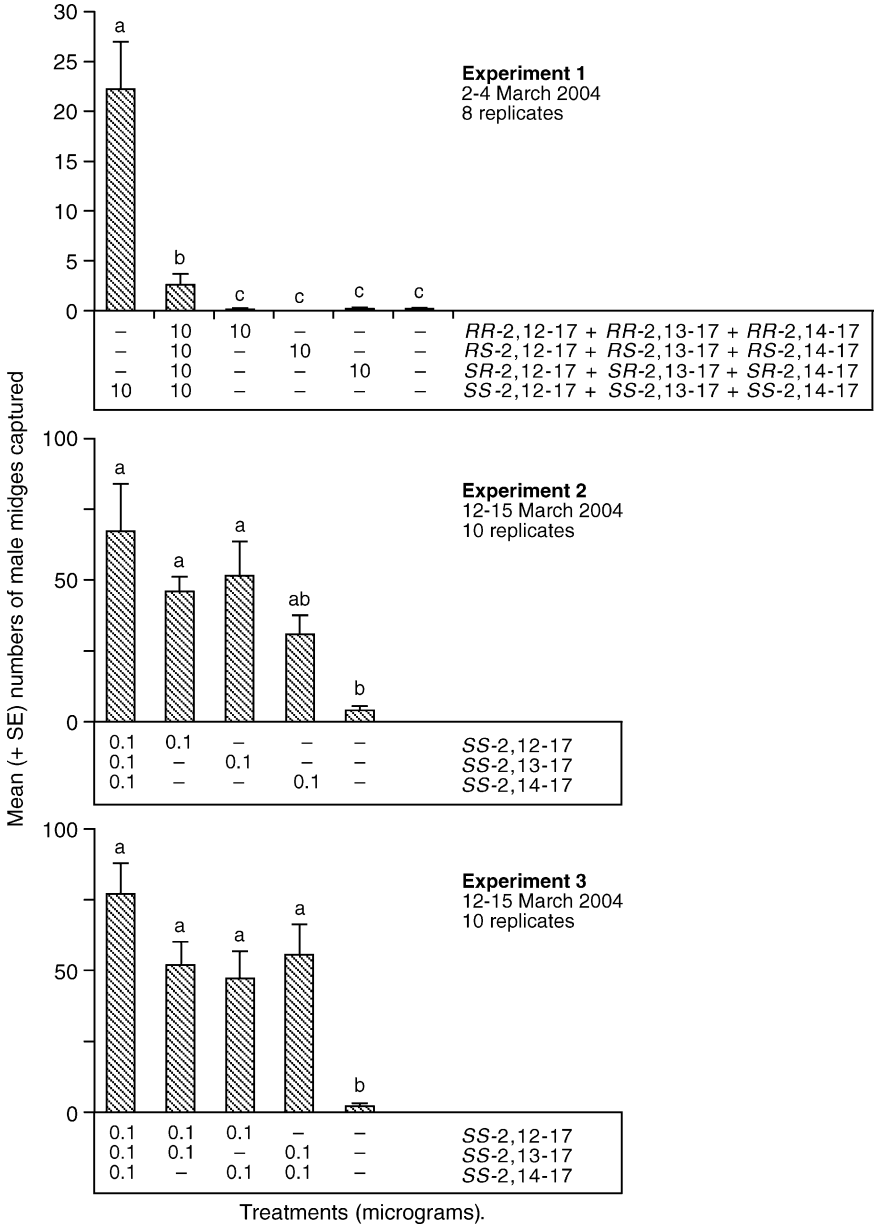

FIG. 3. Mean (+SE) number of male *M. thujae* captured in sticky traps baited with various blends of candidate pheromone components in microgram quantities. Red cedar, *T. plicata*, seed orchard immediately west of Blenkinsop Lake, Victoria, BC. Bars in each experiment with different letter superscripts are significantly different; nonparametric analyses of variance by ranks (Friedman's test) followed by comparison of means (Scheffé test), $P < 0.05$ (SAS/STAT, 1988).

C. pisi, only the *SS*-stereoisomers of the diacetoxy compounds are biologically active, whereas one or more of the other stereoisomers are inhibitory (Figure 3).

The pheromone components of *M. thujae* [(*S*,*S*)-2,12-diacetoxyheptadecane, (*S*,*S*)-2,13-diacetoxyheptadecane, and (*S*,*S*)-2,14-diacetoxyheptadecane] resemble those of other cecidomyiids[1] [(2*S*,10*E*)-2-acetoxy-10-tridecene: Hessian fly *Mayetiola destructor* (Foster et al., 1991; Millar et al., 1991); (*S*,*S*)-2,11-diacetoxytridecane, (*S*,*S*)-2,12-diacetoxytridecane, and 2-acetoxytridecane: *C. pisi* (Hillbur et al., 1999, 2000, 2001); (*S*,*S*)-2,7-dibutyroxynonane: orange wheat blossom midge *Sitodiplosis mosellana* (Gries et al., 2000); (2*S*,4*Z*,7*Z*)-2-acetoxy-4,7-tridecadiene: Douglas-fir cone gall midge *Contarinia oregonensis* (Gries et al., 2002); and (*R*,*S*)-2,7-diacetoxytridecane: *Aphidoletes aphidimyza* (Choi et al., 2004)] but have four more carbon atoms in the chain than most of the cecidomyiid pheromones. The saturated diacetoxy structure of the *M. thujae* pheromone components more closely resembles the pheromone components of the heterogeneric *C. pisi* and *A. aphidimyza* than the unsaturated acetoxy pheromone of the congeneric *M. destructor*. This suggests that one or more of the *M. thujae* pheromone components might be trace components of the sex pheromone blend of congeneric *M. destructor*. An alternative explanation is that *Mayetiola* is primarily a Palearctic genus, with most species being associated with grass host species. This suggests that the genus *Mayetiola* may need revision, with reclassification of *M. thujae* and some other Nearctic species (Gagné, 1989).

The identification of the *M. thujae* pheromone should lead to improved monitoring of *M. thujae* populations and better management decisions in seed orchards. For example, numbers of male *C. oregonensis* captured in pheromone-baited traps in seed orchards in Washington and Oregon were predictive of the level of seed damage at harvest (Morewood et al., 2002). Racemic *M. thujae* pheromone can be readily synthesized (Khaskin et al., unpublished data). If racemic pheromone is suitable for pheromone-based mating disruption of *M. thujae* populations (although the racemate is significantly less attractive than the *S*,*S* stereoisomers) it may be feasible to use the pheromone for control of *M. thujae* in seed orchards.

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[1] While this manuscript was in press, the sex pheromone of the swede midge *Contarinia nasturtii* was published in J. Chem Ecol. 31:1807–1828.

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ELECTROPHYSIOLOGICAL AND BEHAVIORAL RESPONSES TO CHOCOLATE VOLATILES IN BOTH SEXES OF THE PYRALID MOTHS *Ephestia cautella* AND *Plodia interpunctella*

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Abstract—Volatiles from chocolate mediate upwind flight behavior in *Ephestia cautella* and *Plodia interpunctella*. We used gas chromatography with electroantennographic detection and found 12 active compounds derived from three different chocolate types, i.e., plain, nut-containing, and rum-flavored. Eight of the compounds were identified with mass spectrometry, and the activity of three compounds, ethyl vanillin, nonanal, and phenylacetaldehyde (PAA), was subsequently confirmed in both electrophysiological and behavioral assays. In the electroantennogram experiment, PAA and nonanal were consistently eliciting responses in both species and sexes. Ethyl vanillin was active in males of both species, and also in *P. interpunctella* females. *E. cautella* females showed no antennal activity in response to ethyl vanillin. All three volatiles were attractive to *E. cautella* males and *P. interpunctella* females in a flight tunnel. *E. cautella* females were significantly attracted only to ethyl vanillin. *P. interpunctella* males were attracted to PAA. Ethyl vanillin is a novel insect attractant, whereas both nonanal and phenylacetaldehyde mediate behavior in many insect species. A final experiment revealed that a blend of the three volatiles was required to induce landing in the flight tunnel bioassay, and that the landing rate was dependent on dose. The three-compo-

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nent blend attracted both sexes of *P. interpunctella* and females of *E. cautella*, whereas *E. cautella* males were not attracted.

Key Words—Pyralidae, Lepidoptera, GC-EAD, GC-MS, EAG, flight tunnel, food volatiles, ethyl vanillin, phenylacetaldehyde, nonanal.

INTRODUCTION

Adult moths (Lepidoptera) of the Pyralidae family rarely feed, but volatiles from the larval host may still be used by females to locate oviposition sites and possibly by males to locate habitats with females (Ramachandran et al., 1990). A number of studies have identified chemicals mediating both oviposition and flight in pyralid females, e.g., both phenylacetaldehyde (PAA) and terpenes in *Ostrinia nubilalis* (Hübner) (Cantelo and Jacobson, 1979; Maini and Burgio, 1990; Binder et al., 1995; Binder and Robbins, 1997). Female *Amyelois transitella* (Walker) flew toward crude almond oil (Phelan and Baker, 1987), and the mediating chemicals were later identified as oleic acid and linoleic acid (Phelan et al., 1991).

Two pyralid moth species found in indoor habitats, e.g., pet food stores and chocolate factories, are the almond moth, *Ephestia cautella* (Walker), and the Indian meal moth, *Plodia interpunctella* (Hübner). A number of studies have shown that wheat odors induce flight and oviposition in gravid *E. cautella* females (Barrer, 1977; Barrer and Jay, 1980; Gothilf et al., 1993). Similarly, corn (Phillips and Strand, 1994), nuts and almonds (Hoppe, 1981), and walnut oil (Nansen and Phillips, 2003) induce upwind flight and/or oviposition of gravid females of *P. interpunctella*. A 1:1 mixture of acetic acid and isoamyl alcohol (3-methyl-1-butanol), initially developed from host plants of noctuid moths, has been used to trap female *P. interpunctella* in Hungary (Toth et al., 2002). Both males and gravid females of *E. cautella* and *P. interpunctella* fly toward a variety of chocolate products and extracts (Olsson et al., 2005).

Several studies have identified the volatile constituents of chocolate and hundreds of organic compounds were found (Ziegleder and Stojacic, 1988; Schnermann and Schieberle, 1997; Counet et al., 2002). However, their behavioral effect on insects has not been investigated. Our objectives were to identify electrophysiologically active volatiles in extracts of the chocolate varieties that eventually could be used in monitoring traps of the pyralid moths. We tested synthetic references of the identified substances with electroantennograms (EAG) and in a flight tunnel to observe their activity, both electrophysiologically and behaviorally. Finally, we tested a blend of the active volatiles to investigate if it was more behaviorally active than the individual volatiles when presented separately.

METHODS AND MATERIALS

Insects. Laboratory cultures of *E. cautella*, originating from a Swedish chocolate factory, and *P. interpunctella*, provided by the Danish Pest Infestation Laboratory at Lyngby, were used. Larvae were reared on (100 g) of a diet of a 10:2:1 mixture of wheat germ, Brewer's yeast, and glycerol. Insects were separated by sex at the pupal stage, and the adults were kept in separate climate chambers with L17:D7 photoperiod, at 24°C and 60% relative humidity. All insects used in the experiments were 2–5 d old.

Headspace Extracts. Volatiles from 15 g of either plain, nut-containing, or rum-flavored chocolate were collected on a charcoal filter at room temperature for 24 hr, using a closed loop stripping apparatus (Bestmann et al., 1988). The filter was extracted with 80 μl of dichloromethane, and the extract was diluted with 720 μl cyclohexane. The filter was cleaned with a 2:1 mixture of methanol and acetone between collections.

Steam Distillation Extracts. The steam distillation extraction technique was used to obtain more volatiles in higher concentrations from the chocolate samples. Water was boiled at elevated pressure, and the steam transferred to a vessel containing 200 g of chocolate maintained at 100°C. During 2 hr, the steam from the stirred water–chocolate mixture was collected and cooled to room temperature. The collected water layer was extracted three times with diethyl ether. The organic layer (300 ml), with a strong smell of chocolate, was dried with $MgSO_4$ and concentrated under reduced pressure to 2 ml.

Gas Chromatography with Electroantennographic Detection. A Hewlett Packard 5890 series II gas chromatograph with a nonpolar HP-1 column (30 m × 0.25 mm i.d.) was used. Samples were injected splitless, and the injector temperature was 225°C. The carrier gas was hydrogen. The column temperature was kept at 40°C for 2 min then increased by 10°C/min up to 230°C. The final temperature was kept at 230°C for 10 min. The injected sample was split between two outlets allowing simultaneous recording of response of the flame ionization detector (FID) and the electroantennographic detector (EAD). Nitrogen was used as a make-up gas for the split. The antenna was cut off at the first basal segment and at the tip segment, then placed between two glass capillaries filled with saline solution and containing a silver wire (Baker et al., 1991). The antenna was placed approximately 10 mm from the GC outlet tube. Recordings from antennae of virgin females and males were made to determine electrophysiologically active components in the chocolate extracts. Three antennae of each sex and species were tested for each of the extracts. All gas chromatography with electroantennographic detection (GC-EAD) recordings were analyzed with Autospike 32 software (Syntech, The Netherlands).

Gas Chromatography-Mass Spectrometry. Chocolate extracts were analyzed with a Hewlett Packard 5890 series II gas chromatograph equipped with an HP-1 nonpolar column. Helium was used as carrier gas. The initial temperature was maintained at 50°C for 2 min and then increased by 10°C/min to the final temperature 250°C. The effluent was analyzed with an HP5972 mass spectrometer in scan mode. Substances that repeatedly elicited antennal responses were identified by comparing retention times (maximum deviation ± 0.02 min) with synthetic references, and with mass spectra from MS libraries (NBS75k and Wiley275, match quality >90%) and synthetic references.

Chemicals. All synthetic references were >95% pure when checked by the gas chromatography-mass spectrometry (GC-MS) method described above. The chemicals were purchased from Sigma-Aldrich (cyclohexanone, ethyl vanillin, and vanillin), Acros Organics (nonanal), ICN Biochemicals (phenylacetaldehyde), and Fisher Scientific (two isomers of α-pinene and cyclohexanol).

Electroantennograms. Four doses, ranging from 10^{-2} to 10 μg, of the identified substances were tested for their electrophysiological activity. For α-pinene, both isomers were tested to determine which one was electrophysiologically most active. In the case of ethyl vanillin, 100 μg were also tested, to compensate for the lower vapor pressure of this chemical (vapor pressures: ethyl vanillin 8.84×10^{-4} Torr, nonanal 0.532 Torr, and PAA 0.368 Torr). The antenna was mounted and humidified as above. A 10-μl aliquot of a compound to be tested diluted in cyclohexane was applied on a 7 × 13 mm filter paper, which was inserted into a Pasteur pipette. The tip of the Pasteur pipette was inserted in a hole 200 mm from the opening of an 8-mm tube leading to the antenna, and air was puffed for 0.5 s at 5 ml/s flow rate with a stimulus controller (CS-02, Syntech). As positive standard, 5 ng of the four-component pheromone blend of *P. interpunctella* (Zhu et al., 1999) were used for males and 10 μl of headspace extract of rum-flavored chocolate, corresponding to 18 min collection in the closed loop apparatus, were used for females. Ten μl of cyclohexane were used as a negative control to distinguish olfactory from mechanical responses. The stimulation procedure was as follows: first the standard was used, followed by the negative control, and then the standard again. The antenna was then stimulated with the test substances with a standard stimulation every third time. This procedure was repeated for each dose, starting with the lowest to avoid adaptation of the receptors. At least 15-sec intervals were maintained between stimulations. The standardized EAG response of a substance was calculated by dividing the EAG response with the mean of the standard puffed after and before the substance (Hansson et al., 1991). Ten antennae from virgin moths were tested for each species and sex. The EAG recordings were analyzed with Autospike 32 software (Syntech, The Netherlands).

Flight Tunnel Experiments. A 0.7 × 0.7 × 2.0 m flight tunnel was used for all behavioral experiments (Zhu et al., 1999). Light intensity was 10 lx, temperature was maintained at 23 ± 2°C, and relative humidity at 30–60%. All flight tunnel experiments were conducted during the first 2 hr into the scotophase, and each moth was observed for 3 min. Mated females and unmated males were used for all experiments, as mating has a positive effect on female behavior and none on male behavior (Olsson et al., 2005). The chemicals were tested in the flight tunnel by using an ultrasonic sprayer (El-Sayed et al., 1999) at a frequency range of 290–320 kHz, to avoid the disturbing effects on moths hearing the ultrasound (Skals et al., 2003). The chemicals were released at 50 ng/min (10 ng/μl at 5 μl/min) rate after quantification (standard 10 ng decyl acetate) of the active components in the behaviorally active chocolate extracts (Olsson et al., 2005). The released dose was never more than 10 times greater than those found in the chocolate extracts, thereby minimizing the risk of arrestment behavior in response to too high concentrations. Cyclohexane was used as a negative control.

In a subsequent flight tunnel experiment, a blend of ethyl vanillin, nonanal, and phenylacetaldehyde was tested. Gas chromatography was used to analyze the three chocolate types extracted with the two different techniques and to determine the ratio between the volatiles in the blend used. Because nonanal was present in all extracts, it was set as a reference for the comparison, and given the value of 1 in the blend. A 2:1:2 blend of ethyl vanillin, nonanal, and phenylacetaldehyde was chosen as representative for authentic chocolate odors. The blend was released at three doses ranging from 2.5 (e.g., 0.5 ng/μl at 5 μl/min, in which 0.5 ng/μl equals 0.2 ng ethyl vanillin, 0.1 ng nonanal, and 0.2 ng PAA/μl) to 250 ng/min, and tested for both species and sexes. For all tests orientated upwind, flight at least halfway to the odor source and landing on the source were observed.

Statistics. EAG data were analyzed with ANOVA followed by Tukey's *post-hoc* test at $P < 0.05$ level in SPSS 10.0 for Macintosh. The flight tunnel data were analyzed at the $P < 0.05$ level with Ryan's multiple comparison test for proportions (Ryan, 1960).

RESULTS

Twelve electrophysiologically active compounds were found when analyzing the different chocolate types with GC-EAD (Figure 1). Some compounds were found in all samples, whereas others varied with both the type of chocolate and the extraction technique used (Table 1). Compounds 1, 3, 7, 8, and 10–12 were identified by comparing their mass spectra with synthetic samples. Compound 9 (Figure 1) was tentatively identified as 3-ethyl-2,5-

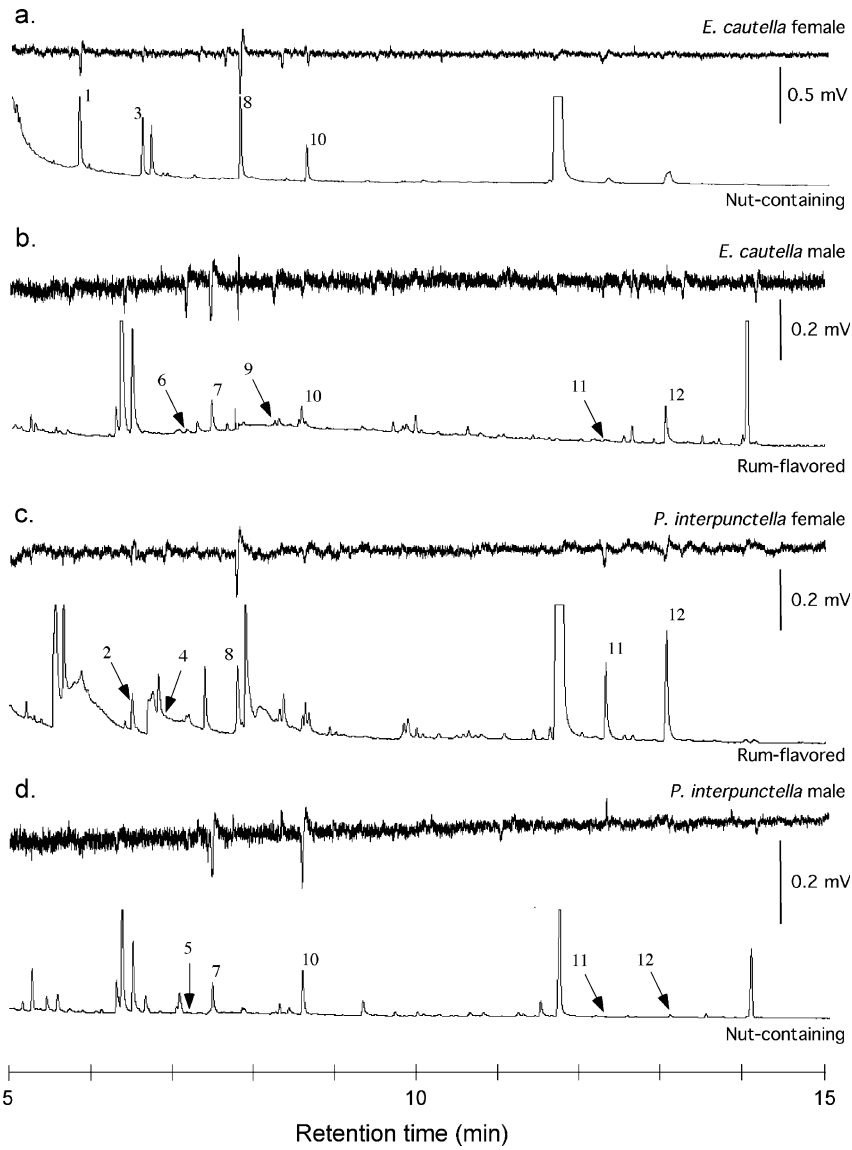

FIG. 1. Examples of GC-EAD runs showing (a) *E. cautella* female response to headspace extract of nut-containing chocolate, (b) *E. cautella* male response to steam distillation extract of rum-flavored chocolate, (c) *P. interpunctella* female response to headspace extract of rum-flavored chocolate, and (d) *P. interpunctella* male response to steam distillation extract of nut-containing chocolate. Chemical names corresponding to the peak numbers are found in Table 1.

TABLE 1. LIST OF ELECTROPHYSIOLOGICALLY ACTIVE SUBSTANCES FOUND IN THREE CHOCOLATE PRODUCTS, DEPENDING ON EXTRACTION TECHNIQUE AND CHOCOLATE TYPE

Peak no.	Chemical	Headspace			Steam distillation		
		Plain	Nut	Rum	Plain	Nut	Rum
1	Cyclohexanone	−	+	−	−	−	−
2	Unidentified	−	−	+	−	−	−
3	α-Pinene	−	+	−	−	−	−
4	Unidentified	−	−	+	−	−	−
5	Unidentified	−	−	−	−	+	−
6	Unidentified	−	−	−	−	−	+
7	Phenylacetaldehyde	−	−	−	+	+	+
8	Cyclohexanol	−	+	+	−	−	−
9	3-Ethyl-2,5-dimethyl-pyrazine	+	−	−	−	−	+
10	Nonanal	+	+	+	+	+	+
11	Vanillin	+	+	+	+	−	−
12	Ethyl vanillin	+	+	+	+	−	+

+: The chemical is present in the extract; −: the chemical is not detected.
Peak numbers correspond to numbers in Figure 1.

dimethylpyrazine, by comparing its mass spectrum with a library mass spectrum. Remaining compounds (peaks 2 and 4–6 in Figure 1) were not identified. *E. cautella* females responded to 11 of the active substances and the males to 10 (Table 2). Both sexes of *P. interpunctella* responded to eight substances each, of which six were in common (Table 2).

In both sexes of both species, the two highest doses (1 and 10 μg) of phenylacetaldehyde and the highest dose (10 μg) of nonanal elicited EAG responses that were significantly different from the control (Figures 2 and 3). *E. cautella* males also responded to the second highest dose (1 μg) of nonanal (Figure 2b). All adults, except *E. cautella* females, responded to 100 μg of ethyl vanillin, and the males of both species also responded to 10 μg of ethyl vanillin (Figures 2 and 3). All other substances and doses were not electrophysiologically active when compared to the control. The difference in EAG response between active and nonactive substances was larger in males than in females for both species.

In the first flight tunnel experiment, males of *E. cautella* responded significantly to nonanal, PAA, and ethyl vanillin, whereas females only flew upwind to ethyl vanillin (Figure 4a). Males were generally more responsive than females, especially to nonanal and PAA where the intersexual differences were significant. In the case of *P. interpunctella*, females responded to nonanal, PAA, and ethyl vanillin, whereas males only responded significantly only to PAA

TABLE 2. SUMMARY OF THE ELECTROPHYSIOLOGICAL ACTIVITY OF THE DIFFERENT SUBSTANCES FOUND IN THE CHOCOLATE PRODUCTS, DEPENDING ON SPECIES AND SEX

Peak no.	Chemical	*Ephestia cautella*		*Plodia interpunctella*	
		Females	Males	Females	Males
1	Cyclohexanone	−	−	+	+
2	Unidentified	+	+	−	+
3	α-Pinene	+	−	−	−
4	Unidentified	+	+	−	+
5	Unidentified	+	+	−	−
6	Unidentified	+	+	+	−
7	Phenylacetaldehyde	+	+	+	+
8	Cyclohexanol	+	+	+	+
9	3-Ethyl-2,5-dimethyl-pyrazine	+	+	+	−
10	Nonanal	+	+	+	+
11	Vanillin	+	+	+	+
12	Ethyl vanillin	+	+	+	+

$N = 3$ for each combination of species and sex. Peak numbers correspond to numbers in Figure 1. +: Electrophysiological activity in at least two out of the three runs; −: no electrophysiological activity.

(Figure 4b). As with *E. cautella* males, *P. interpunctella* males were more responsive than females, with a significantly higher male response to PAA. However, when tested separately, PAA did not induce any significant landing on the source of either sex in either species.

In the second flight tunnel experiment, significantly more *E. cautella* females flew upwind to the intermediate dose (25 ng/min) of the three-component blend, and all doses induced significantly more landings at the source than in the control (Figure 5a). In contrast, *E. cautella* males were not attracted to the blend at any concentration tested. The frequency of upwind flight was positively correlated with the dose for *P. interpunctella* females, as the two highest doses (25 and 250 ng/min) were significantly different from the control (Figure 5b). Male upwind flight behavior was significantly higher at all doses compared with the control. The highest proportions of both *P. interpunctella* males and females landing on source were observed at the intermediate dose (25 ng/min), which was significantly different from the controls (Figure 5b).

DISCUSSION

The attraction of females to chocolate volatiles could be considered as adaptive, because they would guide females to suitable oviposition sites (Olsson

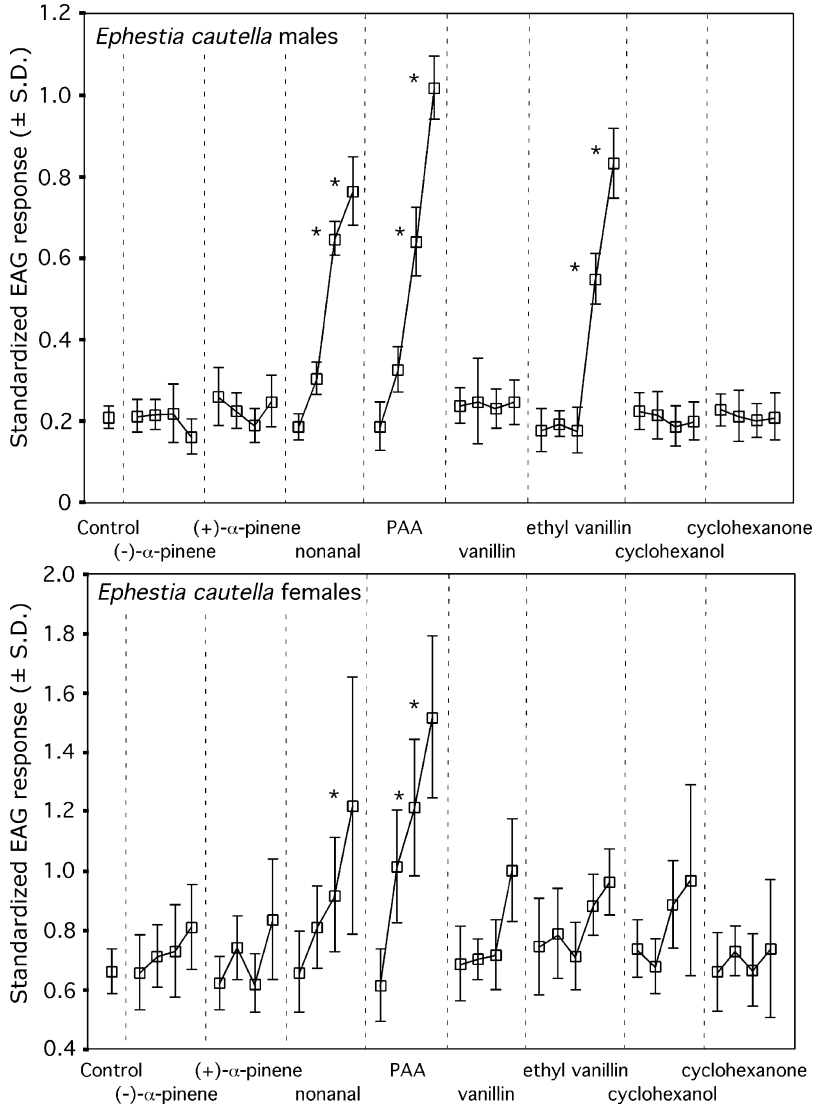

FIG. 2. EAG responses of *E. cautella* males and females to different doses (0.01–10 μg, except ethyl vanillin for which 100 μg were also tested) of chocolate-derived chemicals. *Data points are significantly different from control, when tested at the $P < 0.05$ level with Tukey's *post-hoc* test (ANOVA, males: $F = 73.40$, $df = 33$, $P < 0.001$; females: $F = 7.11$, $df = 33$, $P < 0.001$). Control was cyclohexane.

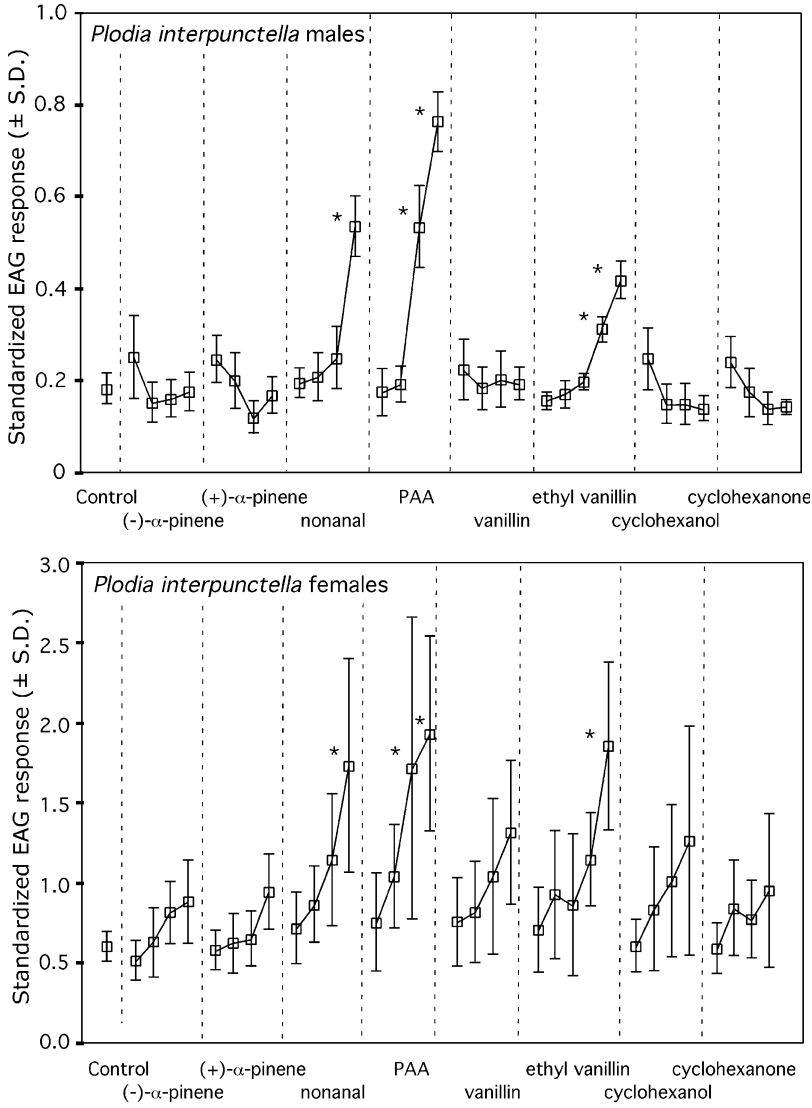

FIG. 3. EAG responses of *P. interpunctella* males and females to different doses (0.01–10 μg, except ethyl vanillin for which 100 μg was also tested) of chocolate-derived chemicals. *Data points are significantly different from the control, when tested at the $P < 0.05$ level with Tukey's *post-hoc* test (ANOVA, males: $F = 38.60$, $df = 33$, $P < 0.001$; females: $F = 4.70$, $df = 33$, $P < 0.001$). Control was cyclohexane.

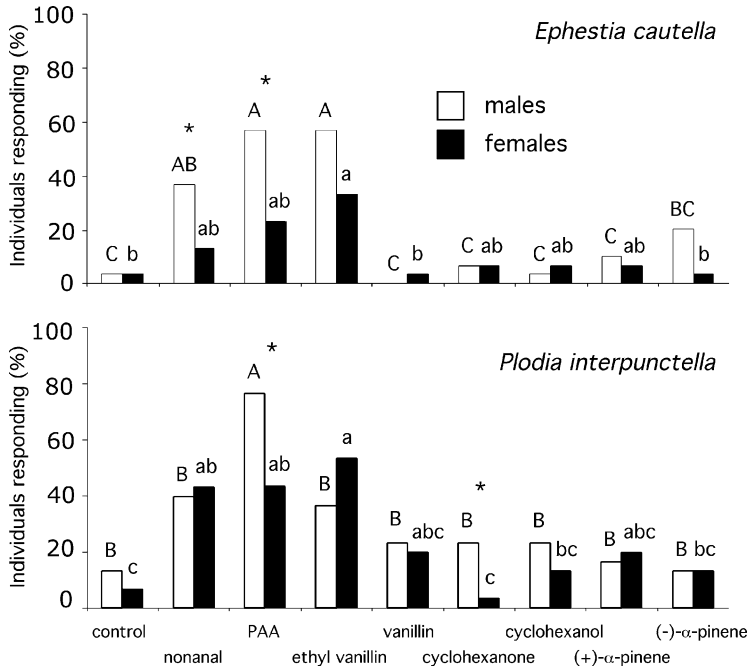

FIG. 4. Proportion of *E. cautella* and *P. interpunctella* males and females flying upwind in response to individual chemicals released at a rate of 50 ng/min. Bars with the same letters do not differ significantly at $P < 0.05$ level (Ryan's test). *Significant intersexual difference for that chemical. Control was cyclohexane.

et al., 2005). For males, a positive response could lead them to sites containing receptive females, and thus increase their reproductive fitness. The findings show that different chocolate products contain substances that elicit both electrophysiological and behavioral responses in both *E. cautella* and *P. interpunctella*. However, there are a number of interspecific and intersex differences in both the EAG and behavioral responses that are somewhat unexpected. A good example of differences between the EAG and behavioral responses is seen with *E. cautella* females and ethyl vanillin. While ethyl vanillin stimulation resulted in no significant EAG response, it did induce upwind flight, possibly due to the different techniques used to deliver the test material in the two experiments. Ethyl vanillin has a low volatility, so fewer molecules may be released in the EAG setup, resulting in low responses as fewer receptors are activated (Schiestl and Marion-Poll, 2002). In contrast, we used a sprayer in the wind tunnel so the volatility of ethyl vanillin would not influence release rates.

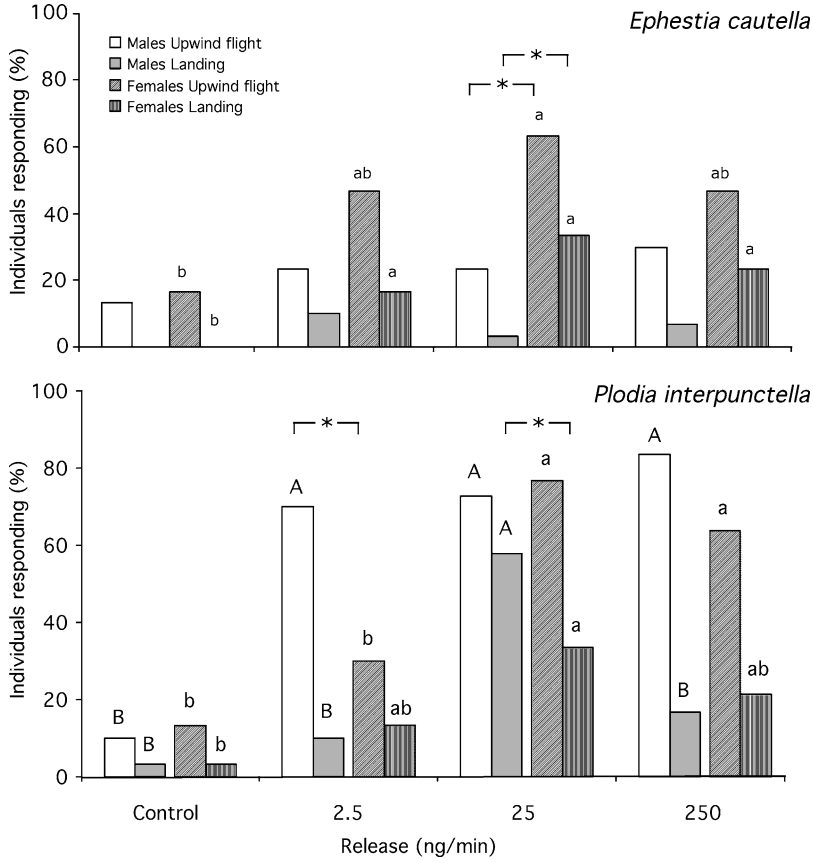

FIG. 5. Proportion of *E. cautella* and *P. interpunctella* males and females flying upwind and landing in response to a three-component blend of ethyl vanillin, nonanal, and phenylacetaldehyde, released at three different doses. Bars within the same category with the same letters do not differ significantly at $P < 0.05$ level (Ryan's test). *Significant intersexual difference within each behavioral step and dose. Control was cyclohexane.

Both sexes of *P. interpunctella* and *E. cautella* females exhibited better upwind flight to the blend than to individual components; *E. cautella* males responded to the individual components but not to the blend. Similarly, more males than females landed in response to the intermediate dose of the blend. This is surprising given that chocolate is more attractive to females than males (Olsson et al., 2005). Therefore, additional experiments will be required to elucidate the biological importance of these differences, including the possibility that there are still some unidentified compounds that elicit behavior.

Ethyl vanillin, identified from samples of milk chocolate, is a synthetic form of vanilla flavor not naturally occurring outside stored product environments (Hurst and Martin, 1982). The observed responses suggest that this is an adaptation to a man-made compound, which would allow moths to locate food sources suitable for larval development.

PAA is an attractant for several other lepidopteran species, e.g., the abovementioned *O. nubilalis*, for both sexes of *Trichoplusia ni* (Hübner) (Creighton et al., 1973), *Autographa californica* (Speyer) (Landolt et al., 2001), and *Pseudoplusia includens* (Walker) (Meagher, 2002). It was also used in the impressive multispecies survey of 72 different moths (Cantelo and Jacobson, 1979). Our results suggest that nonanal and PAA, both chocolate volatiles (Schnermann and Schieberle, 1997; Counet et al., 2002), might be useful in traps for monitoring females of the two indoor moth species and may work as a pheromone synergist enhancing male attraction to pheromone traps.

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PHYTOTOXICITY OF *Phytolacca americana* LEAF EXTRACTS ON THE GROWTH, AND PHYSIOLOGICAL RESPONSE OF *Cassia mimosoides*

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Abstract—We examined the allelochemical effects of control soil, native soil (treated soil), and leaf extracts of *Phytolacca americana* (pokeweed) on the germination rate and seedling growth of *Cassia mimosoides* var. *nomame*. We also studied the resulting changes in root-tip ultrastructure and peroxidase isozyme biochemistry. *P. americana* leaf extract inhibited seed germination, seedling growth, and biomass when compared to control and treated soil. Root and shoot growth in treated soil was stimulated relative to control soil, but root growth was inhibited by 50% in the leaf extract treatment. Biomass of *C. mimosoides* seedlings grown on leaf extract was reduced sevenfold when compared to the control seedlings. The amounts of total phenolic compounds in the leaf extract, treated soil, and control soil were 0.77, 0.14, and 0.03 mg l^{-1}, respectively. The root tips of *C. mimsoides* treated with leaf extracts of *P. americana* showed amyloplasts and large central vacuoles with electron-dense deposits inside them when compared to control root tips. The activity of guaiacol peroxidase (GuPOX) in whole plant, roots, and shoots of *C. mimosoides* increased as leaf extract increased; maximum activity was observed in extract concentrations of 75% and higher. Root GuPOX activity was three times higher than in shoots. Therefore, we conclude that inhibition of *C. mimosoides* growth is related to the phenolic compounds in the *P. americana* leaf extract and the ultrastructure changes in root-tip cells and increased GuPOX activity is a response to these allelochemicals.

Key Words—Allelochemicals, enzyme activity, phenolic compounds, *Phytolacca americana*, seed germination, seedling growth.

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INTRODUCTION

Allelopathy is an important phenomenon operating in natural and managed ecosystems. Many secondary metabolites, including phenolic, terpenoid, flavonoid, and alkaloid compounds, act as plant allelochemicals. Among these, phenolic compounds are the most abundant under field conditions (Rice, 1984; Williamson, 1990) and are known to affect seed germination, seedling growth, cell division, fungal activity, protein synthesis, and enzyme activity (Thijs et al., 1994; Inderjit, 1996; Seneviratne and Jayasinghearachchi, 2003). The phenolic compound, hydroxamic acid, affected membrane permeability resulting in reduced plant growth; and the interference of hydroxamic acid, 2-benzoxazolinone (BOA), and other hydroxamic acids with auxins inhibited the cell-cycle progression of *L. sativa* (Macias et al., 2003). Rye allelochemicals, BOA and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), caused increased cytoplasmic vacuolation, reduced ribosome density and dictyosomes, reduced number of mitochondria, and reduced lipid catabolism (Burgos et al., 2004). Lovett (1982) found that root-tip cells affected by allelochemicals exhibited increased vacuolation, and the alkaloid of thornapple (*Datura stramonium*) caused an increase in amyloplasts as well as the size and number of microbodies in *Helianthus annuus* root tips (Lovett, 1982; Levitt et al., 1984).

The phytotoxic effects of water-soluble phenolic compounds in *Phytolacca americana* have not been studied in the context of morphological and physiological responses associated with seed germination and subsequent seedling growth. In 1979, *P. americana* was introduced into South Korea and became an important ecological problem in 1993 when it spread throughout the country, displacing many native species. It is a perennial weed that occurs worldwide and has been spreading especially in polluted areas of South Korea (Park, 1995). Under field conditions it interferes with the growth and establishment of competing plant species by releasing water-soluble compounds into the soil (Lee et al., 1997). Furthermore, at low concentrations, extracts of *P. americana* inhibited seed germination, seedling growth, and exhibited antimicrobial activity (Kim et al., 2000). Three *Phytolacca* species in South Korea exhibited allelochemical effects that were related to phenolic compounds (Kim et al., 2005).

Recently, the presence of soluble phenolic compounds was used to screen for disease-resistant cultivars of sugarcane and date palm (Daayf et al., 2003; Nutt et al., 2004). And although there is a great need to determine the effect of allelochemicals on enzyme activity, few studies have related physiological effects to peroxidase activity and gene expression in plant differentiation. Consequently, we studied isozymes in *P. americana*.

Impaired enzyme activity, identified by Rice (1984) as a primary target for phytotoxic activity, may explain the inability to metabolize starch. According to

Alscher and Hess (1995), plants produce enzymes such as peroxidase, catalase, and superoxide dismutase (SOD) for self-protection against external stress. In particular, peroxidase exists in the form of isotype enzymes, which have a different molecular structure while activating the same substrate. During organogenesis, the type of isoperoxidase is more important than the total amount of peroxidase (Scandalios, 1990), and is involved in plant growth as well as in many plant defense mechanisms (Lagimini, 1991). Phenolic compounds act as antioxidants by donating electrons to guaiacol peroxidase (GuPOX) for the detoxification of hydrogen peroxide (H_2O_2) produced under stress conditions (Asada, 1992; Sakihama and Yamasaki, 2002). Phenolic compounds also function cooperatively with ascorbate as antioxidants, particularly in vacuoles and apoplasts where they coaccumulate with GuPOX (Yamasaki et al., 1997; Ren et al., 1999; Xiujuan et al., 2002). Phenolic compounds inhibiting plant growth were reported to increase the activities of proteinase, peroxidase, and catalase (Loebenstein and Linsey, 1961; Gaspar et al., 1985), whereas caffeic acid and salicylic acid reduced phosphorylase and GuPOX activity, respectively (Rice, 1984; McCue et al., 2000).

The objectives of the present study were to: (1) determine the effect of *P. americana* leaf extracts, control soil, and soil from natural *P. americana* patches on seed germination and seedling biomass accretion; (2) examine subsequent growth and ultrastructure changes in *Cassia mimosoides* root tips treated with *P. americana* leaf extract; and (3) determine total peroxidase and GuPOX activity in organs of *C. mimosoides* after treatment with leaf extract from *P. americana*.

METHODS AND MATERIALS

Plant and Soil Collection. Seeds from *C. mimosoides* were collected from Chunan, Chungcheong Province, South Korea, and were used to study seed germination, seedling growth, root-tip ultrastructure changes, and guaiacol peroxidase (EC 1.11.1.7) activity. *C. mimosoides* seeds have a high germination rate, are widely distributed throughout South Korea, and are sensitive to water-soluble leaf extracts. These extracts were obtained from leaves of *P. americana* growing on Mt. Pardal located in downtown Suwon, Gyeonggi Province, South Korea. After removing the organic layer, the upper 5 cm of soil was collected under a patch of *P. americana* (treated soil). Control soil was obtained 2 m away in an area where *P. americana* was not present.

Leaf Extract Study. Two hundred g of fresh *P. americana* leaf were extracted with 1 l of distilled water at room temperature for 48 hr and then centrifuged at 15,000 rpm for 30 min (Centrikon T-1045, Kontron Co., Zurich, Switzerland). The supernatant was collected and stored at 4°C until used. The

leaf extract was filtered through Whatman No. 1 filter paper and used undiluted (100%), and diluted to 75%, 50%, 25%, and 10% concentrations, whereas the control consisted of water for bioassay and the GuPOX activity study. After being selected for uniform size, *C. mimosoides* seeds were sterilized for 3 min in a solution of 5% sodium hypochlorite and then rinsed three to five times with distilled water. Thirty seeds were sown in a Petri dish (90 mm diam.) and treated with a 25% leaf-extract solution. Seeds were incubated at 28°C under 400 μmol m^{-2} s^{-1} fluorescent lighting with 16/8 hr L/D periods for 6 d. Seed germination was assessed daily, while root tips were observed after 3 d. Seedling growth, biomass, and GuPOX activity were measured after 6 d. Enzyme samples were stored in the freezer (−50°C). The experiment was replicated three times. The bioassay method used was a modified version of the procedure described by Lodhi (1976).

Transmission Electron Microscopy of Root Tips. Root tips of *C. mimosoides* were cut to 0.1–0.2 mm in length and dehydrated in a Spurr mixed solution (Spurr, 1969) and stained (Reynolds, 1963). Samples were analyzed by transmission electron microscopy (TEM) at 80 kV.

Guaiacol Peroxidase Assay. The enzyme samples were extracted to measure GuPOX activity. One ml of extraction buffer containing 25 mM KPO_4, 1 mM ascorbate, and 1 mM EDTA was added to 0.3 g of liquid N-frozen root, shoot, and whole plant of *C. mimosoides*, and ground with a mortar and pestle. After being left to stand at 4°C for 20 min, the samples were centrifuged at 13,000 *g* for 20 min (Centrikon T-126, Kontron). The amount of soluble protein was measured with a protein assay kit (Bio-Rad, Hercules, CA, USA) using the method described by Bradford (1976). GuPOX activity was measured using the method described by Amako et al. (1994) and recalculated to units per microgram protein using enzyme kinetics software.

Statistical Analysis. Data were normally distributed, and significant differences between treatments and controls were calculated using one-way ANOVA and Duncan's mean separation test for the measured parameters. The data shown in figures are the mean ± SE.

RESULTS

Leaves of *P. americana* showed high concentrations of total phenolic compounds (0.77 mg l^{-1}), whereas the soil under the plants had concentrations about four times higher than the control soil (0.14 vs. 0.03 mg l^{-1}). Seed germination of *C. mimosoides* was significantly inhibited when treated with *P. americana* leaf extract and in the soil collected from under *P. americana* plants. The germination rate was reduced more in the leaf extracts than in the treated soil (Figure 1, $P < 0.001$). Germination was reduced by 40% in the leaf

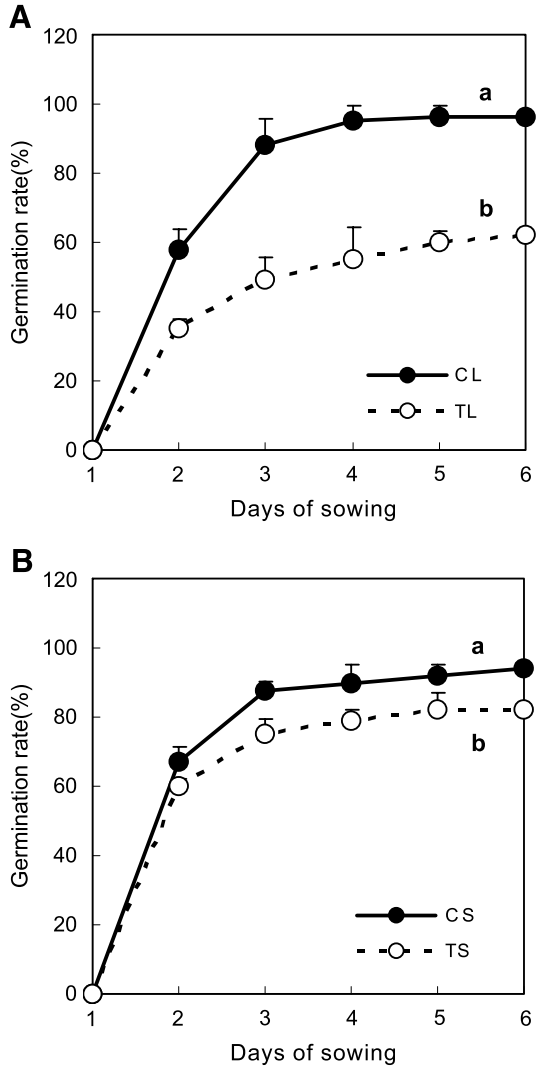

FIG. 1. Comparison of germination rate of *Cassia mimosoides* treated with leaf extract (A) and soil supporting a patch of *Phytolacca americana* (B). CL: control leaf, TL: treated leaf, CS: control soil, TS: treated soil. Means with the same letter are not significantly different (Duncan's multiple range test, $P < 0.001$), ±SE of $N = 3$ measurements each are given.

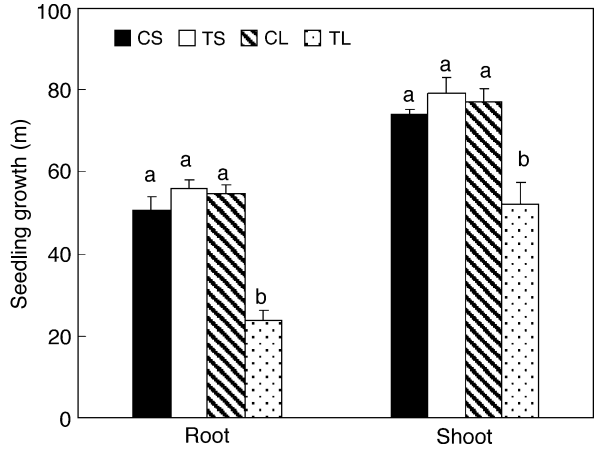

FIG. 2. Comparison of seedling growth of *C. mimosoides* grown with treated leaf extract and soil of *P. americana.* CS: control soil, TS: treated soil, CL: control leaf, TL: treated leaf. Means with the same letter are not significantly different (Duncan's multiple range test, $P < 0.001$), ±SE of $N = 3$ measurements each are given.

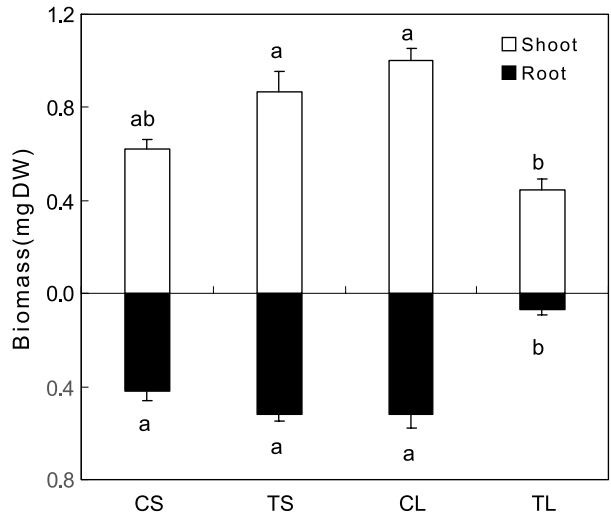

FIG. 3. Comparison of dry weight of *C. mimosoides* seedlings grown with treated leaf extract and soil of *P. americana.* CS: control soil, TS: treated soil, CL: control leaf, TL: treated leaf. Means with the same letter are not significantly different (Duncan's multiple range test, $P < 0.001$), ±SE of $N = 3$ measurements each are given.

extract when compared to the control (Figure 1A), whereas germination was reduced by only 10% in the treated soil (Figure 1B). In seedlings treated with leaf extract, shoot and root growth were significantly reduced compared to that of control (Figure 2, $P < 0.001$). Root growth was especially sensitive to the leaf extract, exhibiting more than a 50% growth reduction. Shoot growth was reduced

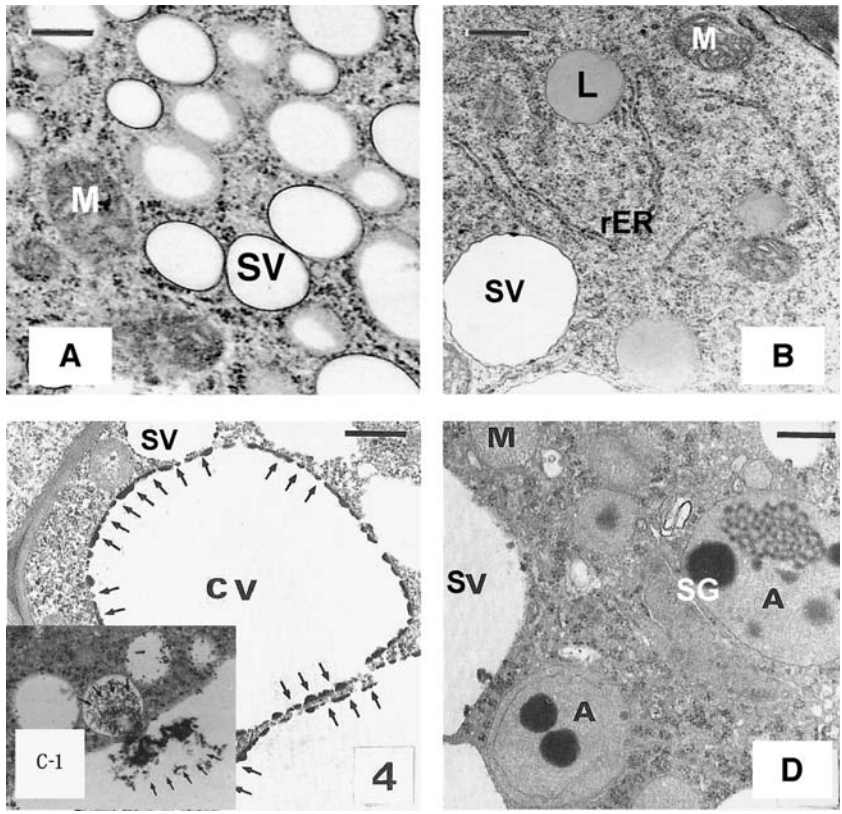

FIG. 4. TEM micrographs of *C. mimosoides* root tips: Control (A and B) and treated (C and D) with leaf extracts of *P. americana*. (A) A large number of small vacuoles and mitochondria are in the parenchyma cell. Bar = 2.4 μm. (B) Lipid bodies, mitochondria, small vacuoles and rER are in the cytoplasm. Bar = 2.4 μm. (C) Electron-dense deposits (arrows) are accumulated in the central vacuole (CV) along the tonoplast of a parenchyma cell. Bar = 1.5 μm, C-1: these arrows point to small vacuoles that are reduced in number, while this amorphous material was discharged into the central vacuole. Bar = 1.5 μm. (D) An amyloplast (A) near the central vacuole (SV) bounded by a double membrane containing two starch grains. Bar = 2.4 μm.

by about 33% in seeds treated with leaf extract (Figure 2). Biomass and seedling growth of *C. mimosoides* plants in treated soil was slightly higher than in the control soil, but the biomass differences were not significant (Figures 2 and 3).

The threshold leaf extract concentration was estimated to be 25% because the greatest changes in the bioassay were observed between 10% and 50% (data not shown). In the root tips from control plants, mitochondria and a large number of small vacuoles were observed in parenchyma cells (Figure 4A). Lipid bodies, mitochondria, and rough endoplasmic reticula (rER) were present in the cytoplasm (Figure 4B). In the root tips from seedlings treated with 25% leaf extract, the size of the central vacuole increased, and electron-dense deposits were observed to accumulate along the tonoplast in parenchyma cells of the stele (Figure 4C). Many small vacuoles joined the central vacuole, whereas amorphous materials were discharged from small vacuoles into the central vacuole (Figure 4C-1). Some cellular organelles were reduced in number, and amyloplasts, including starch grains, appeared in parenchyma cells (Figure 4D).

GuPOX activity dramatically increased in the shoots, roots, and the whole plant with increasing extract concentrations. Root GuPOX activity was three

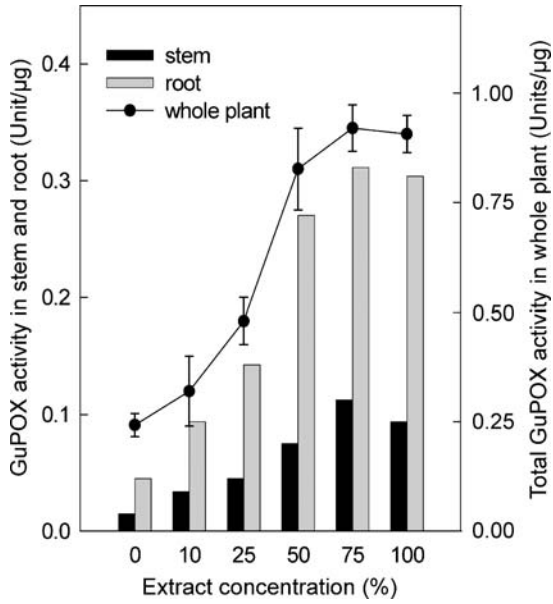

FIG. 5. Comparison of GuPOX activity in root, shoot, and whole plant of *C. mimosoides* treated with *P. americana* leaf extract. Bars represent SE of the mean. Organs treated with extract are indicated by symbols in the legend.

times higher than in the shoots. The highest GuPOX activity was observed in the sample treated with 75% leaf extract, whereas activity in the 100% extract sample was slightly lower. GuPOX activity in the roots exposed to 75% leaf extract increased more than sevenfold over the control (Figure 5).

DISCUSSION

Phenolic compounds found in leaf extracts and treated soil have been reported to reduce seed germination, seedling growth, and biomass (Williams and Hoagland, 1982; Rice, 1984; Einhellig, 1995; Inderjit, 1996) as observed in the present study (Figures 1, 2, and 3). Water-soluble compounds including phenolic compounds released by *Pluchea lanceolata* into the soil were found to significantly decrease seed germination and seedling growth of asparagus bean (*Vigna unguiculata*) (Inderjit and Dakshini, 1992). In the present study, seed germination in treated soil was inhibited (Figure 1B), whereas seedling growth and biomass were only slightly stimulated (Figures 2 and 3). Lodhi (1976) also found increased seedling growth in treated soil containing phenolic compounds. Under field conditions, Rice (1984) suggested that high concentrations of allelochemicals might not be maintained as a result of leaching and microbial degradation. In our study, the observed increase in seedling biomass in response to the presence of soil phenolic compounds has been reported previously (Lodhi, 1976; Rice, 1984). Concentrations of phenolics were found to be higher in leaf extracts (0.77 mg l^{-1}) than in treated soil (0.14 mg l^{-1}). Upon entry into the soil, chemical compounds may be degraded, transformed, and retained depending on the number and types of soil microbes present (Blum, 1995). Thus, bioassays using allelopathic compounds in treated soils may better reflect plant responses such as seedling growth and biomass accumulation, as observed in the present study.

TEM, combined with biochemical assays, suggested possible mechanisms of plant responses to phenolic compounds from *P. americana* leaf extract (Figure 4). In control root tips, a large number of small vacuoles, mitochodrias, rER, and lipid bodies were observed in parenchyma cells (Figure 4A and B). In root tips treated with leaf extract, the arrows point to small vacuoles that were reduced in number, whereas an amorphous material was discharged from the small vacuoles into the central vacuole (Figure 4C-1). It also showed the accumulation of electron-dense deposits along the inside of the tonoplast (Figure 4C). Elakovich and Wooten (1991) reported that plant cells were not affected by osmotic potential below 143 mOsm kg^{-1}. In this research, the osmotic potential of diluted extract 75%, 50%, 25%, and 10% is 72, 46, 18, and 8 mOsm kg^{-1}, respectively. We treated *C. mimosoides* with a 25% concen-

tration of *P. americana* leaf extract. Therefore, the result in Figure 4 suggested that the observed changes in the root tip ultrastructure was not a result of high osmotic potential as reported by Lee and Kim (2000), but may be attributable to phenolic compounds in the leaf extract of *P. americana* that caused the development of starch grains in amyloplasts and electron-dense deposits in the central vacuole. Lovett (1982) and Rizvi and Rizvi (1992) reported a similar response in vacuole size of root tips affected by allelochemicals. Kutchan et al. (1986) also suggested that vacuoles are important in segregating phytotoxic secondary metabolites and play a compartmentalizing role in detoxification (Lovett, 1982; Burgos et al., 2004). The accumulation of electron-dense deposits along the tonoplast has not been previously observed (Figure 4A and B), but is consistent with a detoxification role. The amyloplasts (Figure 4D) observed in the present study may function to accumulate toxic substances for self-protection against *P. americana* leaf extract. The amyloplasts were also found in sunflower (*H. annuus*) root-tip cells following treatment with alkaloids from thornapple (*D. stramonium*) (Levitt et al., 1984; Lovett et al., 1987).

Enzymes play a major role in protecting the plant from oxygen free radicals (Asada, 1992; Sakihama and Yamasaki, 2002; Xiujuan et al., 2002). The isoenzyme form of peroxidase was reported to be the most important one in the differentiation process of plants and many plant defense mechanisms (Scandalios, 1990). Enzyme activity associated with root differentiation was found to increase with stress from toxins, drought, and freezing damage (Alscher and Hess, 1995). Among peroxidase isozymes, GuPOX and AsPOX are representative enzymes that are induced in plants that are mechanically damaged, infected by pathogens, or externally stressed. Lee and Kim (2000) reported that AsPOX was not responsible for the increase in peroxidase activity on *C. mimosoides* treated with *P. americana* leaf extract. Therefore, we investigated GuPOX activity and isozyme forms of peroxidase to understand the physiological response to phenolic allelochemicals. GuPOX activity increased as *P. americana* leaf extract concentration increased. Root activity was three times higher than shoot activity in *C. mimosoides* treated with different concentrations of *P. americana* extract. In the present study, there was a positive relation between GuPOX activity and *P. americana* leaf extract concentration (Figure 5). In response to elevated GuPOX activity, root differentiation was inhibited (Figures 2 and 3). Yoo and Kim (1988) reported that root activity was highly sensitive to external factors. GuPOX activity is known to be sensitive to external stress and plays a major role in protection mechanisms (Gaspar et al., 1985; Ren et al., 1999; Xiujuan et al., 2002). As GuPOX activity increased, inhibition of root growth increased as well. *C. mimosoides* may have been protected by increased GuPOX activity in response to the stress of *P. americana* leaf extract. We suggest that up-regulation of GuPOX activity may be a protection mechanism.

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CHEMISTRY OF THE LICHEN *Hypogymnia physodes* TRANSPLANTED TO AN INDUSTRIAL REGION

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Abstract—Lichens produce a great number of secondary metabolites that participate in ecological interactions and respond to environmental changes. We examined the influence of heavy metal accumulations on lichen secondary metabolism. Thalli of *Hypogymnia physodes* were transplanted for 6 months to the Cracow–Silesia industrial region. Based on heavy metal accumulations in lichen, two of the investigated sites were classified as highly polluted. The highest concentrations of Cd, Pb, and Zn were found in lichens transplanted in the vicinity of a Zn–Pb smelter. Significant accumulations of Cr and Ni were detected in *Hypogymnia* transplanted near a chemical industry. Physodic, physodalic, hydroxyphysodic acids, and atranorin were identified and analyzed in extracts obtained from specimen samples. The most detrimental changes were observed in lichen transplanted into the vicinity of a chemical industry producing chromium, phosphor, and sulfur compounds that contained 340-fold higher Cr levels than control thalli. Decreases in the levels of physodic acid, hydroxyphysodic acid, and atranorin were detected, and one additional polar compound (probably product of degradation of lichen acids) appeared in the extract. The content of physodalic acid increased in every thalli sample transplanted, suggesting a possible role of this compound in defense against stress caused by accumulated pollutants. The levels of physodic acid decreased in thalli from both of the most polluted sites compared to those of the controls—but were not changed in thalli transplanted to less polluted sites. Our results illustrate that lichen compounds are sensitive to heavy metal accumulation and could be used as biomarkers in environmental studies.

Key Words—Air pollution, heavy metals, sulfur, secondary metabolites, biomarkers.

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INTRODUCTION

Lichens are the symbiotic association between a fungus and a photosynthetic partner such as an alga or cyanobacterium. They are perennial and maintain a uniform morphology over time. They grow slowly, have a large-scale dependence on the environment for nutrition, and do not shed parts during growth. The lack of epidermis, stomata, and cuticular waxes makes these organisms incapable of controlling gas exchange and allows absorption of different contaminants over the entire surface of the organism (Conti and Cecchetti, 2001). Thus, for over 40 years, lichens have been widely used in monitoring air pollution whether as bioindicators or bioaccumulators in environmental quality evaluation of industrial surroundings (Pilegaard, 1979; Bargagli et al., 1997; Loppi et al., 1997; Garty, 1988; Jezierski et al., 1999), urban areas (Nimis, 1986; Gonzales et al., 1998; Budka et al., 2002), protected areas (Glenn et al., 1995), and even on national scale (Puckett and Finegan, 1980; Herzig et al., 1989; Freitas et al., 1999). In areas that lack naturally grown lichens resulting from excessive air contamination, lichen transplantation is applied as a standard method to study airborne metal and sulfur pollutants (Conti and Cecchetti, 2001; Budka et al., 2002). This method was first introduced by Brodo (1961). The cosmopolitan epiphytic lichen *Hypogymnia physodes* (L.) Nyl. has been considered as a suitable lichen monitor of atmospheric pollution in view of its relative resistance both to heavy metals and sulfur contamination (Bennett, 2000; Jeran et al., 2002).

Lichens are well known for being rich in structurally unique phenolic derivatives with unclear biological roles (Caviglia et al., 2001; Stark and Hyvärinen, 2003). Only 50–60 of the 630 known lichen secondary metabolites have been found in other fungi or higher plants (Elix, 1996). These compounds represent usually 0.1–5% of dry weight; however, they can comprise up to 20% of thalli dry weight (Romagni and Dayan, 2002; Molina et al., 2003).

The significant investment in energy and carbon dedicated to the production of lichen secondary metabolites suggests their important physiological and ecological roles. Lichen phenolic substances probably regulate interactions between mycobionts and phytobionts in the thallus (Molina et al., 2003) and may increase the permeability of phytobiont cell walls (Huneck, 1999). Lichens are slow-growing organisms and need long-term defense against both lower and higher plants, microbes, and herbivores (Huneck and Yoshimura, 1996). Up to 50% of the lichen species studied synthesize substances with some degree of antimicrobial activity (Romagni and Dayan, 2002). Lichen substances can influence the growth and development of different fungal species, including wood-decaying, pathogenic, and parasitic species growing on the thallus (Lawrey, 1983; Goldner et al., 1986). Many of these are effective growth inhibitors of higher plants (Lawrey, 1983; Huneck, 1999) and

can act as antifeedants for insects and other animals (Clark et al., 1999; Huneck, 1999; Molina et al., 2003). Lichens typically grow under extreme conditions of temperature, humidity, intensity of light, or excessive exposure to heavy metals. The Acarosporetum was found to be the most metal-resistant lichen community (Beck, 1999). Some lichen metabolites are considered to be stress metabolites synthesized in response to biotic and abiotic stimuli. These substances may protect thalli from the dangerous toxic actions of free radicals produced by oxidative stress exposure (Huneck and Yoshimura, 1996; Caviglia et al., 2001).

Lichen secondary metabolites have been investigated mostly for chemotaxonomic purposes and in connection with their potential as phytomedicines and natural biopesticides (Huneck and Yoshimura, 1996; Peres and Nagem, 1997; Huneck, 1999; Dayan and Romagni, 2001; Manojlovic et al., 2002). Only a few studies have considered changes in lichen secondary metabolites caused by environmental stress (Calatayud et al., 2000; Caviglia et al., 2001; Conti and Cecchetti, 2001; MacGillivray and Helleur, 2001). Because these compounds may play a role in the adaptation of lichens to their environment, as well as in ecological interactions, they are suitable candidates for the detection of detrimental changes in ecosystems caused by contamination (Jezierski et al., 1999; Caviglia et al., 2001).

A given stressor might cause an increase in the production of a lichen acid, or the normal level of lichen acids might be reduced as they react or decompose in response to stress (MacGillivray and Helleur, 2001). Because the roles of lichen secondary metabolites have not been well established, lichen transplantation seems to be a relevant model system for such investigations. Moreover, lichen compounds in thalli transplanted to polluted areas have not been studied so far.

The goal of the present investigation was to establish the accumulation of metals and sulfur as well as a number of selected secondary metabolites profile in lichen *H. physodes* (L.) Nyl. transplanted to sites with different pollution exposure.

METHODS AND MATERIALS

Test Sites. The studies covered the Cracow–Silesia industrial region in southern Poland (Figure 1). This area has a population density of ca. 280 persons/km. It is one of the most polluted areas in Europe, and still has among the highest emissions of heavy metals in Poland. The investigated sites were located in the vicinity of major emission sources from industrial plants, all of which have been placed on the list of industries with the most harmful impact on the environment in Poland (Bereś et al., 2003). As a northwesterly wind predominates in this region of Poland, the locations of the test sites were selected

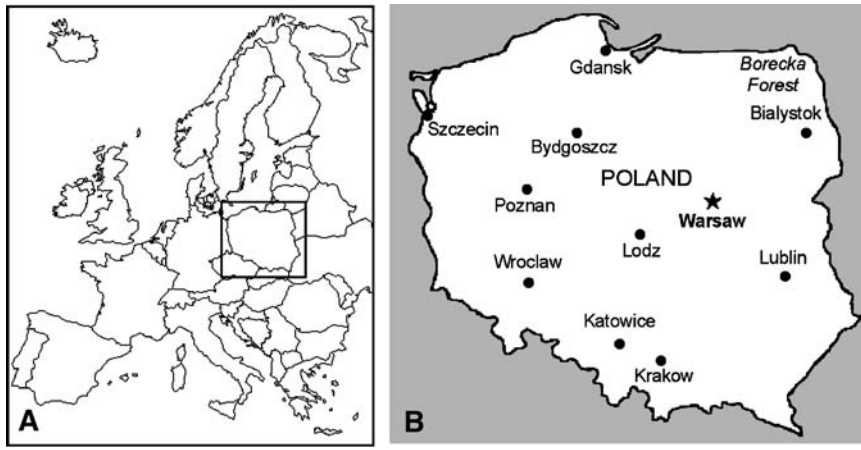

FIG. 1. A) The geographical location of Poland within Europe. B) Major cities in Poland, including the location of the unpolluted Borecka forest where the original lichen samples were collected (northeast of Poland), and the Cracow region where the tests were conducted.

southeast of the emission sources. The following sites of area 200 m^2 were chosen for the study:

1. Bukowno (Figure 2). This site is in the immediate vicinity of the "Bolesław" Zn–Pb smelter. This industry was founded in 1949 and mainly involves mining of zinc and lead, as well as production of their concentrates. In 1990, this industry emitted over 17, 7, and 0.3 tons of Zn, Pb, and Cd, respectively. Since then, successively lower emission levels have been observed. In 2000, emission of particulates reached 0.753, 0.0022, and 0.0002 tons of Zn, Pb, and Cd, respectively (data provided by the Zn–Pb smelter).
2. Młoszowa (Figure 2). The main pollution source in this site (the Trzebinia S.A. refinery) was established at the end of the 19th century. It produces fuels, asphalts, paraffin, and oils. The quality of the environment at this site may also be affected by emission from the "Siersza" S.A. power plant in Trzebinia. This study area is also relatively close to the A-4 Cracow–Katowice highway.
3. Jankowice (Figure 2). This site is affected by emission from the chemical industry "Dwory" S.A. in Oświęcim. This industry emitted 743,000 tons of gaseous pollutants (mainly PAHs, Cl, and S compounds) in 2000 (Bereś et al., 2003).
4. Alwernia (Figure 2). This site is located in the immediate vicinity of the "Alwernia" S.A chemical industry, which was established in 1924.

Currently, the industry produces chromium, phosphorus, and sulfur compounds. Emission of dusts from "Alwernia" in 2000 reached 78,000 tons, including 1.7 tons of Cr. In 2002, the industry emitted 1.3 tons of Cr and 263 tons of gaseous pollutants (data provided by "Alwernia" S.A.).

Lichen Transplantation. Control lichens for transplantation were collected in the Borecka Forest located in the northeastern part of Poland (Figure 1), far from large metropolitan areas or other pollution emission sources (Śnieżek, 1997). Samples of the epiphytic foliose lichen *H. physodes* (L.) Nyl., growing on branches of hazel tree (*Corylus avellana* L.), were collected and transplanted to the various study sites on April 15, 2002. Ten branches, ca. 30 cm long, containing about 10–15 g wet weight of the thalli in total, were brought to each site and hung on selected trees at a height of ca. 1.5 m (Figure 3). After 6 months, thalli were separated from the bark and analyzed. Untreated controls consisted of samples collected and transplanted within the Borecka Forest.

Elemental Analysis of Thalli Tissues. Thalli collected from the various test locations were oven-dried to constant weight at 60°C and ground in an electric mill. Samples weighing 0.3–0.5 g were digested in $HNO_3/HClO_4$ (4:1) on a water bath at 80°C for about 3 wk (until totally clear). Cd, Cr, Ni, and Pb levels

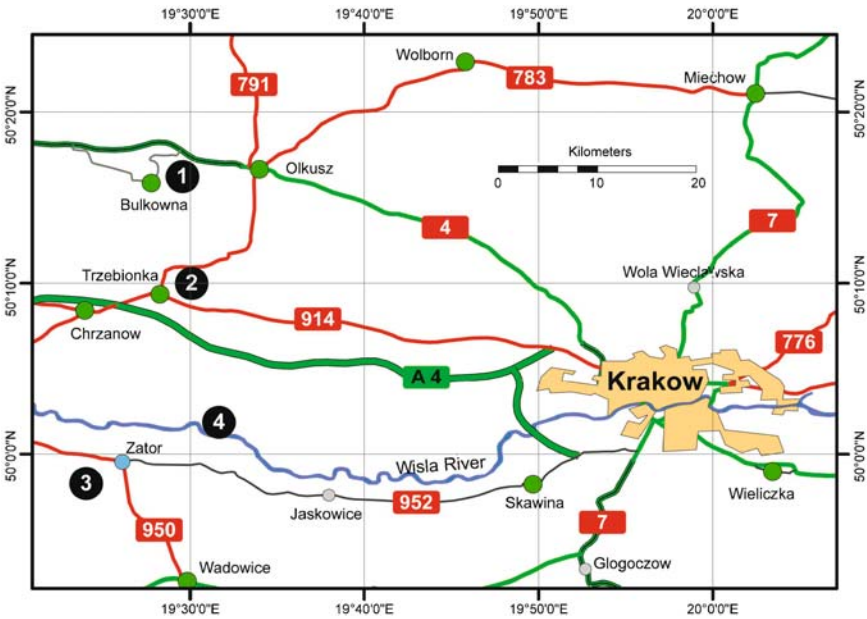

FIG. 2. The Cracow region showing the locations of the various test sites.

FIG. 3. Transplanted lichens hanging from a birch tree.

were determined by using an Aanalyst 800 Perkin-Elmer atomic absorption spectrophotometer with a graphite furnace. Cu, Fe, and Zn concentrations were determined with an IL 251 flame spectrophotometer. Results were expressed in $\mu g\ g^{-1}$ of dry weight. The accuracy of the analysis was checked against standard reference material, namely, Tomato Leaves SRM 1573a.

Total levels of sulfur in transplanted lichens were detected with the turbidimetric method by Butters-Chenry (Nowosielski, 1968). Homogenized thalli (0.2 g) were treated with 75% nitric acid and left for 24 hr at 18°C, evaporated, and treated with magnesium nitrate. This treatment was followed by oxidization at 450°C and treatment with 25% nitric acid, 50% acetic acid, 85% orthophosphoric acid, and 2% barium chloride. The resulting opaqueness was measured by colorimeter (Spectromom 204) at 290 nm. Concentrations of sulfur were calculated according to a $MgSO_4$ standard curve. Results were expressed in $\mu g\ g^{-1}$ of dry weight.

Analysis of Secondary Metabolites in Thalli. After transplantation, visually similar thalli (1.5–2 g of air-dried thalli) were extracted three times for 15 min with 100% ethanol (about 30 ml/g of lichen) with the use of a sonicator. Extracts were filtered and evaporated on a rotary evaporator. Crude extracts (2.5 mg) were diluted in 3 ml of methanol [high-performance liquid chromatography (HPLC) grade]. For separation and analysis of the secondary metabolites, 20 μl of samples were injected onto an analytical HPLC. The HPLC system consisted of a Waters 717 with autosampler, a Waters 600 controller, a Waters photodiode array detector, and 4.6 × 250 mm analytical column (Water Spherisorb 5 μm ODS).

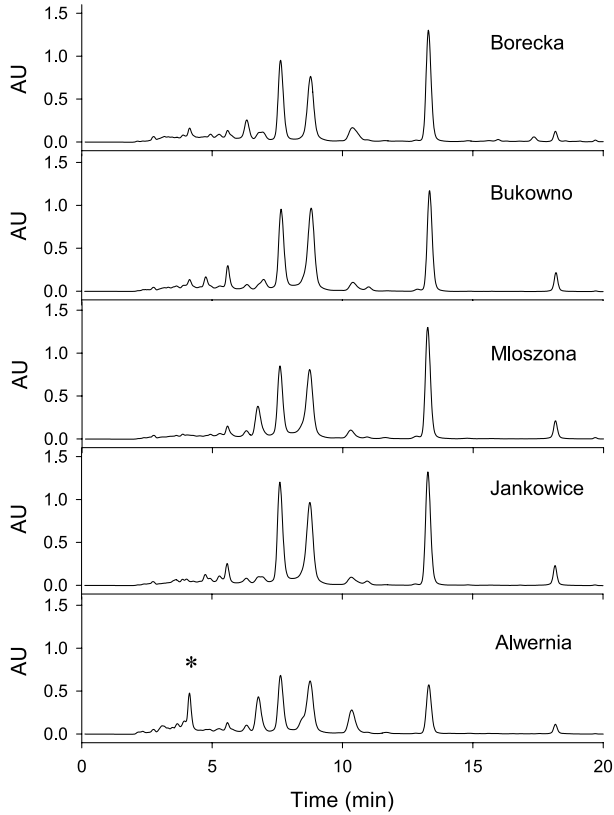

FIG. 4. Chromatograms of crude extracts from *H. physodes* samples from the various test locations. The retention time of hydroxyphysodic acid (7.7), physodalic acid (8.7), physodic acid (13.5), and atranorin (18.1) in the crude extracts matched those of the analytical standards at 7.6, 8.8, 13.3, and 18.2 min, respectively. The identities of the peaks were confirmed by comparing the UV spectra in the crude extract to that of the analytical standards. The polar peak in Alwernia chromatogram is denoted by an asterisk.

The best separation was obtained with 1% phosphoric acid in water (**A**) and methanol (**B**) (Feige et al., 1993, modified) using a gradient from 75 to 100% **B** over 15 min. The flow rate was 1 ml/min and pressure 1500–2000 mm Hg. Elution of the secondary metabolites was monitored at 254 nm. Concentrations of compounds were estimated and/or compared based on peak areas. Compounds were identified by comparison with standards (retention time and UV absorption). Except for the absolute amount, the percentage of total peak area in the whole extract was given. Crude extracts constituted 15–18% of the air-dried weight of thalli. Chromatographic separation revealed three major metabolites (Figure 4) identified as hydroxyphysodic acid (Rt = 7.6 min), physodalic acid (Rt = 8.8 min), and physodic acid (Rt = 13.3 min). Additionally, a smaller peak was recognized as atranorin (Rt = 18.2 min). The chemical structures of these compounds are given in Figure 5. Lichen standards were previously isolated from *H. physodes* at the Center for Research on Natural Products, University of Mississippi. The structures were identified by mass spectrometry and nuclear magnetic resonance. Standard deviations of the HPLC analysis reproducibility were lower than 2.5%.

Statistical Analysis. Means and standard deviations were calculated for elements and secondary compounds. Statistical differences in the concentrations of Cd, Fe, Ni, S, Zn, and lichen acids in thalli transplanted to different sites were established using ANOVA on logarithmic data, followed by Tukey honestly significant difference for equal sample sizes. For determination of statistical differences in Cu, Cr, and Pb concentrations, Kruskal–Wallis nonparametric tests were applied followed by paired comparisons of the mean ranks (Sachs, 1984).

RESULTS

Accumulation of Elements. Six months after transplantation of the lichens, the highest concentrations of Cd, Pb, and Zn were found in samples transplanted to the Bukowno location, in the vicinity of the Zn–Pb smelter, relative to both control thalli and thalli transplanted to the other sites (Table 1). Pb concentrations did not increase in the other locations, relative to the control thalli (about 10 $\mu g\ g^{-1}$). However, Pb reached 124 $\mu g\ g^{-1}$ in lichen transplanted to Bukowno (Table 1). Similarly, Zn accumulation was rather low in all of the studied sites, except for Bukowno where it increased from 55 $\mu g\ g^{-1}$ (control thalli) to 583 $\mu g\ g^{-1}$ (Table 1). Accumulations of Cd were found in all of the transplanted lichens. The level of this element in thalli from Bukowno was ca. 14-fold higher than control thalli. The level increased only 2–2.5-folds in thalli transplanted to the other sites studied (Table 1).

Significant accumulations of Cr and Ni were detected in *H. physodes* transplanted to the Alwernia location (Table 1). The level of Cr 6 months after transplantation to this site was as much as 343-fold higher than in control

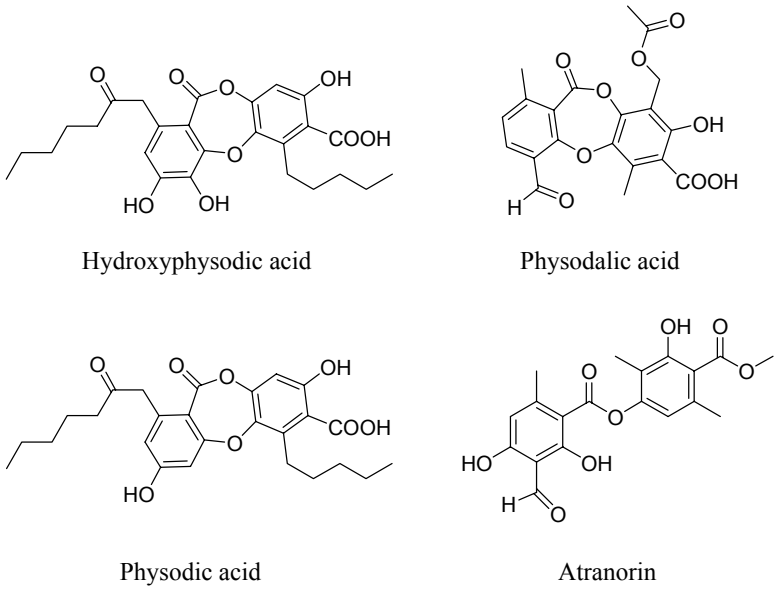

FIG. 5. Structures of the secondary metabolites detected in *H. physodes*.

lichens (Table 1). Ni increased to 9.18 μg g^{-1} compared to 1.08 μg g^{-1} in the control. Additionally, the concentration was slightly increased in thalli from the Bukowno location, but was not statistically different from the rest of the sites (Table 1).

Increases in Cu and Fe concentrations occurred in all thalli after transplantation, but the highest accumulations were noted in *Hypogymnia* samples from Bukowno and Alwernia (Table 1).

Finally, there were significant accumulations of sulfur in all transplanted lichens. Sulfur concentrations ranged from 1406 to 2188 μg g^{-1} in thalli transplanted to the polluted sites compared to 1237 μg g^{-1} in control thalli (Table 1).

Secondary Metabolites in Transplanted Thalli. Significant differences in the quantity of secondary metabolites were found between control thalli from Borecka Forest and thalli transplanted to the polluted areas, as well as among lichens transplanted to areas of different pollution levels.

The most significant decrease in physodic acid (−58% relative to control thalli) was detected in thalli transplanted to Alwernia. The level of physodic acid decreased also in transplanted lichens transplanted in Bukowno (−13% relative to the control thalli). The contents of this compound were not changed in *Hypogymnia* transplanted to Młoszowa or Jankowice (Figure 6A).

TABLE 1. CONCENTRATION OF ELEMENTS IN *Hypogymnia physodes* TRANSPLANTED TO THE CRACOW–SILESIA INDUSTRIAL REGION

Locations	Elements (μg g^{-1} dry weight)							
	Pb	Zn	Cd	Cr	Ni	Cu	Fe	S
Borecka	9.0 ± 0.4a	55 ± 7a	0.54 ± 0.02a	0.22 ± 0.04a	1.08 ± 0.19a	3.7 ± 0.4a	350 ± 47a	1237 ± 33a
Bukowno	123.7 ± 20.0b	583 ± 56c	7.70 ± 0.47d	0.79 ± 0.06b	1.91 ± 0.12b	10.8 ± 0.3d	1306 ± 164c	1842 ± 397b
Młoszowa	11.2 ± 1.5a	68 ± 6ab	1.37 ± 0.05c	0.48 ± 0.06b	1.36 ± 0.19ab	7.5 ± 0.4bc	720 ± 106b	1960 ± 109b
Jankowice	11.3 ± 1.2a	55 ± 4a	1.02 ± 0.12b	2.89 ± 0.54b	1.38 ± 0.06ab	5.9 ± 0.3b	597 ± 38b	1999 ± 242b
Alwernia	12.7 ± 3.1a	77 ± 5b	1.29 ± 0.10bc	75.85 ± 4.62c	9.18 ± 1.42c	9.9 ± 1.8cd	1202 ± 368c	1665 ± 109ab

Data represent mean ± SD. Means within a column with different letters are statistically different in the element levels between studied sites at $P < 0.05$ for $N = 5$.

The level of physodalic acid significantly increased in all transplanted lichens compared to control thalli from Borecka Forest (Figure 6B). The highest concentrations were in lichens transplanted to Bukowno and Alwernia (63 and 13% higher than in control samples, respectively; Figure 6B). Additionally, increases in the percentage contribution of physodalic acid in the crude extract were observed in samples from Borecka Forest (17.1% increase), in Bukowno (25.8% increase), and in Alwernia (20.6% increase) (Table 2).

Concentrations of atranorin increased in thalli transplanted to Jankowice (by 36%), Bukowno (by 30%), and Młoszowa (by 16%) (Figure 6C). A decrease in atranorin level was observed in lichen samples transplanted to

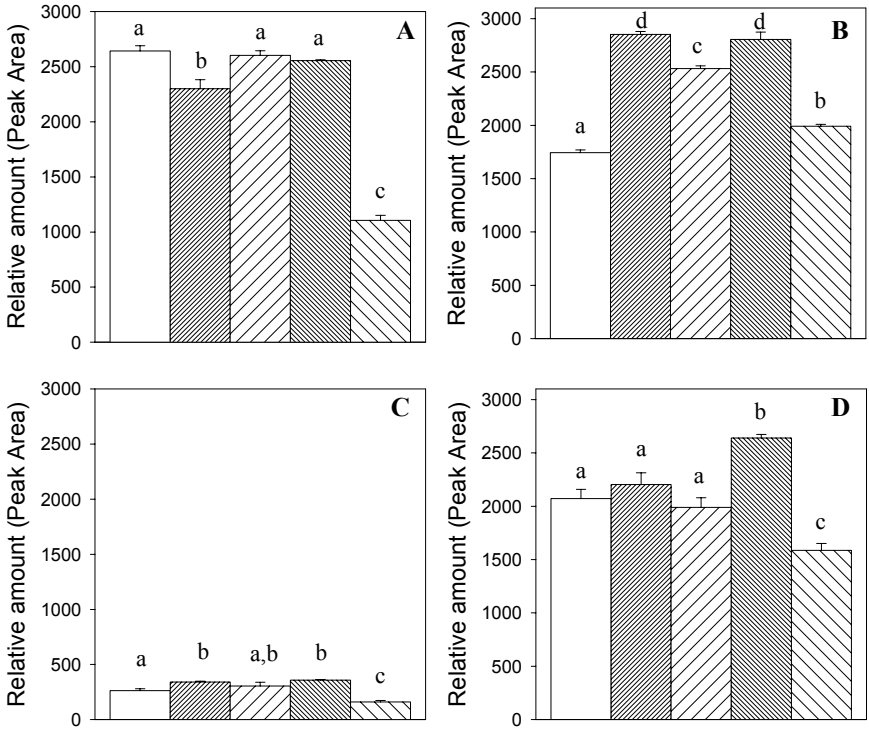

FIG. 6. Concentrations of (A) physodic acid, (B) physodalic acid, (C) atranorin, and (D) hydroxyphysodic acid in lichen *H. physodes* samples transplanted and acclimated to various levels of environmental pollution. = Borecka (control), = Bukowno, = Młoszowa, = Jankowice, and = Alwernia. Controls consisted of samples that had been collected and transplanted within the Borecka forest site. Data represent mean ± SD. Bars with the same letters are not statistically different based on ANOVA ($P < 0.05$), $N = 3$.

TABLE 2. RELATIVE AMOUNTS (%) OF THE SECONDARY METABOLITES IN THE EXTRACT ISOLATED FROM *H. physodes* FROM VARIOUS TEST LOCATIONS

Location	Unidentified (Rt = 4.4) (%)	Hydroxyphysodic acid (%)	Physodalic acid (%)	Physodic acid (%)	Atranorin (%)
Borecka	–	18.4	17.1	23.5	2.3
Bukowno	–	20.0	25.8	25.8	3.1
Młoszowa	–	18.7	23.7	24.4	2.9
Jankowice	–	21.7	24.4	22.2	3.1
Alwernia	12.1	16.5	20.6	11.5	1.7

Relative peak area was used.

Alwernia. Similar tendencies of this compound were noted on a percentage basis (Figure 6C, Table 2).

An increase in hydroxyphysodic acid concentration was observed in *H. physodes* transplanted to Jankowice forest (Figure 6D). However, the percentage in the crude extract was not changed (Table 2). In lichen transplanted to Alwernia, thalli contained lower levels of hydroxyphysodic acid than control thalli (Figure 6D).

Crude extracts obtained from lichens transplanted to Alwernia contained a lower percentage of physodic acid, hydroxyphysodic acid, and atranorin. Additionally, an unidentified peak (yellow oily compound) with a retention time (Rt) of 4.1 was observed in *H. physodes* samples from Alwernia and constituted 12% of the crude extract (Table 2).

DISCUSSION

Accumulation of elements in transplanted *H. physodes* revealed significant spatial diversification of air pollution in the investigated Cracow–Silesia industrial region. According to our results, sites were classified as either heavily polluted (Bukowno and Alwernia) or moderately polluted (Młoszowa and Jankowice), with less harmful impact of neighbor industries on the environment. Areas with high levels of air pollution are directly influenced by emissions from the Zn–Pb smelter (Bukowno) and from the chemical industry (Alwernia).

To assess whether our results were unusually high, the obtained concentrations of elements were checked against baseline values for chemical elements in *H. physodes*, the most studied lichen in the monitoring of air quality (compiled by Bennett, 2000; Table 3). We found the highest concentrations of Pb, Cd, and Zn in lichens transplanted near the Zn–Pb smelter, where values for these elements were either equal (Pb) or exceeded means for enriched areas

TABLE 3. STATISTICAL BASELINE VALUES FOR CHEMICAL ELEMENTS IN *H. physodes* (AFTER BENNETT, 2000)

	Elements ($\mu g\ g^{-1}$ dry weight)							
	Pb	Zn	Cd	Cr	Ni	Cu	Fe	S
Background average	19.5	73	0.56	2.11	1.72	6.0	621	738
Enriched average	126.9	427	2.56	43.50	12.28	28.6	3081	1695

"Background"—any site not in the city, nor near a point source, and not under any kind of local pollutant influence. "Enriched"—opposite to these criteria.

(Cd, Zn; Table 3). In the rest of the transplantation sites, concentrations of Zn and Pb were equal or lower than mean levels for background areas. Cd was lower than mean levels for enriched areas (Table 3). Baranowska-Bosiacka et al. (2001) found that Pb and Cd accumulated in *H. physodes* remained mainly on the surface of the thalli, whereas Zn and Cu penetrated into the protoplast. Microparticle-induced X-ray emission (PIXE) analysis of thalli transplanted to polluted sites revealed that Zn was concentrated in the algal layer and lower cortex, whereas Cu and Pb were detected only from analyses of smaller areas in individual layers of thalli (Budka et al., 2002). Although Cd was detected with AAS, it was not visible in PIXE studies that confirmed the surface deposition (Budka et al., 2004). We noted the highest levels of Cr and Ni in *Hypogymnia* transplanted near a chemical factory (Alwernia) producing chromium, phosphorus, and sulfur compounds. Cr values were twice the mean for enriched areas, whereas Ni was close to it. In the rest of our test sites, Cr and Ni levels were equal or lower than the mean for background areas (Table 3). Neither Cu nor Fe exceeded threshold levels, even in the most polluted sites (Table 3). PIXE analysis of transplanted *H. physodes* revealed the highest concentrations of Fe in the lower cortex, which suggests a transport mechanism within thalli or a nonatmospheric origin of this element. Additionally, low concentrations of Fe were found in the upper part of the lichen, which could be from dust distributed on the surface (Budka et al., 2004). Especially high values in relation to data given by Bennett (2000) were noted for S levels. Even control lichen from the Borecka forest had almost twice the S levels than the mean for background areas. These levels exceeded the threshold level in the rest of studied sites (Table 3) and reflect the widespread occurrence of air pollution containing sulfur compounds over Poland.

Before the symptoms of different kinds of stress, including pollution, can be visualized by morphological or ultrastructural changes, less obvious results occur on the molecular or chemical level. These less salient markers have become a subject of interest in recent years as they may be used as early indicators of environmental stress on a target organism before damage occurs.

The potential use of secondary metabolites as markers for environmental stress has been postulated for plants and lichens (Jones and Coleman, 1989; Zobel and Nighswander, 1990, 1991; Zobel, 1996; Rabotti and Ballarin-Denti, 1998; Loponen et al., 2001; Caviglia et al., 2001). Moreover, because secondary substances play a significant role in ecosystem interactions, and actions of different kinds of stressors cause modification in their quantity and composition, which, in turn, influences other ecosystem partners (Larcher, 1995). For this reason, secondary compounds can also be used as warning indicators for the surrounding ecosystem (MacGillivray and Helleur, 2001).

Extracts from *H. physodes* contained several biologically active compounds (Huneck and Yoshimura, 1996) and may influence the growth of other organisms in their surroundings. For example, physodic acid has anticancer, antimutagenic, antiviral, and allergenic activities and may inhibit cell division. Atranorin has anticancer, fungitoxic, and antimicrobial activities and may also influence growth of plants as well as growth and development of insects. Hydroxyphysodic acid has potent insecticidal activity (Huneck and Yoshimura, 1996; Hunek, 1999; Romagni and Dayan, 2002). Therefore, it is possible that changes in concentration may influence the fitness of lichens to their environment. Because all of the described metabolites may play a role in the interaction of a lichen with its biotic environment, any reduction in concentration may be reflective of the health of lichens and lower resistance to herbivore attack, diseases, as well as interactions with lower plants (e.g., algae and bryophytes; Huneck, 1999), and host trees. In another study, the metabolites from epiphytic lichen were found in the xylem of the host (Avalos et al., 1986).

The most detrimental changes were observed in secondary metabolites of lichens transplanted to Alwernia, which accumulated high levels of Cr. Significant decreases in hydroxyphysodic acid, physodic acid, and atranorin were observed in thalli collected 6 months after transplantation. Interestingly, crude extracts of lichens transplanted to Alwernia contained an additional unidentified polar peak in chromatograms (Rt = 4.1, denoted as an asterisk in Figure 4) that constituted as much as 12% of the crude extracts. This peak was not observed in lichen extracts from the other sites, including control thalli. After isolation, this polar fraction eluting as a single peak (Figure 4) was a dark yellowish, oily substance and most probably is the product of degradation of lichen acids caused by accumulated chromium ions. According to Jezierski et al. (1999), intensified free radical production in lichens exposed to air pollution is the result of degradation of lichen acids to β-diketones. The unidentified peak might also be a compound produced by lichens for the purpose of defense against stress associated with the accumulation of pollutants in thalli.

A decrease in physodic acid was observed in lichens transplanted to both of the most polluted sites—the forests in Alwernia and Bukowno. In Bukowno, significant accumulations of Pb, Cd, and Zn were noted in thalli collected after

the transplantation period. However, the decrease was more intense in thalli that accumulated Cr, suggesting the reductive power of this element on the degradation of physodic acid.

Thalli of lichens transplanted to all of the test sites in the Silesia–Cracow industrial region contained higher concentrations of physodalic acid than control thalli. Thus, biosynthesis of physodalic acid seems to be elicited in response to stress caused by exposure to heavy metals. Data found in the literature show physodalic acid as an antimutagenic compound (Romagni and Dayan, 2002). There has been no record of detoxifying activity for this substance. The slight increase in atranorin found in lichens transplanted to Bukowno, Młoszowa, and Jankowice suggests a possible role of this compound in detoxification, or it may be a precursor of other phenolic compounds.

In summary, significant, site-specific changes in chemistry of thalli were found in lichens transplanted to the polluted regions selected in this study. The most detrimental alterations were detected in thalli that accumulated high levels of Cr after transplantation near the chemical industry. Decreases in the levels of physodic acid, hydroxyphysodic acid, and atranorin were detected, and one additional peak appeared in the extract. A decrease in physodic acid concentration was also observed in lichens transplanted to the other most highly polluted site near the Zn–Pb smelter as the effect of accumulations of Cd, Pb, and Zn. An increase in the content of physodic acid in all transplanted lichens suggests a role of this compound in defense against stress caused by accumulated pollutants.

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RAPID COMMUNICATION

1,4-DIMETHOXYBENZENE, A FLORAL SCENT COMPOUND IN WILLOWS THAT ATTRACTS AN OLIGOLECTIC BEE

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Abstract—Many bees are oligolectic and collect pollen for their larvae only from one particular plant family or genus. Here, we identified flower scent compounds of two *Salix* species important for the attraction of the oligolectic bee *Andrena vaga*, which collects pollen only from *Salix*. Flower scent was collected by using dynamic-headspace methods from *Salix caprea* and *S. atrocinerea*, and the samples were subsequently analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD) to detect possible attractants of *A. vaga*. EAD active compounds were identified by gas chromatography coupled to mass spectrometry. Both *Salix* species had relatively similar scent profiles, and the antennae of male and female bees responded to at least 16 compounds, among them different benzenoids as well as oxygenated monoterpenoids and sesquiterpenoids. The strongest antennal responses were triggered by 1,4-dimethoxybenzene, and in field bioassays, this benzenoid attracted females of *A. vaga* at the beginning of its flight period, but not at the end.

Key Words—Floral scent, *Salix*, willows, GC-EAD, oligolectic bees, *Andrena*, GC-MS, flower visitor attraction.

INTRODUCTION

Bees are important pollinators of flowering plants. They visit flowers primarily to take nectar for their food and to collect nectar/oil and pollen for their larvae.

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Many of the about 30,000 bee species worldwide are oligolectic and collect pollen for the larvae only from a particular plant family or genus. So far, little is known about the cues used by the oligolectic bees to find host plants. However, it has been shown that naive bees of oligolectic *Chelostoma florisomne* (L.) rely on flower and especially pollen odors to recognize host plants (Dobson and Bergström, 2000), and floral scent compounds generally may be important for host plant finding by oligolectic bees.

Salix L. is a genus that hosts many different oligolectic bee species, probably because of its readily accessible pollen (Michener, 2000). *Salix* is a woody genus, distributed almost worldwide, but centered in the northern hemisphere (Newsholme, 1992). Plants are dioecious, and insects are important pollen vectors (e.g., Karrenberg et al., 2002). The often strongly scented flowers are borne in catkins. The scent is assumed to attract the pollinators (Tollsten and Knudsen, 1992). In Europe, several species of the genus *Andrena* are specialized on *Salix*, among them *Andrena vaga* Pz. (Westrich, 1989).

Here, we used coupled gas chromatography and electroantennography (GC-EAD) to elucidate the floral scent compounds of *Salix caprea* L. and *S. atrocinerea* Brot. that elicit signals in the antennae of female and male *A. vaga*. The compound that elicited the main signal in the antennae of bees was further tested for attraction in a field bioassay.

METHODS AND MATERIALS

Plant Material and Volatile Collection. Floral scent was collected from male plants of *S. caprea* and *S. atrocinerea* in the Ecological–Botanical Garden of the University of Bayreuth. For each sample, floral scent was collected from 30 to 40 catkins during daytime for 8 hr by using dynamic headspace methods. Flowering branches were cut in the field and placed in water in the laboratory for immediate scent collections (compare with Tollsten and Knudsen, 1992).

Flowering branches were enclosed in an oven bag (Nalophan), and the emitted volatiles were trapped in an adsorbent tube filled with 20 mg of a 1:1 mixture of Tenax-TA 60–80 and Carbotrap 20–40. The air was sucked from the bag over the adsorbent by a membrane pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany). Volatiles were eluted with 80 μl of acetone (SupraSolv, Merck KgaA, Germany) to obtain odor samples for the chemical and electrophysiological analyses (see below).

Electrophysiology. Electrophysiological analyses of the floral scent extracts were performed with a GC-EAD system. Antennae from two females and two males of *A. vaga* were tested. The bees were caught on the 4th and the 21st of April at a nesting site in the Ecological–Botanical Garden. The GC-EAD system consisted of a gas chromatograph (Vega 6000 Series 2, Carlo Erba, Rodano, Italy) equipped with a flame ionization detector (FID) and an EAD

setup [heated transfer line, two-channel universal serial bus (USB) acquisition controller] provided by Syntech (Hilversum, Netherlands). One microliter of an odor sample was injected splitless at 60°C, followed by opening the split vent after 1 min and heating the oven at a rate of 10°C/min to 200°C. The end temperature was held for 5 min. A ZB-5 column was used for the analyses (length 30 m, inner diam 0.32 mm, film thickness 0.25 μm, Phenomenex). The column was split at the end by the four-arm flow splitter GRAPHPACK 3D/2 (Gerstel, Mülheim, Germany) into two pieces of deactivated capillary (length 50 cm, ID 0.32 mm) leading to the FID and to the EAD setup. Makeup gas (He, 16 ml/min) was introduced through the fourth arm of the splitter. For measurements, an excised antenna was mounted between glass micropipette electrodes filled with insect ringer (8.0 g/l NaCl, 0.4 g/l KCl, 0.4 g/l $CaCl_2$). The electrodes were connected to silver wires.

Chemical Analyses. To identify the EAD active compounds, 1 μl of the scent samples was analyzed on a Varian Saturn 2000 mass spectrometer and a Varian 3800 gas chromatograph fitted with a 1079 injector (Varian Inc., Palo Alto, CA, USA). Column and settings were as described in Dötterl et al. (2005). Component identification was carried out using the NIST 02 mass spectral database, or MassFinder 3, and was confirmed by comparison with retention times of authentic standards.

Behavioral Experiment. To test the attractiveness of 1,4-dimethoxybenzene, which elicited the main signal in the antennae of the bees, a two-choice bioassay was conducted in spring 2005 in the Ecological–Botanical Garden near the nesting site of *A. vaga*. One rubber GC septum impregnated with 10 μl of 1,4-dimethoxybenzene (99%, Aldrich) and one blank rubber GC septum were presented on a stand (distance of the septa, 1 m) around noon for 20 min, when activity of bees was high. The reaction of bees was classified as "zigzagging" when the bees flew upwind toward one of the septa up to within 10 cm and as "landing" when the bees had contact with a septum.

RESULTS AND DISCUSSION

Floral odors of *S. caprea* and *S. atrocinerea* elicited clear signals in the antennae of both sexes of *A. vaga* (Figure 1). At least 16 EAD active compounds were found, of which 11 were present in both *Salix* species. The antennae of the bees responded especially to different benzenoids and isoprenoids, and 1,4-dimethoxybenzene consistently elicited the main antennal response. Clear signals were also triggered by different monoterpene oxides, by the nitrogen-bearing compound indole, and by the sesquiterpene (*E*,*E*)-α-farnesene. The antennal responses of female and male bees were similar; however, female bees were more strongly tuned to oxygenated sesquiterpenes, such as (*E*)-nerolidol.

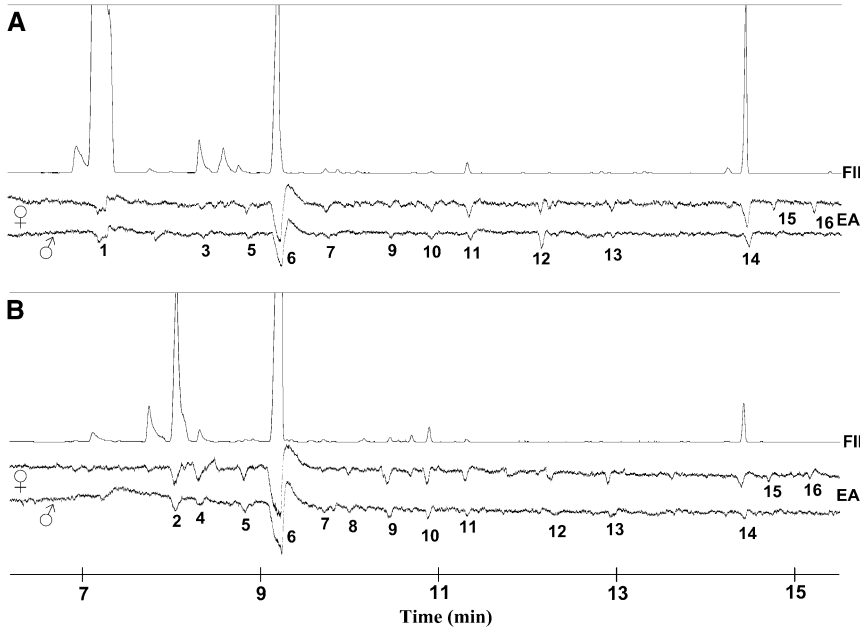

FIG. 1. Coupled gas chromatographic and electroantennographic detection (GC-EAD) of *Salix caprea* (A) and *S. atrocinerea* (B) flower scent samples using antennae of *Andrena vaga* males and females. **1**: (*E*)-β-ocimene; **2**: linalool; **3**: 4,8-dimethyl-1,3,7-nonatriene; **4**: 2-phenylethanol; **5**: 4-oxoisophorone/benzyl nitrile; **6**: 1,4-dimethoxybenzene; **7**: methyl salicylate; **8**: lilac alcohol isomer; **9**, **10**: monoterpene oxides; **11**: indole; **12**: eugenol; **13**: sesquiterpene; **14**: (*E*,*E*)-α-farnesene; **15**: sesquiterpene oxide; **16**: (*E*)-nerolidol.

TABLE 1. NUMBER OF FEMALES OF *Andrena vaga* ATTRACTED TO A SEPTUM IMPREGNATED WITH 1,4-DIMETHOXYBENZENE (DMB) AND TO A BLANK SEPTUM (CONTROL)

	"Zigzagging"		"Landing"	
Date	DMB	Control	DMB	Control
5 April	6	0	0	0
11 April	7	0	3	0
13 April	4	0	1	0
14 April	4	0	0	0
26 April	0	0	0	0
6 May	0	0	0	0

The bioassay proved that 1,4-dimethoxybenzene not only elicits antennal responses but also mediates behavioral responses (Table 1), at least in female bees. They flew upwind toward the septum impregnated with this benzenoid, and some landed on it for a short period of time. After landing, they did not show the nectar drinking or pollen-collecting behavior, which can be observed on *Salix* catkins.

However, in the first 2 weeks of April only, at the beginning of their flight season, females were consistently attracted to 1,4-dimethoxybenzene. End of April/beginning of May bees were not attracted at all. It seems that only freshly hatched, foraging-naive bees were attracted. This observation is consistent with the results of Dobson and Bergström (2000). They found that oligolectic *C. florisomne* relied on protoanemonin, the dominant pollen odor of its host plants (*Ranunculus*), only if the bees were foraging-naive, whereas foraging-experienced bees recognized their host plants on the complex volatile blend of a whole flower, which first has to be learned.

Not a single male of *A. vaga* was attracted by 1,4-dimethoxybenzene, although males also strongly responded to this substance in the GC-EAD study, and males were regularly observed on *Salix* species, probably drinking nectar. Because males of *A. vaga* hatch 2–3 weeks earlier than females (Westrich, 1989), we cannot rule out that they were already foraging-experienced when bioassays were conducted. Possibly, foraging-experienced bees have learned to recognize complex flower scents and do not rely on their innate preferences for single scent compounds (see also Dobson and Bergström, 2000).

1,4-Dimethoxybenzene is found in floral scents of all *Salix* species so far studied (this study and Tollsten and Knudsen, 1992). Furthermore, it is a common compound in orchids pollinated by perfume-collecting euglossine bees (Williams and Whitten, 1983; Gerlach and Schill, 1991), where it is suspected to be a good bee attractant (Williams and Whitten, 1983). 1,4-Dimethoxybenzene is also known to attract the chrysomelid beetle *Diabrotica speciosa* (Ger.) (Ventura et al., 2000).

To summarize, our results demonstrate that floral scent compounds of *Salix* can act as cues for the attraction of oligolectic bees, and that 1,4-dimethoxybenzene attracts female *A. vaga* bees. In oligolectic bees, males often search for females at the host plants; therefore, floral scent could be used by males in combination with female sex pheromones to find females. This is the first study analyzing antennal and behavioral responses of an oligolectic bee to the floral scent of its host plants.

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RAPID COMMUNICATION

(2,3,4,4-TETRAMETHYLCYCLOPENTYL)METHYL ACETATE, A SEX PHEROMONE FROM THE OBSCURE MEALYBUG: FIRST EXAMPLE OF A NEW STRUCTURAL CLASS OF MONOTERPENES

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Abstract—The sex pheromone of the obscure mealybug, *Pseudococcus viburni*, consists of (1*R**,2*R**,3*S**)-(2,3,4,4-tetramethylcyclopentyl)methyl acetate, the first example of a new monoterpenoid structural motif in which the two isoprene units forming the carbon skeleton are joined by 2′–2 and 3′–4 connections rather than the usual 1′–4, head-to-tail connections. This highly irregular terpenoid structure, and the irregular terpenoid structures of related mealybug species, suggest that these insects may have unique terpenoid biosynthetic pathways.

Key Words—Sex pheromone, nonhead-to-tail terpenoid, irregular terpenoid, (2,3,4,4-tetramethylcyclopentyl)methyl acetate, *Pseudococcus viburni*.

INTRODUCTION

The great majority of the more than 30,000 isoprenoid-derived compounds identified from natural sources are formed from 1′ to 4, head-to-tail connections between isopentenyl diphosphate and dimethylallyl diphosphate, the two

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5-carbon building blocks that are connected to form the carbon skeletons of all known terpenoids (Poulter, 1990; Rivera et al., 2001). Further cyclizations and rearrangements then produce the variety of known terpene skeletons. Irregular terpenoids in which the isoprene units are joined in nonhead-to-tail fashion are relatively rare, with most examples being found in members of the plant family Asteraceae (Poulter, 1990).

Its name notwithstanding, the obscure mealybug, *Pseudococcus viburni* (Signoret) (Homoptera: Coccidae), is a common and widely distributed insect that feeds on a variety of plants, including grapevines, glasshouse crops, and ornamental plants (Laflin et al., 2004). The relatively sessile adult females produce a powerful sex pheromone to attract the ephemeral, nonfeeding, winged adult males for reproduction. We report the discovery of a highly irregular, nonhead-to-tail monoterpenoid with a (2,3,4,4-tetramethylcyclopentyl)methanol carbon skeleton, as a female-produced sex pheromone component of the obscure mealybug. In contrast to the 1′–4 linkages of most terpenoids, the ring structure of this compound appears to be constructed from 2′–2 and 3′–4 connections of two isoprenoid units (Figure 1A), providing the first example of an entirely new class of monoterpenoid structures.[1]

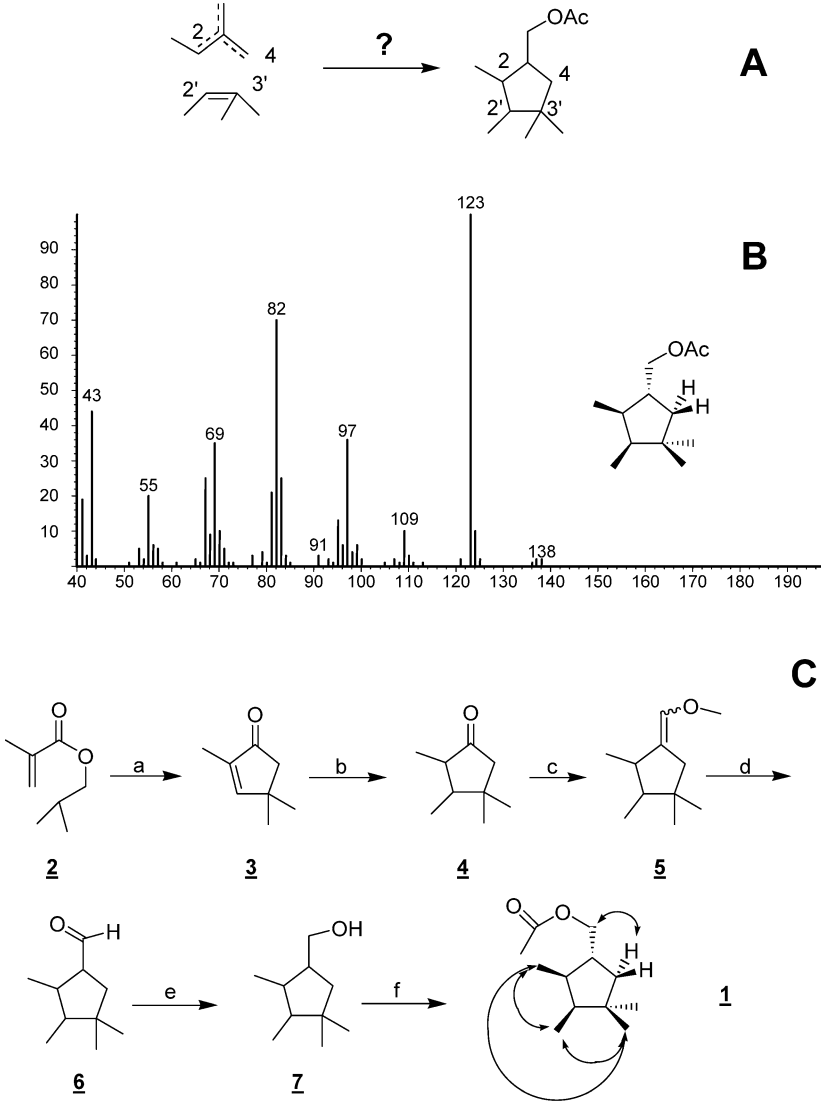
METHODS AND MATERIALS

Mealybugs, originally collected from grapevines, were reared in cohorts by infesting butternut squash fruits (*Cucurbita moschata*) with newly hatched mealybug crawlers. After 1 wk, squash were sprayed with pyriproxyfen (10 ppm in 0.1% aqueous Triton-X) to eliminate males. Treated squash with mature virgin females were aerated in 5-l glass chambers, collecting headspace odors on 2.5 cm × 4 mm ID activated charcoal traps. Collectors were changed every 3–5 d, and eluted with pentane. Control collections were made with uninfested squash, squash with sexually immature females, and squash with mixed-sex cohorts.

An aliquot of extract was reduced with 5% Pd on carbon catalyst in a septum-capped vial with a balloon of H_2 attached. A second aliquot was concentrated just to dryness, then treated with 1 M NaOH in 95% EtOH.

The extracts from ~20 aerations were concentrated and fractionated by preparative GC (2 m × 2 mm ID glass column, 10% SP-1000 on 80/100 mesh Supelcoport, headpressure 240 kPa; program 50°C/1 min, 10°/min to 200°C, hold 20 min). Fractions were collected in glass capillaries chilled with dry ice.

[1] A recent report (Lavoine-Hannegalle and Casabianca, 2004) suggested that 1,1,2,3-tetramethyl-4-hydroxymethyl-2-cyclopentene might be present as a trace component of lavender oil. However, the identification was based solely on interpretation of a mass spectrum and must be regarded as tentative at best, pending isolation and unequivocal identification by other spectral methods.



a. PPA, 100ºC; b. Me_2CuLi; c. $(Ph)_3P{=}CHOMe$; d. H_3O^+; e. $NaBH_4$; f. AcCl, pyridine; prep GC. Arrows on structure 1 show NOE correlations used to confirm the relative configuration. Full ^1H and ^{13}C data are given in the Online Supplement.

FIG. 1. (A) Connections between the two isoprene units making up the skeleton of the pheromone; (B) EI mass spectrum of (1*R**,2*R**,3*S**)-(2,3,4,4-tetramethylcyclopentyl)-methyl acetate; (C) synthesis of (2,3,4,4-tetramethylcyclopentyl)methyl acetate as a mixture of diastereomers.

NMR spectra were taken on an INOVA-400 spectrometer in C_6D_6 or CD_2Cl_2. Mass spectra were recorded on an H-P 5973 mass spectrometer coupled to an H-P 6890 GC, with helium carrier gas, and an HP5-MS column (30 m × 0.25 mm ID).

A field trial was conducted in vineyards near San Luis Obispo, CA, USA, using sticky traps baited with 0.1-mg doses of pheromone in hexane loaded on gray rubber septa (six replicates).

RESULTS AND DISCUSSION

Comparisons of extracts from sexually immature and mature females, and from mature virgin females and mixed-sex colonies by gas chromatography revealed one major compound [Kovat's index (KI) 1308 on a DB-5 GC column] that was exclusively produced by sexually mature virgin females. The compound was unchanged after reduction with hydrogen and palladium-on-carbon catalyst, indicating that the structure contained no carbon–carbon multiple bonds. Hydrolysis with ethanolic NaOH produced an earlier eluting alcohol (KI 1175), indicating the presence of an ester function, with the 133-unit difference in KI values between the parent compound and the derivative suggesting an acetate ester. The KI values of both the alcohol and the parent ester were in the ranges typical of monoterpene alcohols and acetates (Adams, 1995).

The electron impact ionization (EI) mass spectrum of the parent compound (Figure 1B) supported these data. Specifically, a fragment of low intensity at *m*/*z* 138, derived from loss of acetic acid from the parent molecule of molecular weight 198 amu supported the presence of an acetate ester, whereas the tentative molecular formula of $C_{12}H_{22}O_2$, with no C–C double bonds present, suggested a single ring in the structure. Although the base peak at *m*/*z* 123 ($C_9H_{15}^+$) and the rest of the mass spectrum had similarities to database spectra of multiply substituted cyclohexylmethanols, no matches were found with any literature or database spectra. As expected, the mass spectrum of the alcohol derived from hydrolysis of the pheromone was similar to that of the parent compound, with a base peak at *m*/*z* 123.

Based on interpretation of the mass spectrum and considerations of known terpenoid biosynthetic pathways, several structures were postulated and synthesized, but none proved correct. Consequently, crude pheromone extracts collected from multiple cohorts of virgin females were combined to obtain sufficient material for NMR analysis. The pheromone was isolated from the combined extracts (>100,000 female days of collection) by preparative gas chromatography, yielding a few micrograms of purified material, sufficient for a proton NMR spectrum, and limited information from NOE and ^1H–^1H COSY spectra. Interpretation of the proton and partial NOE and COSY spectra

suggested structure **1** (Figure 1C), with the methyl groups on C_2 and C_3 tentatively assigned to the same side of the ring, and the relative stereochemistry at C_1 being impossible to determine from the small quantity available.

To verify the carbon skeleton, a nonselective synthesis was developed that produced all four of the possible diastereomers (Figure 1C). Thus, isobutyl methacrylate **2** was cyclized with polyphosphoric acid (Conia and Leriverend, 1970), yielding 2,4,4-trimethylcyclopent-2-en-1-one **3**. Methylation of enone **3** yielded a 1:4 mixture of *cis* and *trans* 2,3,4,4-tetramethylcyclopentanone diastereomers **4**. Reaction of ketones **4** with (methoxymethylene)triphenylphosphorane followed by hydrolysis of the resulting vinyl ethers **5** gave a mixture of aldehydes **6**, which were reduced with $NaBH_4$ in EtOH to give alcohol **7**. Acetylation produced a mixture of acetate diastereomers, one of which matched the insect-produced isomer. This isomer was isolated by preparative GC, and determined to be the (1*R**,2*R**,3*S**)-diastereomer **1** (Figure 1C) by nuclear Overhauser effect NMR analysis. Full ^{1}H and ^{13}C data NMR data are in the Online Supplement Table A1 (Electronic Supplementary Material is available for this article at http://dx.org/10.1007/s10886-005-9320-0 and is accessible for authorized users). To date, the absolute configuration of the insect-produced compound has not been determined. However, in preliminary field tests, the racemic compound was highly attractive to male mealybugs ($N = 6$; treatment, 211 ± 47 (mean ± SE) males caught; control 2.2 ± 1.6 males caught), indicating that the unnatural enantiomer is not inhibitory.

In addition to the relatively rare examples isolated from plants (Poulter, 1990), several examples of irregular monoterpenoid skeletons have been found in the Insecta. For example, cyclobutane structures with c1′-2′-3-2 connectivity have been isolated from several weevil species, such as the boll weevil *Anthonomis grandis* (Tumlinson et al., 1969). α- and β-necrodols, characterized by a 1,2,2,3,4-pentamethylcyclopentane nucleus with the ring formed from 2′–4 and 3′–2 connections, are produced as defensive compounds by the silphid beetle *Necrodes surinamensis* (Roach et al., 1990). However, mealybugs and the related armored scale insects (Dunkelblum, 1999) appear to be masters of irregular terpenoid biosynthesis. For the six mealybug species for which pheromones have now been identified (review, Millar et al., 2005), all of the pheromone components consist of irregular terpenoids, with all the components except that of the obscure mealybug having a 1′–2 linkage. Even within this unusual group, the obscure mealybug, with its unprecedented 2′–2 and 3′–4 linkages, stands out.

There are further implications. First, because each of these species produces only one or two of these irregular monoterpenoids, the biosynthesis of these compounds by the insects is clearly under tight control. Second, the fact that none of these terpenoid skeletons have been reported from any of the insects' typical host plants, which include well-studied crops such as citrus,

grapes, and tree fruits, suggests that these compounds may be synthesized *de novo*, rather than being derived from minor modifications of compounds sequestered from the host plants.

It is also noteworthy that all of the irregular terpenoid pheromones identified from mealybugs are unique structures. Unlike many other groups of closely related insect species, which create species-specific pheromone signals by using different ratios and subsets of compounds common to some or all members of the group, mealybugs appear to use species-specific chemicals to create unique communication channels, eliminating any possibility of interference in or competition for the pheromone channel. This lack of competition for the channel may explain why these insects are generally insensitive to stereoisomers or other analogs of their pheromones. This is in contrast to insects such as beetles and moths, which are frequently acutely sensitive to even traces of structural analogs of their pheromone components.

The evidence suggests that mealybugs have developed terpenoid biosynthetic pathways for production of their pheromones that are distinct from the typical terpenoid pathways found in other organisms. This ubiquitous insect family will likely prove to be an abundant source of irregular terpenoids, and the enzymes responsible for their synthesis.

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